Stefano Mancuso · Sergey Shabala
Editors

Rhythms in Plants

Dynamic Responses in a Dynamic Environment

Second Edition

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Preface

Rhythmical behaviour is a quintessential pattern of life itself and is believed to play a key role in cell division and morphogenesis, to mediate all kind of movements, and to provide an advantageous strategy for evolution and adaptation of living organisms. Rhythms have fascinated people for more than 2000 years. As early as the fourth 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 notice single movements of parts of plants in a cursory manner, Albertus Magnus in the thirteenth century and Valerius Cordus in the sixteenth century, thought the daily periodical movements of the pinnate leaves of some *Leguminosae* worth recording. Ray in his "Historia Plantarum" at the end of the seventeenth century commences his general considerations on the nature of the plants with a succinct account of *phytodinamical* phenomena mixing up together the movements from irritability and the daily periodical movements; the latter, he says, occur not only in the leaves of *Leguminosae*, but in almost all similar pinnate leaves, and with these periodical movements of leaves, he places also the periodical opening and closing of the flower of *Calendula*, *Convolvulus*, *Cichorium*, and others.

The number of publications on rhythms in plants increased dramatically in the last decades, and the old mystery of the "biological clock" has been tackled from the molecular, genetic, and biochemical perspectives. Yet, most research deals only with measurable rhythms—the so-called hands of the clock mechanism. The clock "pacemaker" still remains a mystery. Many superimposed rhythms are able to coexist in the same cell compartment, all with different periods. How many oscillators does a living cell have? Just one? Or is each of these processes controlled by its own independent oscillator? The situation is similar to that which existed in the physics of elemental particles in the "1960–"1970s, when an avalanche-like increase of discovered particles initiated a revision of theoretical concepts and resulted in the creation of a new paradigm in physics. 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, near astounding pace of discoveries shows how extremely the subject has changed and is reflected in the different chapters of the current book which covers aspects of plant physiology neither recognizable nor quantifiable few years ago.

The second edition of this book provides a timely update on a recent progress in this field and comprehensively summarizes current knowledge of molecular and physiological mechanisms behind circadian and ultradian oscillations in plants, and their physiological implications for growth, development, and adaptive responses to dynamic environment. The book is structured around three major topics:

- Ultradian oscillators
- Circadian oscillators
- Theoretical aspects and modelling

Written by a diverse group of leading researchers, this book will surely spark the interest of readers from many branches of science: from physicists and chemists wishing to learn about multifaceted rhythms in plant biology, to biologists and ecologists dealing with state-of-the-art modelling of complex rhythmic phenomena.

Before we close and let the reader enjoy (we hope!) the content of this volume, we would like to acknowledge the Springer's team (Dr. Andrea Schlitzberger and Dr. Christina Eckey) for their idea to proceed with this publication. We would also like to express our sincere gratitude to all contributing authors who have enthusiastically embraced an idea to go for the second edition of this book. Finally, and most importantly, we are truly grateful to all "frontline people" in our institutions and elsewhere in the world for their enthusiasm and patience in revealing one of the greatest mysteries of the life—THE CLOCK.

June 2015

Stefano Mancuso
Sergey Shabala

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Contents

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Part I
Ultradian Oscillations

Chapter 1
Ultradian Growth Oscillations in Organs: Physiological Signal or Noise?

Tobias I. Baskin

Abstract This review examines ultradian oscillatory growth in the multicellular organs of vascular 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, the regular ultradian movements of plant organs, when they occur, reflect successive responses to mechanical perturbation.

1.1 Introduction

1.1.1 Oscillation as a 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

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(Goldbeter 1996). Expansion of a cell is certainly a complex 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. The following illustrates how expansion could be oscillatory (Fig. 1.1). Suppose water uptake were linked to turgor loss, such that aquaporins would open only when 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. This hypothetical loop illustrates feedback between steps in the growth process. 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 growth mechanisms. Also, I will not treat growth oscillations in single cells, such as pollen tubes or root hairs (the interested reader may consult the review in this volume by Feijo), even though my objective is exemplified beautifully by Castle (1940) who detected an

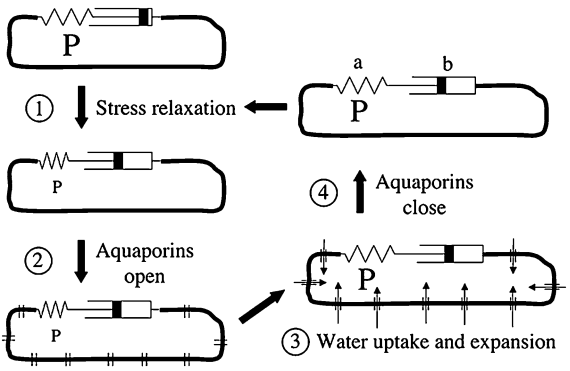

Fig. 1.1 Hypothetical model of diffuse growth giving rise to oscillatory expansion. For a single cell, the mechanical behavior of the cell wall is idealized by an elastic element (*spring*; *a*) and a plastic element (*dash-pot*; *b*) in series. Turgor pressure (*P*) is held by the deformation of the 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

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.

1.1.2 Growth Versus Movement

Unfortunately, the word *growth* is used in two distinct ways. On the 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 cellular level, or indeed to the elemental deformation of a unit area of cell wall. The latter is the direct output of the growth machinery, whereas tip displacement integrates the behavior of the entire organ, often many centimeters long. 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 mechanisms indirectly.

1.2 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 widely believed to be true because it is often assumed, first, that an oscillatory movement called circumnutation is undergone by the stems and roots of essentially *all* plants and second, that circumnutation is a coherent oscillation in growth. 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). By *large* amplitude, I mean that the lateral displacement is much greater than the stem diameter. A large amplitude displacement is not required for a regular period (Schuster and Engelmann 1997; Adolfson et al. 1998). This regular behavior reflects an obvious circumnutation, clearly adaptive for vines and climbing plants, but happening also in species such as sunflower (*Helianthus annuus*) and arabidopsis (*Arabidopsis thaliana*) where a plausible reason for the behavior is synchronization of growth among cells.

However, the tips of stems and roots of many plants move in erratic trajectories with small amplitude (i.e. equal or even less than organ diameter) and erratic period (Heathcote and Idle 1965; Spurný et al. 1978; Barlow et al. 1994; Shabala and Newman 1997). To claim that circumnutation is a property of all growing organs is

to assert that the large and regular movements of the *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. Here, I will restrict the word *circumnutation* to movements with a salient and regular period.

Whether large or small, stem movements are widely ascribed to differential growth. But this need not be the case. In many plants, movements of leaves have ultradian periods, similar to those of 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.

Reversible volume changes have been implicated in circumnutation. For example, circumnutation continues for a few periods following decapitation of pine (*Pinus sylvestris*) hypocotyls 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 (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). Conceptually, this is if the pulvinus were spread throughout the bending stem, I will call this arrangement a *diffuse* pulvinus.

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 equally universal oscillations in growth. In addition, a major topic of research on circumnutation has been to determine to what extent this movement can be explained by gravitropism. One explanation, formulated into an explicit model 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 role of gravitropism in circumnutation continues to be debated (Johnsson 1997; Hatakeda et al. 2003; Tanimoto et al. 2008; Johnsson et al. 2009). Insofar as gravitropic bending is accepted as being based on differential growth, those circumnutations powered by a diffuse pulvinus can be distinguished from gravitropism mechanistically.

Nevertheless, circumnutation can involve oscillations in growth. Growth rate oscillations, 180° out of phase on either side of the circumnutating stem, occur in the epicotyl of pea (*Pisum sativum*; Baskin 1986) as well as in the sunflower hypocotyl (Berg and Peacock 1992; Fig. 1.2). 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

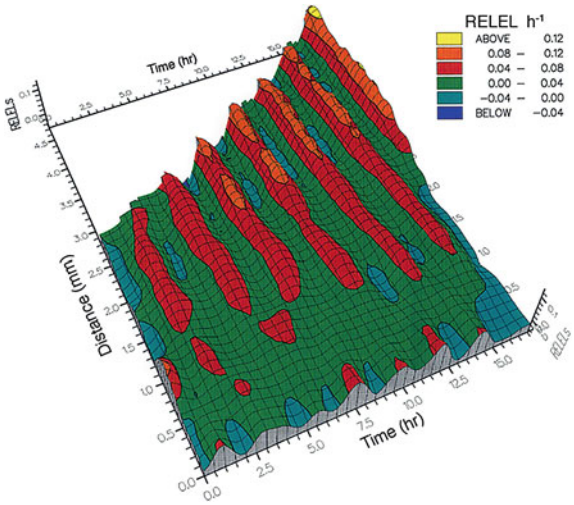

Fig. 1.2 Three-dimensional plot of displacement and elemental elongation versus time for a circumnutating sunflower hypocotyl (modified from Berg and Peacock 1992). The hypocotyl was marked at 2-mm intervals and growth rates recovered from marks digitized at 15-min intervals. Data were interpolated to 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

2-mm zones and found that differential expansion is responsible for most of the bending (Fig. 1.2). Interestingly, these authors did record negative elemental elongation rates, implying that contractions (hence reversible volume changes) contribute to the oscillatory movement. Even a total absence of contraction cannot exclude a contribution from elastic changes because reversible and irreversible processes are readily superimposed (Proseus et al. 1999; Fig. 1.1). Therefore, while circumnutation can depend on out-of-phase growth oscillations, as in pea and sunflower, circumnutation can alternatively depend on reversible volume changes (diffuse pulvinus), as in French bean. With so few examples documented, it is unjustified to assume that circumnutation invariably reflects differential growth.

Surprisingly, regular and relatively large amplitude oscillations of organ position can result from underlying processes that are constant. The peduncle of the aquatic angiosperm *Vallisneria asiatica* circumnutates with huge amplitude, driven by the interaction of the water surface with the unidirectional (i.e. non-oscillatory) rotation of the stem (Kosuge et al. 2013). When arabidopsis is grown on an inclined and impenetrable surface, the root will grow in a sinusoidal pattern (*root waves*) that has been attributed to circumnutation (Migliaccio et al. 2013) and a regular growth oscillation presumed. However, in an elegant analysis, Thompson and Holbrook (2004) showed that the undulating wavy pattern represents buckling of the root and results from gravitropism and friction between the root tip and the substrate. No

oscillation in growth is needed, and in fact, the tip displacement rate of the root fluctuates erratically, again illustrating how a regular, oscillatory pattern (the shape of the root) can be built up without an oscillation in either reversible or irreversible expansion.

As an aside, 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.

1.3 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 around the circumference. Symmetrical entrainment would give rise to oscillations in tip displacement velocity. I will call these *axial* oscillations, because they are in line with the longitudinal axis of the organ. Additionally, it is common for stems and roots to move laterally through a distance on the order of the organ's diameter. Insofar as entrainment might not be perfectly symmetrical, oscillatory lateral displacements of small amplitude might plausibly indicate synchronized linear oscillations in expansion rate. How prevalent are well-synchronized, ultradian oscillations in movement?

In seeking to answer this question, I will consider first measurements of velocity (both lateral and axial) followed by direct measurements of expansion.

At first sight, ultradian oscillations seem prevalent, reported in many papers. Oscillations in velocity have been claimed with periods of 170 min in rice roots (Iijima and Matsushita 2011), 20–70 min in the arabidopsis inflorescence stem (Degli Agosti et al. 1997; Jouve et al. 2000), 80–120 min in the azuki bean (*Vigna angularis*) stem (Gotô and Chiba 1983), and 60 and 270 min for a maize (*Zea mays*) 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 (*Vigna radiata*) hypocotyls (Prat and Parésys 1995; Prat et al. 1996). In the above examples, although periods were assigned, their significance was rarely tested statistically and the records typically bristle with periods, implying temporal instability. In other examples, the fluctuations were not analyzed temporally but appear aperiodic by eye (Jiang and Staude 1989; Behringer et al. 1990; Yang et al. 1993; Liptay et al. 1995). In general, these fluctuations in axial velocity on a scale of a few hours are far less regular than those seen in circumnutation and arguably represent erratic feedback rather than an endogenous rhythm.

In roots, various types of lateral movement fluctuations have been recorded in the ultradian range, but almost always with small amplitudes, wandering trajectories, and poorly defined periods (Spurný 1966; Spurný et al. 1978; Hasenstein 1991; Barlow et al. 1994; Shabala and Newman 1997; Thompson and Holbrook 2004). For maize roots, small amplitude (few hundred microns) lateral oscillations have been reported with reasonably regular periods of 8 or 90 min (Walter et al. 2003; Vollsnes et al. 2010; Popova et al. 2012), but in other publications, maize roots grow with scarcely perceptible lateral deflections (Erickson and Sax 1956; List 1969). The clearest example I know of sustained lateral oscillations in roots is for rice with about a 1-h period (Hayashi et al. 2004).

Fluctuations in axial velocity might be commonly observed, but they can be separated from growth, at least to some extent. First, erratic fluctuations are often absent, even from high-resolution traces (e.g. Stolarz et al. 2008; Durnham-Brooks et al. 2010). The steady lateral oscillation of the tip of the rice root, mentioned above, vanishes under aluminum treatment, at a concentration that has no discernable effect on growth rate (Hayashi et al. 2004). Erratic oscillations in stem tip velocity occur in 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 *C. rubrum*, tomato (*Solanum lycopersicum*) stems growing under a regular photoperiod and having diurnal oscillations in axial velocity retain erratic higher-frequency fluctuations but only in some individuals (Kerckhoffs et al. 1997). Finally, in a report showing fluctuations in stem tip velocity in the seedlings of five species, with apparent periods ranging from 3 to 120 min, similar fluctuations were detected when the position transducer was attached near the (non-growing) base of the hypocotyl (Kristie and Jolliffe 1986), implying that the source of these fluctuations is distinct from growth.

Turning now to direct measurements of expansion, the story is similar: Fluctuations in expansion rate are usually erratic and are sometimes entirely absent. There is a massive body of work where a position transducer is used to record the growth of excised stem segments. Strictly speaking, this reports the velocity at which one end of the segment is moving relative to the other end; nevertheless, the segments are typically about 1 cm long and contain growing tissue exclusively, attributes that make the reported velocity data reasonably close to an elemental expansion rate, at least compared to data from attaching the transducer to the tip of an intact organ. Growth in these records is usually constant (e.g. Penny et al. 1974), sometimes fluctuates erratically, and only rarely oscillates regularly.

Position transducers involve mechanical attachment and might suppress small fluctuations. Instead, growth is measured more reliably and less invasively from images. In spatial analyses of root growth, elemental elongation rates throughout the growth zone fluctuate erratically (Erickson and Sax 1956; List 1969; Salamon et al. 1973; Chavarría-Krauser et al. 2008; Shih et al. 2014). Sometimes, the spatial profile of elemental elongation in maize is bimodal (Walter et al. 2002, 2003) and that of arabidopsis is multimodal (van der Weele et al. 2003). These bumps might indicate a regular oscillation in elongation rate as a cell traverses the growth zone;

instead, they might indicate a programmatic distinction among regions of the growth zone. Likewise, in the ultradian range, coleoptiles grow without apparent fluctuation (Baskin et al. 1985) and leaf expansion fluctuates erratically (Wiese et al. 2007; Mielewczik et al. 2013). Interestingly, in the later paper, one of the leaves undergoes clear ultradian growth rate oscillations during one night, but these involve substantial contraction, suggesting that they reflect an elastic phenomenon.

It is perhaps premature to take the absence of evidence as evidence for absence; research on growth rhythmicity has focused all but exclusively on diurnal rhythms (Walter et al. 2009). As a result, most published growth data are averages over individuals, an expedient used to reinforce and hence identify the phase relation between growth and an external cycle (typically light). Unfortunately, averaging will smooth out ultradian rhythms insofar as they are endogenous and hence out of phase among individual plants.

Taken altogether, this survey suggests that unmistakable, ultradian growth oscillations, as reported for pea epicotyls (Baskin 1986) and sunflower hypocotyls (Berg and Peacock 1992), are the exception, rather than the rule. Organs do undergo lateral movements of minor amplitude and have fluctuations in their overall extension rate, but these fluctuations are erratic and possibly due to processes other than growth. Some organs grow without a trace of ultradian fluctuations. Admittedly, distinct oscillations might emerge from a dedicated study using high-resolution methods; however, given the guiding concept of facile entrainment of neighboring oscillators, one expects a robust output. To my knowledge, there is no example of an ultradian growth oscillation shown to be symmetric (i.e. not out of phase on different sides of the stem) and having the temporal stability characteristic of circumnutation.

1.4 The Power of Bending in Plants

Pronounced, ultradian growth oscillations, although not ubiquitous, do occur and require explanation. The aforementioned 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 the 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 liable to interact.

Nevertheless, the overshoot model was recently supported 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; Tanimoto et al. 2008). Furthermore, circumnutation in arabidopsis stems is strongly if not absolutely

suppressed in microgravity (Johnsson et al. 2009). However, in many environments, circumnutation, though having a well-defined period, is small in amplitude and hence unlikely to generate a significant gravitropic signal (e.g. Hatakeda et al. (2003) report wild-type amplitudes of ~200 μm for the circumnutation of the arabidopsis inflorescence). Furthermore, despite lacking the ability to reorient when rotated, these mutant stems, as well as stems in microgravity, grow vertically (the morning glory stems eventually fall over and adopt a lazy habit). One would expect the non-gravitropic stems to meander when subject to the random deviations needed to initiate an overshoot cycle. That a plant can respond by suppressing circumnutation has been documented for the etiolated rice coleoptile in response to red light (Yoshihara and Iino 2005). Conceivably, a similar response occurs in morning glory stems and arabidopsis inflorescences when gravitational responsiveness has been diminished genetically or by travel beyond the pull of the Earth.

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. Darwin and 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, when after many rotations, 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. 1.3). In some cases, the stems reversed a second time, again as if the alternating stimuli had continued. Gravitropic (or phototropic) overshoot cannot explain these results because stopping the clinostat rhythm led to the stems bending down (or away from the light); instead, it suggests that the stems were also 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. 1.2). Likewise, the peduncle of the cyclamen (*Cyclamen hederifolium*) fruit 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

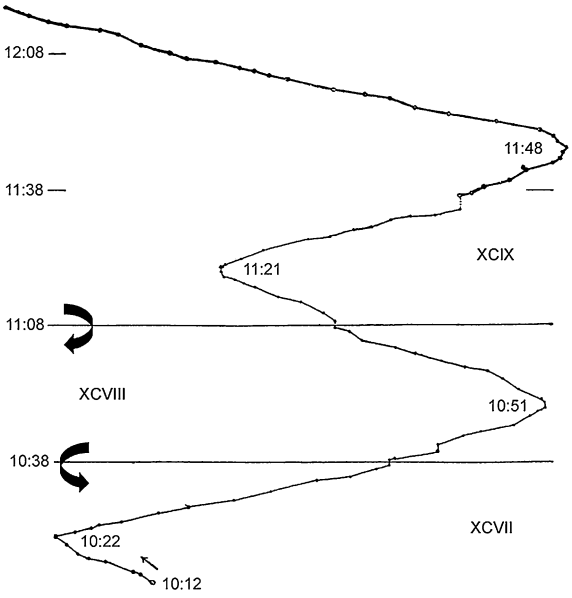

Fig. 1.3 Trajectory showing the existence of a response to bending (redrawn from Darwin and Pertz 1903). Time flows from the *bottom* to the *top*, indicated in hours:minutes by *Arabic numerals*. The *horizontal coordinate* shows the position of the stem tip in arbitrary units. A mustard (*Raphanus* 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

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 documented. For example, in dandelion (*Taraxacum officinale*) peduncles, a modest and transient (5–10 min) lateral stress elicits a vigorous growth response (Clifford et al. 1982). An ingenious series of experiments was 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). This work demonstrates 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.

1.5 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 are uncommon and those that occur in some cases are not due to growth and in others are too erratic to be called oscillations. 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, or the superposition of elastic changes related to water flow.

To settle this issue, measurements of relative elongation at cellular and sub-cellular resolution are crucial. Also useful would be to look for growth oscillations in single 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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Chapter 2
Nutation in Plants

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Abstract This chapter aims to explore and describe the physiological aspects 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 explain 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, thus interpreting 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. A revised two-oscillator model is also 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. The role of hormones will be finally discussed, with particular attention to the effect of ethylene in controlling nutation.

2.1 Overview of Nutations: Definition and Kinematic

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

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intervals, but the organ's instantaneous growth direction usually oscillates slowly around that mean. From a distal viewpoint, the plant organ tip, or an elongating cylindrical plant organ, describes an ellipse, a circle- or pendulum-like movements about the plumb line, which can alternate between a clockwise and counterclockwise 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 (Migliaccio et al. 2009). This oscillating growth pattern was well known to nineteenth-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. 2.1). Thus, circumnutational oscillations are manifestations of the radially asymmetric growth rate typical of elongating plant organs (Fig. 2.2). These do not include tropic processes occurring in response to a directional cue, such as gravity or light, or nastic movements, which occur in response to external factors but are independent of the position, i.e. the closing of leaves at night. These various forms of movements usually occur together; for example, it has been shown that gravity amplifies the circumnutatory response in *Arabidopsis thaliana* (Johnsson et al. 2009).

Darwin's (1875) close observation of the behaviour of 'climbing plants', which 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 not only occurs 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

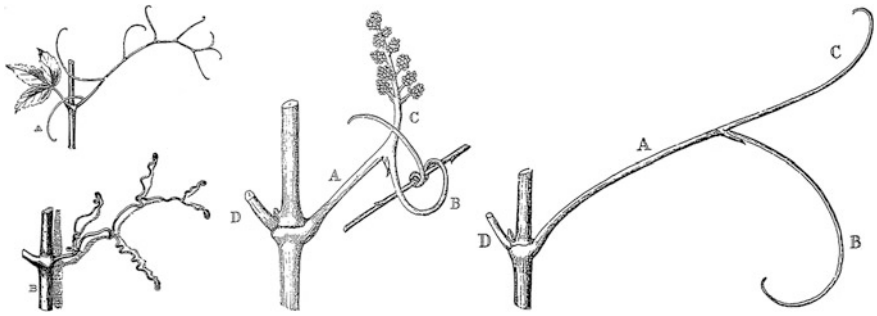

Fig. 2.1 Some sketches illustrating Darwin's close observation of the behaviour of 'climbing plants' (extracted from Darwin 1875)

Fig. 2.2 *Brassica oleracea* and circumnutation of the hypocotyl and cotyledons (extracted from Darwin and Darwin 1880)

species, the plant organs involved and the developmental stage of growth. Shoots of climbing plants (e.g. *Dioscorea batatas*, *Ipomoea quamoclit* 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 counterclockwise (Fig. 2.3). 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). In some cases, the oscillating rhythm is connected with circadian cycles, as shown in *Helianthus* (Niimura et al. 2005; Stolarz et al. 2008; Stolarz 2009). Therefore, appropriate methods are needed to fully reveal the

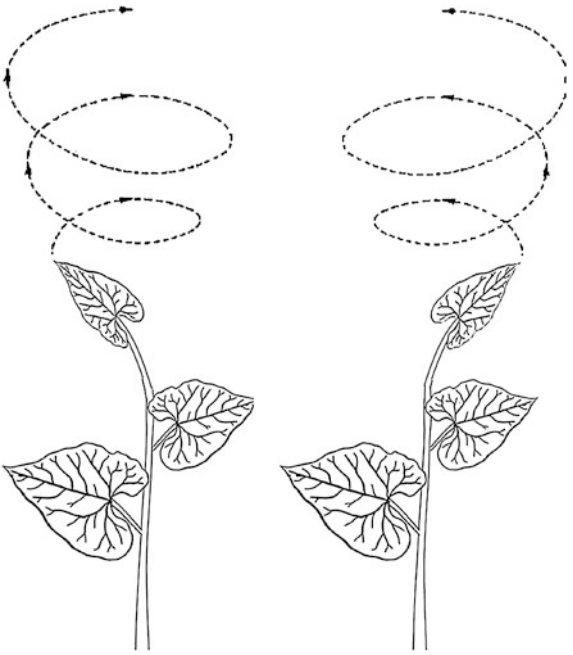

Fig. 2.3 Clockwise and counterclockwise oscillations of *Phaseolus vulgaris* L

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 the vigour of circumnutations. It may be significant that these effects can occur only within 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.

2.2 Circumnutation Can Be Explained by Theories and Models

Unequal rates of growth on different sides of an organ represent the primary process behind circumnutation: the side with the most rapid growth pushes the apex of the organ over, bending it towards the least active side (Migliaccio et al. 2013). The active area then proceeds around the organ, typically along a helical path, which can be right-handed or left-handed (Johnsson 1997). Circumnutation is therefore 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. 2.4; see Forterre (2013) for an extensive review). 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. This is also associated with defects in microtubule patterning (microtubules are transversely oriented in spirals with respect to the longitudinal axis of the cell in elongating root cells), which can cause the epidermal and cortical layers to lag in expansion rates such that cell file rotation increases (Roy and Bassham 2014). 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 pulvinar 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 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).

Currently, two main models for circumnutation have been proposed.

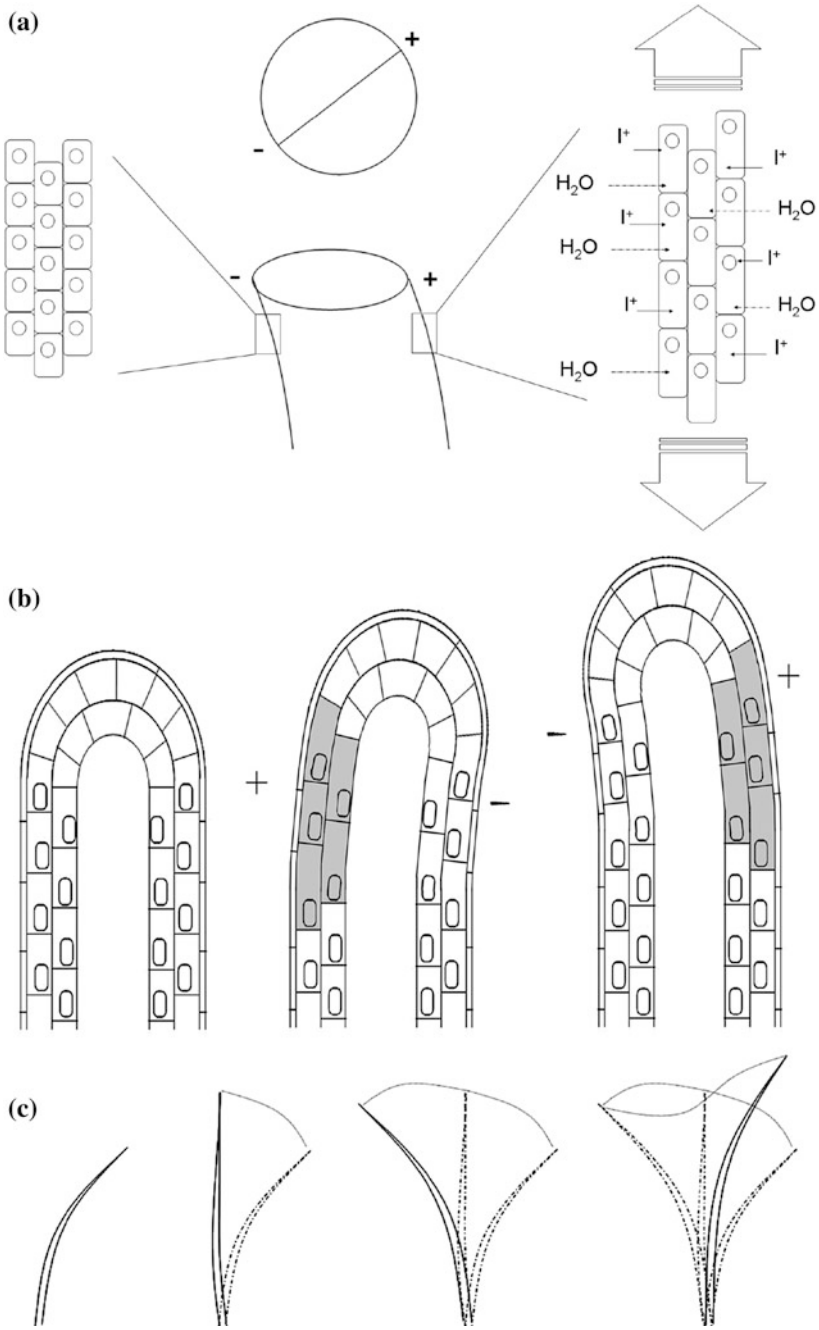

Fig. 2.4 Schematic representation of cellular turgor changes (**a**), differential growth rate (**b**), and at two opposite sides of a shoot and its effects on circumnutation (**c**)

2.2.1 Model I: '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 nineteenth-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, *TOC*1 (mutant which shortens the period for all circadian processes analysed to date) and *ELF*3 (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.

2.2.2 Model II: '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. 2.5). 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 (Brown 1993) and the ISS (Correll and Kiss 2008; Paul et al. 2012), however, revealed that

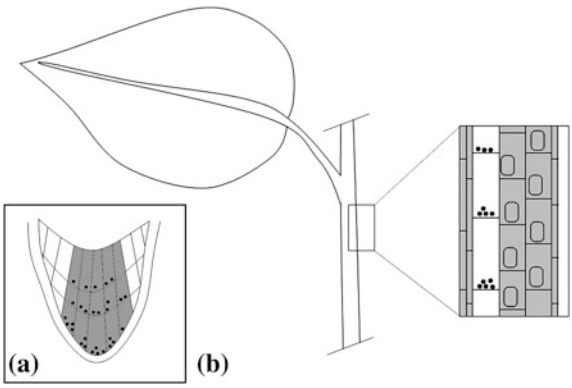

Fig. 2.5 Diagram of a root (**a**) and a stem (**b**), illustrating the regions of gravity perception

gravity is not an absolute requirement either for the initiation or for the continuation of circumnutatory movements, leading to the conclusion that the inner oscillator and gravitropism seem to operate independently. The patterns of circumnutation observed during microgravity experiments clearly show that gravity is not an essential component of the mechanism that drives this type of root growth (Paul et al. 2012). It has been hypothesized for a long time that circumnutation is primarily a result of a correlation between gravitropism and the tactile interaction with a surface (thigmotropism) (Simmons et al. 1995). And although the specific metabolic drivers are not fully known, differential expression among cultivars has proposed the existence of a certain amount of unknown candidate genes that may contribute to cultivar-specific phenotypes, as well as confirming several known to be involved with circumnutation (Vaughn and Masson 2011). Regardless of the underlying influences, the statement that gravity contributes to circumnutation deserves therefore reconsideration.

Kitazawa et al. (2005) demonstrated that gravisensing endodermal cells are required for shoot circumnutation in morning glory (*Pharbitis nil*). They 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) analysed the shoot circumnutation of two agravitropic mutants of Arabidopsis, *sgr*2 and *zig*/*sgr*4, 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.

2.2.3 The 'Mediating' Model

To overcome the differences between the models previously described, Johnsson et al. (1999) proposed a revised model which combines the two models: 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 re-initiated 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.

2.3 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 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), 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-handed 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 *rgr*1 (*r*educed root *gr*avitropism), found that the frequency of the waving pattern and circumnutation was the same in *rgr*1 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 thigmotropism had been excluded. Using wild-type ecotypes and different gravitropic mutants (auxin transport mutants such as *aux*1 and *eir*1; auxin physiology mutants such as *axr*1; handedness mutants such as 1-6*C*), they observed that wild-type Arabidopsis roots made large movements of circumnutation only to the right-hand side, but auxinic mutants, such as *aux*1 and *eir*1, showed a lack of regular chiral circumnutation: auxinic mutants are disturbed not only in their gravitropic response (*aux*1 and *eir*1 are totally agravitropic) but also in their chiral circumnutational movement. Furutani et al. (2000) isolated two mutants, namely *spr*1 and *spr*2, which presented a left-handed symmetry with their coils growing anticlockwise, and the left-handed waves dominant in the slanting. The mutant genes were successfully cloned, with the proteins SPR1 and SPR2 identified. When bound to GFP, SPR1 is first localized to the plus end of microtubules, and SPR2 is localized along the length of the cortical microtubules (Sedbrook 2004). The isolation of mutated *spr* genes (*lefty*1 and *lefty*2, Marinelli et al. 1997), which encode for α-tubulin 6 and α-tubulin 4, respectively, gave an evidence for the involvement of tubulins in guiding the symmetry of the mutants. This result was further confirmed by Collings et al. (2006) on the basis of a research using the *mor*1 mutant, which is involved in the control of the synthesis of microtubules arrays, spindle, and phragmoplast, and consequently of the whole karyokinesis process.

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).

2.4 The Role of Plant Hormones: Auxin and Ethylene

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.

Recently, the role of ethylene in controlling the circumnutation was explored by several studies. Kim et al. (2011) found that the nutation response was constitutive in *ctr1-2* mutants maintained in air. On the contrary, ethylene-stimulated nutations were eliminated in *ein2-1* mutants and *etr1-7* loss-of-function mutants. When *etr1-7* mutant was transformed with a wild-type genomic ETR1 transgene, the nutation phenotype was set again, thus supporting a strong requirement for ETR1.

Interestingly, loss-of-function mutations in the other receptor isoforms had no effect on nutations in ethylene-stimulated seedlings. On the contrary, they constitutively nutated in air. These evidences lead to the development of a model where all the receptors are involved (but not strictly required) in ethylene-stimulated nutations, but the ETR1 receptor is needed, with a contrasting role from the other receptor isoforms in this nutation phenotype. Further experiments showed a lack of nutations when Arabidopsis seedlings were treated with 5 mM AVG, an inhibitor of ethylene biosynthesis. There are also clear differences that can be highlighted between the inhibitory effect of ethylene on hypocotyl growth and its role in stimulating the nutations (Binder et al. 2004a, b). Finally, the chain of biochemical and transcriptional events by which ethylene causes nutation is not well defined and still under investigation. Hatakeda et al. (2003) showed that auxin transport inhibitors, such as NPA, have been shown to block nutations. Similarly, Kim et al. (2011) found that NPA blocks nutations but at the same time has no measurable effect on growth inhibition caused by ethylene. This supports the assertion that, whereas nutations require growth, growth does not require nutations, hypothesizing that ethylene promotes nutations by differentially altering local auxin levels in the zone where nutational bending is observed. Given the complexity of interactions between hormones in plants, it is also likely that other hormones could be involved with ethylene-stimulated nutations. As previously shown, the regulation of microtubule orientation seems to be a mechanism controlling nutations (Furutani et al. 2000). As ethylene directly affects microtubule orientation (Steen and Chadwick 1981), this could be considered another potential mechanism to control nutations by ethylene. However, many other hormones affect microtubules (Shiboaka 1994), and therefore, the interaction between ethylene and microtubules in controlling nutation is not completely defined yet.

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Chapter 3
Rhythmic Stem Extension Growth and Leaf Movements as Markers of Plant Behavior: How Endogenous and Environmental Signals Modulate the Root–Shoot Continuum

Johannes Normann, Lars Lehner, Marco Vervliet-Scheebaum, Josef Svoboda, Anezka Albrechtová and Edgar Wagner

Abstract With the short-day plant *Chenopodium rubrum* and the long-day plant *Chenopodium murale*, growth and behavior 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 movements (LM) change their phase relationship in a specific way. Flower induction correlates with 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-inducing signal. Changes in LEAFY (a florigenic transcription factor) and aquaporin expression can also be recorded during this phase. The perception of a flower-inducing dark period leads to a change in electrochemical, hydraulic signaling between the leaves and SAM, thereby determining polarity in the whole plant and paving the way for "florigen". Leaves from flowering plants can be grafted on non-induced plants

Dedicated to the 80th anniversary of Hubert Greppin.

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(short- or long-day species) to induce flowering in the recipient plant. Flowering could even be induced using a different donor and recipient species (inter-species signaling). 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 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 the 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, in particular the root and shoot meristems (RAM and SAM).

3.1 Introduction

3.1.1 Rhythmic Environmental Conditions and Their Impact on the Biosphere

In the inter-tidal zone, biological rhythms related to the solar and lunar day are important for the well-being of many animals (Al-Musawi and Wagner 2012). The impact of lunisolar tidal force on land plants has been reviewed by Barlow and Fisahn (2012) showing quite some circumstantial evidence for the control of behavior by solar and lunar gravitational force. There is experimental evidence that tree stem diameters fluctuate with tide (Zürcher et al. 1998).

Rhythmicity is one of the characteristics of life which expresses itself at all levels of organization, from unicellular systems to man. Rhythmic phenomena in the physiology, development, and behavior of all living systems show period lengths ranging from fractions of a second to hourly, daily, and even annual cycles (Cumming and Wagner 1968; Cumming 1967a; Lloyd and Rossi 1992).

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 laboratory 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, depending on latitude and season (Svoboda and Hošek 1976). In contrast to biological rhythms showing other frequencies, circadian rhythms (CRs) are temperature compensated and

Table 3.1 "Rhythmic phenomena in seedlings of *Chenopodium rubrum* L. (in brackets period lengths of sub-peaks)" (from Wagner et al. 2000, complemented after Bünning 1977)

Phenomenon	Period length (h)
Photoperiodic light sensitivity	30
Betacyanine accumulation	24–30 (15)
Betacyanine turnover	24–30
Adenylate kinase activity	30 (15)
Energy charge (ATP + $^1/_2$ADP/ATP + ADP + AMP)	21–24 (11–13)
NADPH/NADP ratio	21–24
Dark respiration	21–24
Chlorophyll accumulation	15
Net photosynthesis	15
Triose phosphate dehydrogenase activity	15
Malate dehydrogenase activity	12–15
Glutamate dehydrogenase activity	12–15
Glucose-6-phosphate dehydrogenase activity	12–15
Gluconate-6-phosphate dehydrogenase activity	12–15
Pyridine nucleotide, pool size [NAD(H_2); NADP(H_2)]	12–15 (6)
Turgor-controlled growth phenomena:	
– stem extension rate	23–27
– leaf movements	23–27

For a summarizing publication, see Wagner et al. (1975)

almost totally unsusceptible to chemical manipulation. It is the stability, or homeo-dynamics, of period length which qualifies the circadian rhythm as a precise physiological timer, and this being the essence of Bünning's (1973, 1977) theory of the physiological clock published in a series of monographs between 1958 and 1977 (see Bünning 1973, 1977).

The evolution of CRs dates back to the beginning of the oxygenation of the atmosphere 3–4 billion years ago. The development of photosynthesis resulting in the production of oxygen necessitated the development of a temporal metabolic compartmentation. Cyanobacteria have a temperature-compensated rotary circadian clock capable of operating even in vitro (Nakajima et al. 2010). With increasing oxygen in the atmosphere, cyanobacteria probably developed multi-cellularity with temporal metabolic compartmentation (Schirrmeister et al. 2013).

In parallel to the evolution of oxidation–reduction cycles, peroxy-redoxin proteins became universal markers of circadian rhythms in all domains of life (Edgar et al. 2012). Besides the redox status of living systems, the level of adenylates (ATP, ADP, and AMP) is the other side of energy metabolism representing the so-called energy charge (AEC), as proposed by Atkinson (De la Fuente et al. 2014; Table 3.1). More recently, it has become evident that CRs persist in redox reactions in the absence of transcriptional cycles (Wu and Reddy 2014).

The pattern of timing devices seems even more important taking into account the observation of temperature compensated and entrainable enzymatic oscillations in the range of minutes (Morré and Morré 1998).

In a comprehensive analysis of energy metabolism in *Chenopodium rubrum*, we could demonstrate that adenine and pyridine nucleotide pool size levels are rhythmic. The ratios of reduced to oxidized pyridine nucleotides as well as energy charge, calculated according to Atkinson, show a semi-circadian pattern.

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 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, with the light intensity decreasing much faster than the temperature. 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 is the 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 generally different from 24 h. If, under constant environmental conditions, an oscillation with a periodicity of exactly 24 h is observed (as an experimental artifact), then subtle geophysical synchronizers, so-called zeitgebers, might be involved (Bünning 1977).

In some 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 de-synchronization 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 synchronizers are apparently sufficient for resynchronization or re-phasing of metabolic activities (Highkin and Hanson 1954; Highkin 1960; Hillman 1956; Went 1944, 1958). This shows that the photosynthetic apparatus can profit even from subtle changes in light intensity and temperature. 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 *C. rubrum* L. at various locations in North America (Fig. 3.1; Table 3.2). 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 periodic leaf movements (LM) (Bünning 1935, 1977). The

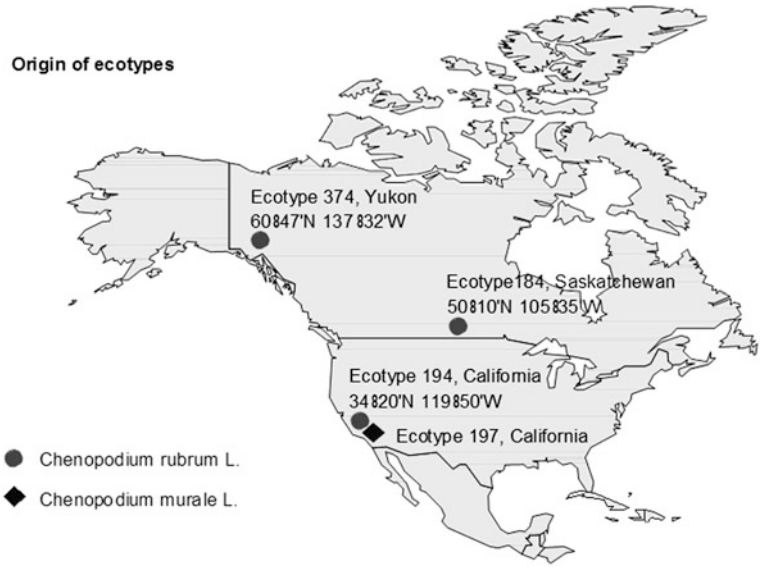

Fig. 3.1 Geographic location of latitudinal ecotypes of *Chenopodium* spp. selected by B.G. Cumming in North America (Cumming 1967b). *C. rubrum* L. is a short-day plant, *C. murale* L. a long-day plant

Table 3.2 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 1967b) 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

genetic determination of period length provides the temporal frame for physiological and behavioral patterns which are necessary for adaptations of organisms and populations to environmental constraints, as evident for three latitudinal ecotypes of *C. rubrum* (Table 3.2; cf. Cumming 1967b; Tsuchiya and Ishiguri 1981).

3.1.2 Metabolic and Genetic Determination of Rhythmic Behavior on the Global Scale—Evolution of the Biosphere

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 characterized 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 (Cyanobacteria, cf. Nakajima et al. 2010; Schirrmeister et al. 2013). Bünning's work paved the way for complex research in circadian and rhythmic processes for many decades.

Until recently, the molecular genetic analysis of CRs in pro- and eukaryotes focused on transcriptional–translational control loops (TTCL) as a basis for mechanisms of 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 (Albrechtová et al. 2006; Bonzon et al. 1981, 1983; Hendricks 1963; Wagner and Cumming 1970; Wagner et al. 1975).

3.1.3 Metabolic Control of Timing—The Importance of Rhythms in Phosphorylation and Redox Potential

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

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). Ultradian oscillations of enzyme activities in plants and animals might be at the basis of circadian time keeping (Morré and Morré 1998).

In view of the redox side of circadian control of energy metabolism, it might be relevant that all ECTO-NOX (ENOX) proteins oscillate and thus might fulfill the role of ultradian oscillators of the biological clock (Wagner et al. 1975; Bonzon et al. 1981, 1983; Merrow and Roenneberg 2001; Morré et al. 1999; Tu et al. 2005; Morré and Morré 2013).

Thus, 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 (Volkov 2006, 2012; Baluška et al. 2006a, b).

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 behavior enables sophisticated adaptation of individual cells in cyanobacteria, whole plants, or populations of plants. 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.

3.1.4 Metabolic and Signaling Networks

Comprehensive studies of redox cycling and adenylate-energy charge in cells and tissues have been performed in human beings and animal models.

Some results of such studies have been described in "Systems Biology of Metabolic and Signaling Networks: Energy, Mass and Information Transfer" (Aon et al. 2014).

The demonstration of a diurnal rhythm in compound surface-membrane potential (Wagner et al. 1998) and evidence of correlation between action potentials and rhythmic LM as well as stem growth of *C. rubrum* (Wagner et al. 2006a, b) 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). 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 macro-parameters, 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.

3.2 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 1967b; Tsuchiya and Ishiguri 1981). The precise regulation in photoperiodism displayed by *Chenopodium*, a plant showing obligate photo-periodic control for its short- and long-day ecotypes, and the availability of such ecotypes for further experimentation have led to extensive studies of rhythmic behavior, flower initiation, and energy metabolism (Table 3.3; Bünning 1977; Wagner et al. 1975).

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. 3.2.

Table 3.3 Period length of organ-specific circadian rhythms and photoperiodic control of flowering in *Chenopodium rubrum* L. (complemented from Wagner et al. 1993, after King 1975)

Leaf	**Shoot Apex**		**Root**
Perception of photoperiod **30 h**	Cell division **20 h**		Exudation **21–24 h**
Level of flowering	**Level of flowering**		
Timing of inductive 12-h dark span	Timing of 6-h pulse to induced plants with or without leaves		
	glucose	0.4 M	
	GA_3	10^{-6} M	
	EtOH	0.1 %	
21–24 h	**21–22 h**		

3.2.1 Rhythmic Changes in Inter-Organ 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 focused on inter-organ communication between the signal-perceiving leaves and the target tissue (stem/apex) implied in the control of flowering.

To study the whole-plant behavior, time-lapse photography was used to investigate the 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 (Lecharny and Wagner 1984; Lecharny et al. 1985; Ruiz-Fernandez and Wagner 1989, 1994), simultaneously analyzing LM via a video system (Fig. 3.2).

An undamped circadian rhythm in the SER was observed in continuous light. Total stem elongation depends on precise cooperation in the stem elongation of the single internodes. In one specific experiment deals with three internodes, the first internode completed growth while the second and third internodes both continued to contribute to total SER (Fig. 3.3).

The fraction of stem elongation resulting from the 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 et al. 1985; Lecharny and Wagner 1984). In the elongating stem, the internodes sustain an undamped circadian rhythm in SER as they move upward.

The cell-surface-continuum as discussed by McKenna et al. (2014) might be the proper “substrate” for this rhythmic behavior.

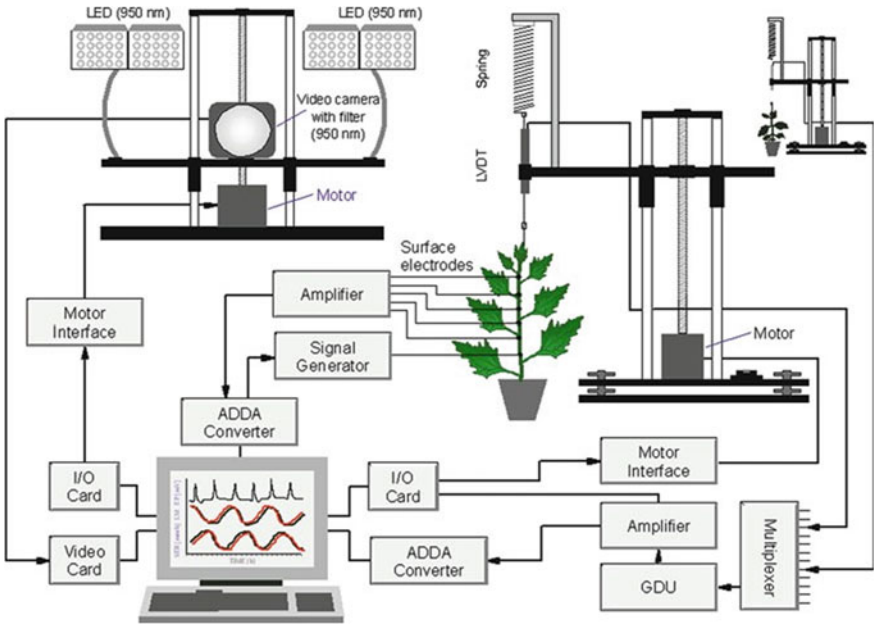

Fig. 3.2 Flow diagram of the experimental setup. The stem elongation measuring and analyzing system is based on linear variable differential transformers (*LVDT*). Twelve individual plants and environmental variables such as light intensity and temperature (device not shown) can be measured simultaneously. The leaf movement measuring and analyzing system is based on video digitizing. Small pieces of aluminum 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 digitized picture as white pixels on a black background, are recorded continuously. To achieve the recordings in light as well as in darkness, the markers are irradiated with LEDs of 950 nm; the camera has a filter passing of 950 nm light only. Abbreviations: *GDU* generator demodulator unit; *I/O* input/output; *D/A* digital/analog; and *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. From (Normann et al. 2007; Wagner et al. 2006a, b)

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 3.4), respectively. The period lengths of LM mirror the kinetics in SER, displaying similar increases of

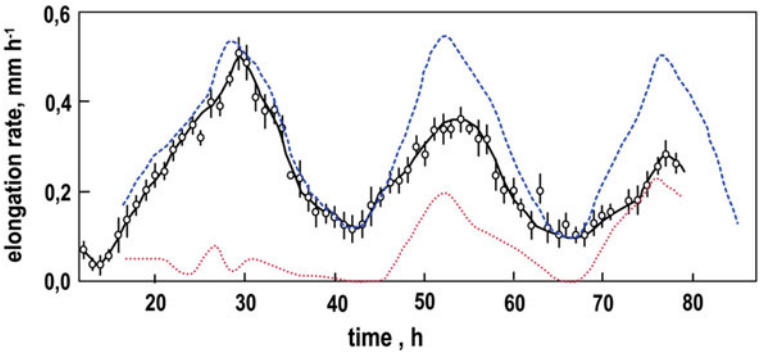

Fig. 3.3 Internode and total stem extension rate of *C. rubrum* seedlings grown in white light. After standard germination conditions, plants were grown for two weeks in white light (40 W m^{-2}) at constant temperature (24 °C). After 4 days in 20 W m^{-2}, they received 8 h darkness followed by the same lighting conditions as prior to darkness. SERs of second (-*o*-) and third (—) internodes are compared to total stem extension rate (…). Mean ± SE of 6 plants (from Lecharny and Wagner 1984)

Table 3.4 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

	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)

n denotes sample size

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. 3.4). Both parameters display clear movement and growth patterns with photoperiod-specific reactions to "light-on" and "light-off" signals. To analyze the patterning quantitatively, the ratios of integral growth in the dark over integral growth in the 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 plants (LDP) could be determined. Flower induction

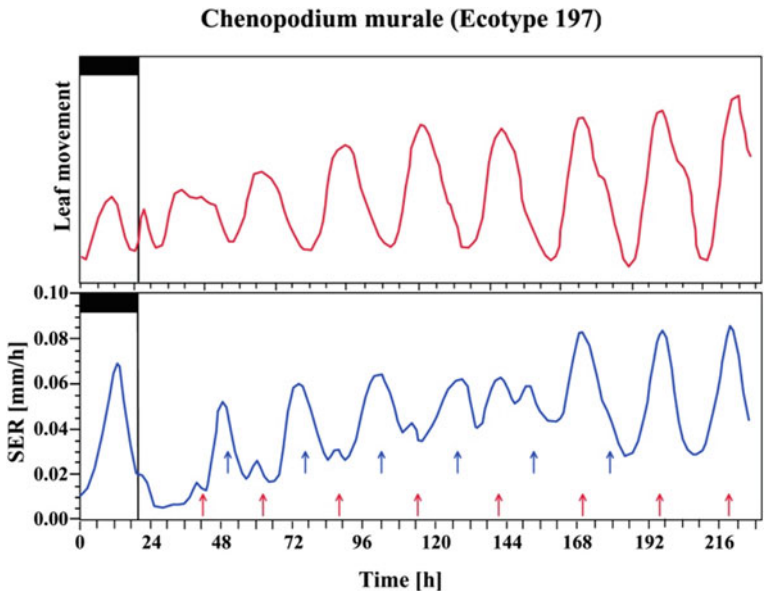

Fig. 3.4 Comparison of leaf movement (*upper graph*) and stem extension rate (*SER*, *lower graph*) of vegetative *Chenopodium murale* being induced to flower during exposure to constant light. The *lower arrow line* in the lower graph indicates a component of the oscillatory pattern which is out of 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)

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. 3.5.

The above observations also hold true for the kinetics of LM. This is hardly surprising, as rhythmic LMs in *Chenopodium* spp. do not depend on differential turgor changes of flexor and tensor cells (Satter and Galston 1973).

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 surfaces 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 using a microdrop recorder (Schwenke and Wagner 1992; Table 3.3). A hydraulic-electrochemical integration seems to be the basis for inter-organ communication in plants, possibly with a feedback from the root apical meristem (RAM) (Schwenke and Wagner 1992; Ohya et al. 2005).

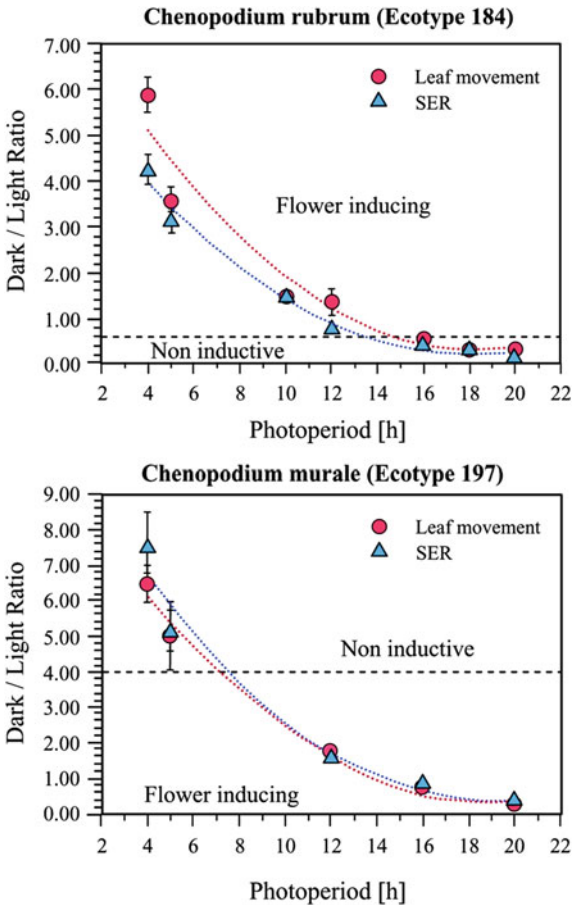

Fig. 3.5 Kinetics of the ratio of integral growth during the dark period over integral growth in the light period for the stem extension rate (*SER*, *triangles*), and the corresponding ratio for leaf movement (*circles*) under various photoperiods. In *C. rubrum* (*upper graph*), flower-inducing photoperiods are correlated with ratios above 0.6 (*dotted line*), whereas, in *C. murale* (*lower graph*), flower-inducing photoperiods are correlated with ratios below a threshold value of 4 (*dotted line*)

3.2.2 Local Hydraulic Signaling: The Shoot Apex in Transition

Photoperiodic flower induction involves a reorganization of organogenesis at the shoot apical meristem (SAM)—from vegetative phyllotaxis to floral development. Floral transition includes molecular signaling 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).

The state of membranes might be modulated by diurnally oscillating phospholipids with respect to the trafficking of flower-inducing factors (Nakamura et al. 2014). Mitochondrial cardiolipin/phospholipid trafficking (Schlattner et al. 2014) or the involvement of sugars by trehalose-6-phosphate signaling (Wahl et al. 2013) could also be involved.

In this way, the "physiological state" of a plant controls signal transduction, and vice versa. Very little is known regarding the 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 analyze 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 signaling network regulates organogenesis, involving molecular, biophysical, and biochemical pathways of signal transduction. During phase change from vegetative to reproductive development, the communication between the leaf, stem, and SAM changes (see Table 3.4). 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 non-inductive for flowering (Fig. 3.5). 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 et al. 2004; Albrechtová and Wagner 2004). The expansion of the meristem results from cell enlargement, rather than changes in cell division and therefore is presumably based on water uptake (Ohya et al. 2005). The phyllotactic 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 AQPs found in other plants (Albrechtová and Wagner 2004). In the leaves and the SAM, its expression differs significantly between vegetative and flowering plants (Albrechtová and Wagner 2004). Involvement of AQPsat 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 (Wahl et al. 2013) 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 SAM. It is anticipated that hydraulic changes at the SAM leading to flower initiation are mediated by specific hydro-electrochemical communication between the leaves, the SAM, and the RAM (Ohya et al. 2005). All organ systems show rhythmic activities with specific phase relationships and are covered by the plant cell wall, i.e., the plasma membrane-cytoskeleton-continuum (McKenna et al. 2014; cf. Table 3.3).

Turgor-dependent volume changes, stretch-activated membrane channels (Kung 2005), and correlated changes in membrane potential might all be the essential parts 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.

3.2.3 Membrane Potential as the Basis for Hydro-electrochemical Signaling, Inter-Organ 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 localized 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 (Kloda and Martinac 2002; Lang and Waldegger 1997).

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 (Wagner et al. 1975) 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, and roots) involves frequency-coded (electric) signals (Wagner et al. 1998). Furthermore, spontaneous compound surface-membrane action potentials were shown to correlate with the turgor-controlled hydraulic growth movements of leaves and SER, which are controlled by the photoperiod. Due to its mechanisms of action, hydraulic and electric signaling is intrinsically coupled (Wagner et al. 2006a, b; McKenna et al. 2014).

3.3 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). CRs in redox and phosphorylation state are considered to be the 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 organization of energy metabolism are very early achievements to avoid oxidative damage during light conditions (Fig. 3.6).

From a detailed analysis of rhythms in enzyme activities involved in compartmental energy metabolism with and without feeding of sugars (cf. Frosch and

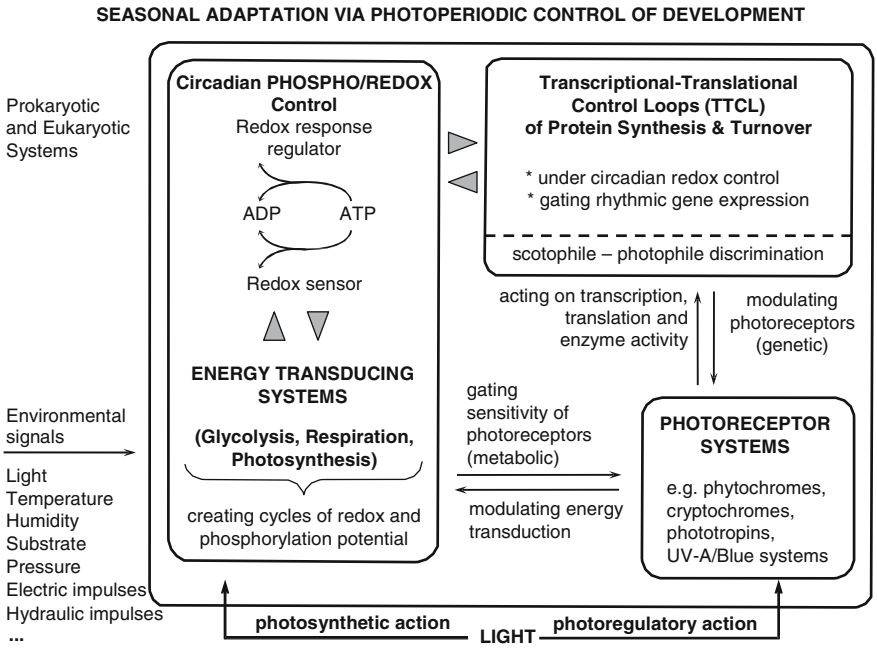

Fig. 3.6 Flow diagram of the postulated regulatory network involved in the generation of circadian regulation of metabolism and behavior at the cellular level. The regulatory loops generate a circadian rhythm in energy transduction (Wagner and Cumming 1970; Wagner et al. 1975), resulting in cycles of 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 3.2), 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

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 with photophosphorylation in the cyanobacteria (Huang et al. 1990) and green plants (Wagner and Cumming 1970; Wagner et al. 1974a, b; Wagner 1976a, b, 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, lead to differential gene activation and expression (Dolmetsch et al. 1998; Li et al. 1998; Love et al. 2004).

Taking into account the symplastic organization 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 organization 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 CRs in growth, like LM (see Satter and Galston 1973) and SERs as universal markers of systemic behavior (Aimi and Shibasaki 1975; Wagner et al. 1998).

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Chapter 4
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 two and a half decades.

4.1 Introduction

4.1.1 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 originated inside the plant and not in the light from the Sun, 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 workings, and increasingly intricate designs were invested in the

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movement-monitoring devices. During the eighteenth and the nineteenth 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 on the one hand (Pfeffer 1877) and the concept of an internal oscillator—an endogenous biological clock—for which the leaf movements serve as the "clock hands". In the twentieth century, biological clock began to be studied also in animals. Beatrice Sweeney presented a detailed and vivid account of this thought evolution (Sweeney 1987).

Among the best studied leaf 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, as well as to map out the signalling paths. They altered the illumination regimes, varied the light intensity and quality and applied various pharmacological agents to the pulvinus (e.g. see the reviews by Satter and Galston 1981; Mayer et al. 1997; 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 just the leaf displacement. The forces involved in the movement have been measured (Gorton 1990; Irving et al. 1997; Koller 2001), immunohistochemistry has been applied (e.g. in the cellular immunogold localization of phytochrome, the photoreceptor affecting leaf movement; Moysset et al. 2001), the related distribution of various ions and other elements was 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), and 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 the movement is executed and how the endogenous rhythm—and external signals, mainly light—affects the pulvinar "motor", have been collected in a small but a thorough compendium on the pulvinus (Satter et al. 1990). During the 25 years that followed, 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 the leaf movements, revised since the former edition (Moran 2007b), are the main focus of this chapter.

4.1.2 The Types of Leaf Movements

Leaf movements can be repetitious and rhythmic (Fig. 4.1a), or provoked (Fig. 4.1b). Stimulated movements can be classified according to their directionality: *tropic* movements are related to the direction of the stimulus that caused them, while *nastic* movements are unrelated. Thus, leaf unfolding in response to the

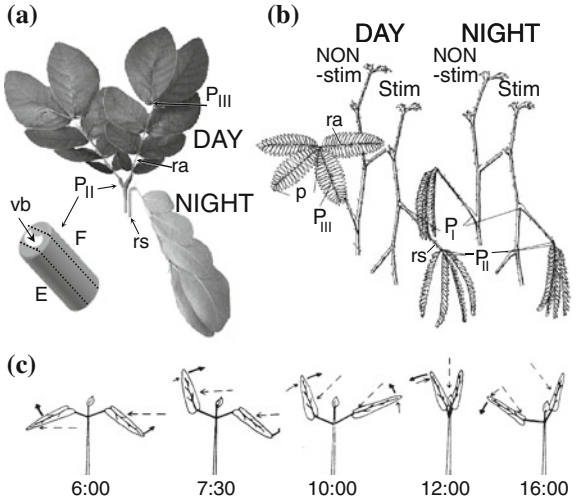

Fig. 4.1 Types of leaf movements. **a** Nyctinastic movement of the terminal pinnae of the compound leaf of *Samanea saman* (Jacq.) Merrill, between open during the DAY and folded during the NIGHT. *Inset:* a schematic drawing of a pulvinus: *E* extensor, *F* flexor, *vb* vascular bundle, P_{II}–P_{III} secondary and tertiary pulvini, *ra* rachilla, *rs* rachis (with permission, Moshelion et al. 2002b). **b** Seismonastic and nyctinastic leaf movement of *Mimosa pudica* L., *p* pinnae. P_I primary pulvinus; other abbreviations as in A (with permission, Fromm and Eschrich 1988a). **c** Primary (laminar) leaves of *Phaseolus vulgaris* L., showing paraheliotropism in the field (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)

turning on of diffuse light is *photo*nastic and leaf folding with the onset of darkness —*scoto*nastic; the turning of leaves towards directed light is termed *photo*tropic and towards the sun—*helio*tropic (Fig. 4.1c). Movement in response to touch–like 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 *M. pudica* leaf upon shaking the plant is *seismo*nastic and upon exposure to the heat of a flame—*thermo*nastic; the turning of leaves upwardly 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, *Mimosa* primary pulvinus exhibits also nyctinasty, seismonasty and thigmonasty, while the secondary pulvinus does not respond to seismonastic stimuli (Fig. 4.1b, Fromm and Eschrich 1988a). *Samanea* leaf movements are largely nyctinastic and insensitive to touch and shaking.

Rhythmic leaf movements can be related to growth and be *non*-reversible, like those of cotyledons of the *Arabidopsis* seedlings or the leaves of the 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 of pulvinar tissues (see below), the irreversibility of these growth processes is thought to be related to the interstitial deposits in cell wall material and decrease in wall extensibility (Wetherell 1990, and references therein).

Rhythmic nyctinastic leaf movements occur in mature, non-growing leaves and are completely reversible, like those found in many of the legumes (*Samanea saman, Accacia lophanta, Albizzia julibrissin, Phaseolus vulgaris, Desmodium gyrans* and the previously mentioned *M. pudica*) and also in some plants of a few other families, such as wood sorrels (*Oxalidaceae*) and mallows (*Malvaceae*).

It is of interest to mention in this context the diurnal movements of flower petals, which underlie the repetitive opening and closing of some flowers and which may persist for several days. This petal movement also involves an "osmotic motor", like in leaves, either in differentially elongating cells in growing petals, or in non-growing petals, in cells which change their size reversibly, by loss of water during the day and refilling during the night (reviewed in great detail by van Doorn and van Meeteren 2003; van Doorn and Kamdee 2014).

The reversible leaf movements originate in the pulvinus (Fig. 4.1a), a mature, specialized motor organ at the leaf base. The identity of the pulvinus as a motor organ is genetically determined, as discovered through pulvini-devoid, non-moving mutants of three orthologs of the same gene: *elongated petiolule1* (*elp1*) of *Medicago truncatula*, *apulvinic* (*apu*) of *Pisum sativum* and *sleepless* (*slp*) of *Lotus japonicus* (Chen et al. 2012; Cortizo and Laufs 2012; Zhou et al. 2012).

The daily persistence of the leaf movements 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 shorter by more than one hour 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 comprise additionally one or more *ultra*dian rhythms (with a period significantly shorter—between tens of minutes to several hours Millet et al. 1988; Engelmann and Antkowiak 1998).

Light has a profound effect on the rhythmic leaf movement, and it is also easily quantifiable. Therefore, it is a most widely used stimulus to perturb (and "entrain") the leaf movement rhythms, to change their phase and to alter their period. Changing these two rhythm properties is a criterion for having affected the internal "oscillator". Red, far-red and blue light (BL) have different effects on the rhythm (reviewed by Satter and Galston 1981; Sweeney 1987; Moran 2007a, b). Curiously, petals of *Calendula arvensis* flowers with diurnal rhythm of opening and closure

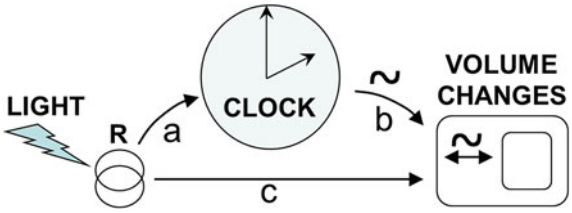

Fig. 4.2 Light stimulates cell volume changes. A model of clock-mediated (circadian) and clock-independent (acute) pathways. **a** Light, perceived by one or more light receptor(s), *R*, affects the clock. **b** The clock governs volume changes, imparting fluctuations (∼) in activity or abundance to the pathway intermediates. **c** Light affects directly the volume changes (a *bidirectional arrow*)

could be entrained by light during the bud stage, but the rhythm phase became fixed when they matured (van Doorn and van Meeteren 2003, and references therein).

Acute versus circadian. It is important to note that the same light 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 ("masking") 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 (Fig. 4.2), the clock-resetting stimulus acts along an input pathway to the clock, altering the 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: (a) 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 (b) 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.

4.2 The Mechanism of Leaf Movement: The Osmotic Motor

4.2.1 Volume Changes

4.2.1.1 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 "flexor", which appears contracting ("flexing") longitudinally at the same time. During leaf closure, the opposite 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, causes the curvature of the pulvinus without affecting its length (Koller and Zamski 2002). It appears that extensors and flexors differ also in the extent of the movement-driving pressures they generate. For example, in *P. vulgaris* laminar pulvinus, the excision of 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 were unchanged, Millet et al. 1989).

4.2.1.2 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 shrank under continuous light for over 200 h with a 28 h period, resembling the period of the original pulvinar cells in situ in similar conditions (Mayer and Fischer 1994). Extensor protoplasts seemed to exhibit the same rhythm, but, curiously, they cycled with the same phase as the flexors, at least during the first 70 h, as if their internal clock shifted by 180^{o} relative to their original in situ rhythm. Notwithstanding, 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).

Protoplasts isolated from *Samanea* flexors also swelled and shrank rhythmically, in continuous dim light, in phase with the in situ intact cells, and both flexor and extensor *Samanea* protoplasts reacted to white light (WL) illumination during the dark period as did the in situ intact cells in the pulvinus (extensors swelled and flexors shrank, Moran et al. 1996).

Thus, the isolated pulvinar protoplasts "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 the isolated pulvinar protoplasts. No less importantly, they also contain the light signal receptors, for both circadian entraining and acute signalling (see also Sect. 4.3.2.1 below).

4.2.2 The Ionic Basis for the Osmotic Motor

4.2.2.1 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 the exception that in contrast to guard cells, in the intact pulvinus 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; noting that plasmodesmata permeability, even to ions, may be regulated, Wigoda et al. 2014).

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 (Kim et al. 1992, 1993; Suh et al. 2000) and the proton-motive force for the uphill uptake of Cl^-, possibly via a proton–anion symporter (Satter et al. 1987). The hyperpolarization also opens the gates of K^+-influx channels. Eventually, K^+ and Cl^- accumulate in the cell vacuole. In the absence of external Cl^-, 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 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.

Unlike in leaf pulvini, during rhythmic flower opening, rather than ions, the osmoticum driving water movements into the flower petals consists mainly of sugars mobilized from stored polysaccharides (starch and/or fructan) and/or imported sucrose. In some cases, the petal movements occur without proton pump involvement (van Doorn and van Meeteren 2003, and references therein), which may be explained by the internal source of the produced osmoticum.

4.2.2.2 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 about 24-h rhythm between −85 and −40 mV (extensor) and between −100 and −35 mV (flexors), with the extensors "sinusoid" preceding that of flexors by about 8 h (Racusen and Satter 1975). Membrane potential varied also in response to light signals which caused leaf movement (see Sect. 4.3.2.1 below, and Racusen and Satter 1975, and

also Sect. 4.3.2.2). 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. 4.2.3.5 below). Membrane potential was also used to learn about the early effects of the hormone salicylic acid (SA) (Saeedi et al. 2013, and Sect. 4.3.4.1 below).

4.2.2.3 Mechanisms Underlying Volume Changes

Ions involved in leaf movements. Results of X-ray microanalysis in pulvini suggested 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 allowed 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; 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 *M. 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 (Fromm and Eschrich 1988b). During reswelling, the extensors accumulated the labelled material (Fromm and Eschrich 1988a).

Could cytoskeletal elements—actin filaments, microtubuli—perform actively the *fast* shrinking (as suggested already by Toriyama and Jaffe 1972)? While 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, indicating, additionally, the involvement of its phosphorylation by a tyrosine kinase (distinct from a serine/threonine kinase, Kanzawa et al. 2006). Interestingly, depolymerized actin in guard cells was required for stomatal opening and for the activity of K^+-influx channels, and this was independent of the activity of the H^+-ATPase (Hwang et al. 1997; Eun and Lee 2000), suggesting that actin may perhaps be involved not only in the "dramatic" movements of the pulvinus, but also in the regulation of its "mundane", nastic movement.

4.2.3 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 little information about the tonoplast transporters of the pulvinar motor cells. Somewhat more detailed are the observations about the function in situ of a few plasma membrane transporters in the pulvini. Their partial characterization is described below.

4.2.3.1 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). 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). Red light or dark, following BL, activated the H^+ pump in flexors (acidifying the flexor apoplast) 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 like 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).

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

4.2.3.2 H^+/Cl^- Symporter

The presence of an H^+/anion symporter has been suggested based on the 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).

4.2.3.3 K^+-Release Channels

These channels are presumed to mediate K^+ efflux from pulvinar motor cell during their shrinking. Patch-clamp studies revealed depolarization-dependent, K^+-release (depolarization-dependent, 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 that for Rb^+ and much higher than that 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 channel gating, but the *overall* effect of cytosolic Ca^{2+} on these channels was rather minor (Moshelion and Moran 2000). In 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 interpreted as increasing cytosolic Ca^{2+} (cytosolic Ca^{2+} concentration was not measured in these experiments, Stoeckel and Takeda 1995). Also, in difference to *Samanea* K_D channels, they were not blocked by external La^{3+} and Gd^{3+} at a concentration 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 min illumination with BL and a *decrease* in their activity within a few minutes of darkness, preceded by a brief red-light pulse (Fig. 4.3, Suh et al. 2000). No circadian control, however, was evident in the responsiveness of the flexor K_D channels to BL. The authors resolved the blue-light effect into two: (a) membrane depolarization-dependent K_D channel activation (a consequence of a blue-light-induced arrest of the proton pump) and (b) 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, TXXTT/VGYG, the *Samanea* predicted protein sequence of SPORK1 is similar to SKOR and GORK, the only *Arabidopsis* outward-rectifying *Shaker*-like K channels. *SPORK*1 was expressed in all parts of the pulvinus and in the leaf blades (mainly mesophyll; Fig. 4.1), as demonstrated in Northern blots of total mRNA. *SPORK*1 expression was regulated diurnally and also in a circadian manner in extensor and flexor, but not in the vascular bundle (rachis) or in the leaflet blades (Moshelion et al. 2002b). While 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.

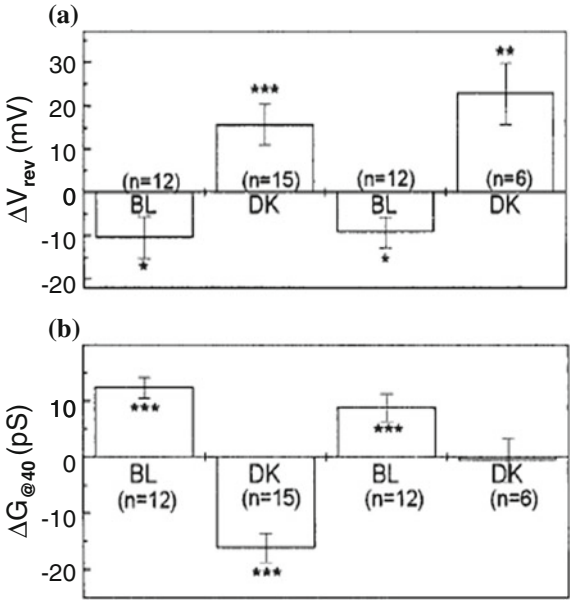

Fig. 4.3 Blue light enhances the activity of the K_D *Samanea* channels in flexor protoplasts. **a** Light-induced shift of the membrane potential, manifested as shifts of the reversal potential, V_{rev} of K_D-channel currents in single cell-attached membrane patches during alternating between blue 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* is the 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 a 40 mV depolarization relative to the V_{rev} of the patch (mean ± SE). The *asterisks* and *n*, as in (**a**) (with permission, Suh et al. 2000)

4.2.3.4 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 K^+ influx during cell swelling which is concurrent with external acidification. This was particularly surprising in view of the external acidification-*promoted* K^+-influx channels in guard cells (Blatt 1992; Ilan et al. 1996). Yu et al. (2001) were able to resolve this paradox by quantitative comparisons of the actual versus 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 detect specifically 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 "lights-on" signal during early morning hours and in flexors anticipating a "lights-off" signal 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 2nd half of the night of a normal day cycle, BL 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), BL 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 were *SPICK*1 and *SPICK*2, and their predicted protein sequences were homologous to AKT2, a weakly inward-rectifying *Shaker*-like *Arabidopsis* K channel. *KAT*1 and *KAT*2, 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 was regulated diurnally (SPICK2 in extensor and flexor, SPICK1 in extensor and rachis) and their expression in the extensor and flexor was 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.

4.2.3.5 Ca^{2+} Channels

A K_D channel rundown (gradual loss of activity) by increased hyperpolarization was used as an indicator—as an 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, but see a comment in Sect. 4.2.3.3 above).

4.2.3.6 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 *P. vulgaris* (Iino et al. 2001) are not conclusive, as NPPB (an inhibitor used in the above study) has been shown also to inhibit plant K^+-release channels with an even higher affinity (Garrill et al. 1996).

4.2.3.7 Mechanical Stretch-Activated Channels

Stretch-activated channels (SACs) were detected by patch clamp in cell membranes in virtually all cell types assayed, including prokaryotes (see, for example, references mentioned 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.

4.2.3.8 Water Channels (Aquaporins)

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 in 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 (e.g. 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 homolog 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 *SsAQP*2-expressing oocytes than in *SsAQP*1-expressing oocytes, and *SsAQP*1 was found to be glycerol permeable. In the oocytes, *SsAQP*2 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 *SsAQP*2 found in pulvini. The transcript levels of both aquaporins 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, like 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.

4.2.4 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; Campbell and Garber 1980). However, very little is known about vacuolar transporters in pulvini.

4.2.4.1 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 was accompanied by a more than threefold increase in the abundance of the H^+-ATPase per length unit of membrane (Fleurat-Lessard et al. 1997).

4.2.4.2 Ion Channels

*SPOCK*1, a homologue of the Arabidopsis *KCO*1 (two-pore-in-tandem K-signature channel) cloned from the *Samanea* cDNA pulvinar library (Moshelion et al. 2002b), likely represents, like the Arabidopsis TPK1 (formerly KCO1, Czempinski et al. 2002), a K^+ selective voltage-*in*dependent VK vacuolar channel (Bihler et al. 2005). *SPOCK*1 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 (and not in the rachis or the leaflet blades, Moshelion et al. 2002b). While the vacuole reservoir of ions most likely participates in the pulvinar cell volume changes, SPOCK1 is a likely K^+-release pathway across the tonoplast during the diurnal leaf movements of *Samanea*.

4.2.4.3 Aquaporins

γ-tonoplast intrinsic protein (TIP) 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 the abundance of the aquaporin (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 aquaporins gene, *TIP*1;1, was cloned from *Mimosa* cDNA library, and its product, expressed in a 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.

4.3 Mechanisms of Regulation

Membrane transporters are the end point in the signalling cascades regulating pulvinus movement. This regulation is rather complex (Fig. 4.4) and includes a large number of factors, such as light, circadian clock, hormones, and temperature. Such regulation occurs at both transcriptional and post-translational levels (Fig. 4.4).

4.3.1 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. Notwithstanding, this may be also one of the ways the clock affects transporters, for example *gating* their responsiveness to acute stimuli (see 4.2.3.4 above).

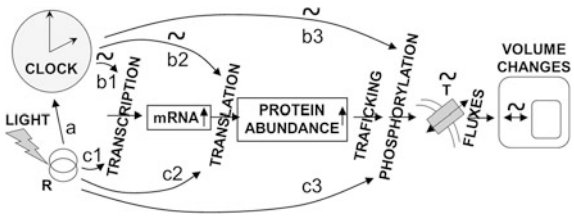

Fig. 4.4 Regulation of membrane transporters in the pulvinus at the levels of transcription, translation and protein modification (a schematic model). *bn* are clock output signalling pathways, and *cn* are signalling pathways from the light-activated receptor, *R*. *T* is the transporter protein in the membrane. The processes affected are indicated. The other signs are as in Fig. 4.2. Feedback loops are not indicated

4.3.1.1 Phosphorylation of the Proton Pump

The recently discovered immunologically undistinguishable three iso-phototropins of the *P. vulgaris* pulvinus (see 4.3.2.2 below, and Inoue et al. 2005) were identified as the first element in the phototransduction cascade in a shrinking pulvinar motor cell (Fig. 4.5). 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 BL induced the phosphorylation of the phototropins and the dephosphorylation of the H^+-ATPase. Three results 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. 4.5a); the phosphorylation and dephosphorylation exhibited similar fluence rate dependencies on BL (Fig. 4.5b); and 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 the H^+-ATPase *de*phosphorylation (Fig. 4.5c–f). This indicated that H^+-ATPase *de*phosphorylation is 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 just *the opposite* from what occurred 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 opposite reactions of the 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 brief, transient increase in activity, Okazaki et al. 1995) and activation of H^+ secretion in *Samanea* extensors (like in guard cells, Shimazaki et al. 1985). Thus, it may be concluded that the whole pulvinus of *Phaseolus* reacts to BL like the *Samanea* flexor.

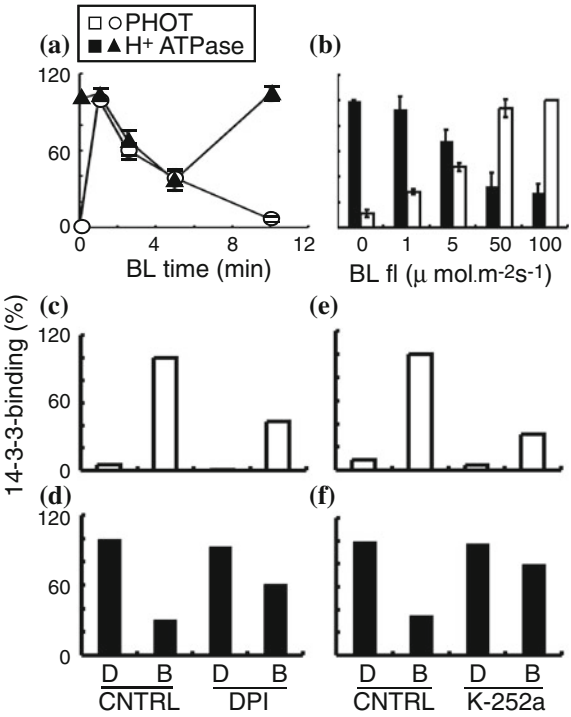

Fig. 4.5 Pulvinar phototropins mediate the dephosphorylation of the plasmalemmal H^+ATPase by blue light. **a** Time courses of recombinant 14-3-3 protein binding to phototropin and to H^+-ATPase (as a measure of their phosphorylation status) in pulvinar microsomal membranes in response to a blue-light pulse (30 s at 100 μmol m^{-2} s^{-1}; mean ± SE, n = 3). **b** The dependencies of 14-3-3 protein binding to the H^+-ATPase and to phototropin on blue-light fluence rate (a representative of 3 similar experiments). **c–d** The effect of 1-h preincubation of excised pulvini in flavoprotein inhibitor, *DPI* (100 μM) in the *dark*. **e–f** The effect of similar pretreatment with Ser/Thr protein kinase inhibitors, K-252a (10 μM) (with permission, Inoue et al. 2005)

4.3.1.2 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 BL implicated a voltage-independent component, which could be a phosphorylation (see 4.2.3.3 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 hydrolyzable analog, 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-hydrolyzable ATP analogue, AMP-PNP (5′-Adenylylimidodiphosphate), did not substitute for ATP. H7 (1-(5-IsoquinolinesulphonyI)-2-methylpiperazine), a

broad-range kinase inhibitor, blocked reversibly 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 suggested that the activation of the outward-rectifying K channels by depolarization depended 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. A support for the latter notion originated in a study, where the addition of $PtdInsP_2$ (phosphatidylinositol(4,5)bisphosphate) replaced MgATP in restoring the "rundown" 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 cells protoplasts (i.e. the activity of their K_H channels) was assayed in whole-cell patch-clamp assays (see 4.2.3.4 above). The promotion of phosphorylation was achieved using okadaic acid, OA, an inhibitor of protein phosphatase type 1 and 2A. High levels of phosphorylation (300 nM of OA) inhibited K_H channel activity, while low levels of phosphorylation (5 nM of OA) promoted channel activity in flexors, but did not affect them in extensors (Yu et al. 2006). This difference between flexor and extensor in the susceptibility of their K_H channels 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 4.2.3.4), raised in cultured insect cells (Sf9), was phosphorylated in vitro by the catalytic subunit of the broad-range cyclic-AMP (cAMP)-dependent protein kinase (PKA, Yu et al. 2006). While 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.

4.3.1.3 Phosphorylation of Water Channels

The water permeability of frog oocytes expressing solely MpPIP2;1, one of the two *Mimosa* plasma membrane aquaporins (see 4.2.3.8), 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).

4.3.2 The Perception of Light

Plant photoreception has been reviewed recently (e.g. Wang et al. 2014; Christie et al. 2015; Wang and Wang 2015). Our focus here is on photoreception related to leaf movement. How are the different light stimuli perceived in the pulvinus? Are the acute and clock signals (Fig. 4.2) perceived via different receptors? What are they? Physiological experiments delineated broad classes of receptors and biochemical–molecular tools are just beginning to be applied in this area.

4.3.2.1 Phytochrome

Phytochrome-mediated responses. A hallmark for a phytochrome-perceived red-light (and sometimes, blue-light) signal is its reversal by far-red light. Light triggers phytochrome holoproteins to interconvert between the R-absorbing form (Pr) and the FR-absorbing form (Pfr), which represents the biologically inactive and active form, respectively (Wang and Wang 2015, and references therein).

Phytochrome mediates 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). At the same time, 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. Pfr) appeared to be unnecessary for darkness to initiate shrinking (Kim et al. 1992, 1993).

In the pulvinar protoplasts of *P. vulgaris*, the Pfr form of the phytochrome had to be present for the shrinking response to be induced by the BL. Far-red light abolished the BL responsiveness, but 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 4.2.2.2).

Molecular identity. Phytochrome is a multi-gene protein (in *Arabidopsis,* it is denoted PHYA through PHYE) with a linear tetrapyrrole 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) was detected by immunoblotting pulvinar sections using an antibody to mustard (*Sinapis alba* L.) PHYA (CP2/9). In 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 this latter notion is the low-fluence irradiance, at the range of 1–1000 μmol m^{-2} s 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, but 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 or 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).

4.3.2.2 Blue Light Photoreceptor

Blue light-mediated responses. BL, perceived by an unknown photoreceptor in pulvini, can also shift the rhythm of leaf movement, although this requires hours-long illumination. 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).

Unlike in *Samanea*, in *P. vulgaris* BL 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. 4.1c). In accordance with this, in protoplasts isolated from the *Phaseolus* laminar pulvinus, BL evoked shrinking, without distinction between the extensor and flexor cells, but it required the presence of pfr of phytochrome (i.e. red-light preillumination, Wang et al. 2001).

The action spectrum of the depolarization recorded in the *Phaseolus* laminar pulvinus (concomitant with initiation of the shrinking signalling) peaked at 460 nm with lower peaks at 380–420 nm. Almost no sensitivity was observed at wavelengths shorter than 360 nm and longer than 520 nm (Nishizaki et al. 1997).

This earlier study failed to notice any red- and far-red light effects on the depolarization of the motor cell, thus excluding phytochrome participation in this movement.

A similar action spectrum was found for both diaheliotropic and paraheliotropic movements of greenhouse-grown soya bean (*Glycine max)* seedlings. The action spectrum of the movements of the pulvini of the unifoliolate leaves—performed with interference filters—peaked between 410 and 440 nm and between 470 and 490 nm (Donahue and Berg 1990). Indeed, BL was found necessary for these movements. Thus, spectroscopic studies suggested that the pulvinar BL receptor is similar to the receptor involved in the general phototropic responses (reviewed by Briggs and Christie 2002; Christie et al. 2015).

Molecular identity. Three genes of phototropins, *PvPHOT*1*a*, *PvPHOT*1*b* and *PvPHOT*2, have been cloned from the bean pulvinus, and their protein products were demonstrated to be the pulvinar BL receptor(s) for the acute responses (Inoue et al. 2005). Their *Arabidopsis* homologs, 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 appear 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 as follows: 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*. There, 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 BL 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 be still phase shifted by (unspecified, but apparently white) light; i.e., while nearly "blind" for developmental responses, the quadruple mutant perceived a light cue for entraining the circadian clock (Yanovsky et al. 2000).

4.3.3 Intermediate Steps

The most established second messenger in plant signalling is cytosolic Ca^{2+} (Hetherington and Brownlee 2004), which has also been the central focus in the in most of the studies of signalling in the pulvinus, with attempts to confirm it as a part of the phosphatidylinositol (PtdIns) signalling pathway. The possible target effectors of Ca^{2+} may be calmodulin (CAM), actin and annexins; these have just begun to be examined in pulvini.

4.3.3.1 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 *P. vulgaris* primary pulvinus suppressed its circadian movements (strongly depending on the phase of application, Kayali et al. 1997). Various CAM antagonists (chlorpromazine (CPZ), trifluoperazine (TFP), calmidazolium and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), but not W5, the inactive analog 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 pseudoacacia* 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 two-hour pulses of BL, but only TMB-8 produced a PRC almost identical to the BL 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, two-hour 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. opposite to that of BL (Gomez and Simon 1995). Ca^{2+} signalling is also implicated in temperature sensing (see 4.3.4.4 below), and it could provide the means of convergence of various signals perceived via different receptors.

Pharmacological alteration of acute responses. Acute effects of red light and BL 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 *P. coccineus* during their swelling and shrinking in a regime of 9 h light to 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 D, 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 of "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, while Ca^{2+} itself is necessary, it is not sufficient for shrinking, and "light off" signal provides this additional required element (Mayer et al. 1997).

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 *M. 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 thus for a mobilization from intracellular stores (Moyen et al. 1995). The BL-induced movement of the primary pulvinus of *Mimosa* is similar to the seismonastic response in 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 deetiolated *Arabidopsis* seedlings. There, 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 BL (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}1), phot2 alone induced phospholipase C (PLC)-mediated phosphoinositide signalling (Harada et al. 2003).

Circadian Ca^{2+} oscillations seem almost 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, in plants, they have been documented so far only in tobacco (*N. plumbaginifolia*) and in *Arabidopsis* seedlings (Fig. 4.6, 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); thus, this is not obvious 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).

4.3.3.2 PhosphatidylInositides (PIs)

Although the animal paradigm cannot be applied uncritically to plants, the general scheme for signal propagation via the PI pathway has 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 (only phosphatidylinositol *tris*phosphate is not produced in plants). The changes in free cytosolic Ca^{2+} concentration, when attributed to mobilization from internal stores, suggested the activation of the PtdIns pathway, in particular the hydrolysis of $PtdInsP_2$ by PLC into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$). This was 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 $InsP_3$ in motor cells of leaf-moving organs (Morse et al. 1987; Kim et al. 1996; Mayer et al. 1997).

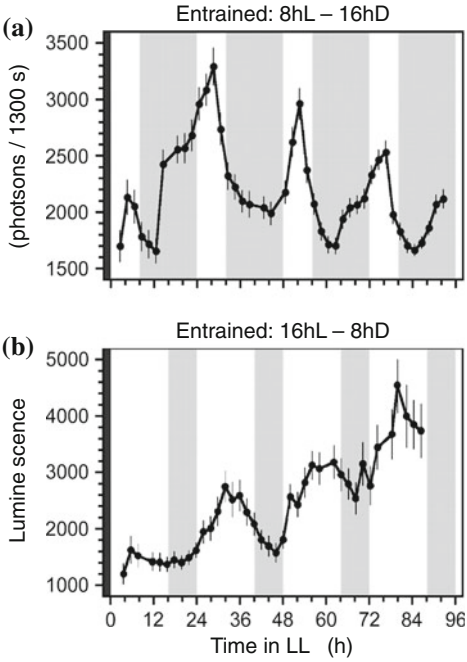

Fig. 4.6 Circadian oscillations of $[Ca^{2+}]_{cyt}$ in *Arabidopsis* seedlings entrained to different photoperiods. Aequorin luminescence emitted by seedlings kept under 110 μmol m^{-2} s^{-1} constant light (LL). Shown are measurements from seedlings entrained in 8L/16D (**a**) and 16L/8D (**b**) for 11 d before LL. During the entrainment light period, the photon flux density was 60 μmol m^{-2} s^{-1}. *Points* represent the mean bioluminescence of 12 seedling clusters ± SE. *Open areas* indicate the subjective day, and *shaded areas* indicate the subjective night (with permission, Love et al. 2004)

In addition to affecting the activity of PLC, light could affect other enzymes. For example, in etiolated sunflower hypocotyls, light transiently down-regulated the activity of PIP5-kinase consequently down-regulating the level of its product, $PtdInsP_2$ (Memon and Boss 1990). In protoplasts isolated from BY2 cultured tobacco cells with genetically and pharmacologically manipulated $PtdInsP_2$ and $InsP_3$ levels, the activity of the K^+-release channel NtORK1 was inversely correlated with the $PtdInsP_2$ level (Ma et al. 2009). Remarkably, in those protoplasts, in which the expression of the human type I PIP 5-kinase increased the levels of $PtdInsP_2$ and $InsP_3PIP$, the osmotic water permeability of the plasma membrane was a few fold 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 an increased activity of aquaporins, due to the elevated levels of inositol phospholipids (Ma et al. 2014).

PIs in the leaf-moving motor cells. 15 s of WL illumination to the *Samanea* 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 BL, to the pulvinar flexors, during the 2nd part of the night) increased $InsP_3$. 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. 4.7 and Kim et al. 1996; 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 $InsP_3$ levels (Kim et al. 1993, 1996). The authors concluded from these results that a PLC-catalysed hydrolysis of phosphoinositides, possibly activated by a G protein, was an early step in the signal transduction pathway by which BL and darkness closed K^+-influx channels in (the appropriate) *Samanea* pulvinar cells (Kim et al. 1996).

4.3.3.3 Annexins

Annexins are Ca^{2+}-, phospholipid- and protein-binding proteins, conserved evolutionarily between animals and plants, with increasingly broad range of revealed signalling functions, including extracellular reception (Gerke and Moss 2002;

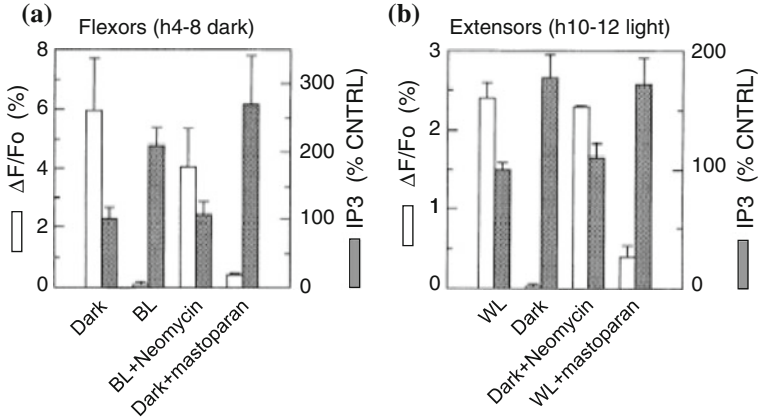

Fig. 4.7 Inositol 4,5 trisphosphate ($InsP_3$) and K^+ permeability of protoplasts in response to shrinking signals. Protoplasts were isolated during light period and transferred to growth chamber at time of normal "light off" (means ± SD of 3 or 4 separate experiments, each done in duplicate). **a** flexor responses to blue light (BL) at hours 4–8 of *dark* period. Cells were treated with indicated agent, and change in the fluorescence of membrane potential indicator dye 3,3′-dipropylthiodicarboxycyanide iodide was measured as a function of time after addition of 200 mM K^+. $\Delta F/F_0$ is the values of fluorescence changes (relative to baseline) observed 30 s after addition of K^+. An increase in $\Delta F/F_0$ indicates that K^+ can enter the cell and depolarize membrane potential; K^+ channels are presumed to be open. No change in $\Delta F/F_0$ indicates that K^+ cannot enter cell; channels are presumed to be closed. $InsP_3$ levels were assayed in extracts prepared from similarly treated protoplasts. Values are presented as percent of untreated controls and represent $InsP_3$ 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, and not blue light, served as a signal. WL, white light (with permission, Moran et al. 1996)

Cantero et al. 2006), and including nucleotide-induced oligo- (possibly tri-) merization of annexin 6 to form active ion channels (of unspecified selectivity Kirilenko et al. 2006). In plants, annexins were predicted to form hyperpolarization-activated Ca channels (Hofmann et al. 2000; White et al. 2002).

Annexins may also mediate Ca^{2+} effects. Eight annexin genes have been found in *Arabidopsis* (Cantero et al. 2006). Recently, annexin 1 of *Arabidopsis* has been suggested to be the ROS-activated Ca^{2+} channel of the plasma membrane. *Arabidopsis* annexin 1 mediates a plasma membrane calcium-permeable conductance in roots that is activated by reactive oxygen species. Recombinant annexin 1 forms a very similar conductance in planar lipid bilayers, indicating that this protein could facilitate directly the in vivo conductance (Davies 2014).

Annexin protein isolated from *Mimosa* was found to bind in vitro to a phospholipid and to F-actin in the presence of calcium, and its amount was 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 or mechanical stimuli, also know to involve Ca^{2+} signaling). Annexin amount increased also at night, and its 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 rather associated with nyctinastic transitions (but see also Sect. 4.2.2.3).

4.3.4 Regulation by Other Effectors

4.3.4.1 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, and pharmacological agents aimed to increase the cytoplasmic Ca^{2+} concentration promoted this opening, and those aimed to decrease it, or to decrease its effect, inhibited (although verapamil and nifedipine, common Ca^{2+} channel blockers, were ineffective, Bourbouloux et al. 1992).

IAA and ABA applied to protoplasts isolated from the laminar pulvinus of *P. 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 shrank 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), in accord with the accepted view of the "osmotic motor". No receptors for the hormone function are known in pulvini.

Salicylic acid (O-hydroxy benzoic acid, SA), applied in solution to the excised primary pulvinus of *M. pudica* (at 0.1–1 mM final concentration), triggered a hyperpolarization of the cell membrane in a concentration-dependent manner, as recorded by a microelectrode impaled into the abaxial (extensor) part of the pulvinus (Saeedi et al. 2013). At 1 mM, the hyperpolarization occured in 15 ± 5 s (!), peaked at 15 ± 5 min and lasted approximately 90 min. Benzoic acid, the SA direct biosynthetic precursor, gave the same general result, though with roughly half the amplitude. In contrast, other benzoic acid derivatives induced depolarization and the SA immediate breakdown product did not have any effect on the membrane potential (Saeedi et al. 2013). The observed hyperpolarization could be most simply interpreted here either as due to SA-stimulated activity of the plasma membrane proton pump, or due to the activation of K^+-release channels (both would be mutually exclusive in a motor cell). Since the primary pulvinus of *Mimosa* reacted to SA application by bending, like in a seismonastic response (*ibid.*), which would be underlain by extensor shrinking, the latter is more plausible. Thus, could SA have activated K^+-release channels directly?

Contrasting with this, very rapid and quite specific (albeit not very sensitive) response to SA, was a slower response (on the order of hours) of external alkalinization, monitored in a bath with excised and sliced pulvini, which can be interpreted as cessation of proton pump activity (*ibid.*). Both responses would be consistent with SA inducing leaf folding.

In contrast to the pulvinus bending in *Mimosa*, in *Cassia fasciculata* pulvini, benzoic acid, SA and other benzoic acid derivatives promoted light-induced opening and inhibited dark-induced closure (Saeedi and Roblin 1987). Are these effects plant dependent or conditions dependent?

Nitrous oxide. The involvement of nitrous oxide (NO—the gaseous signalling molecule with well-established physiology in animals) in nyctinastic leaf closure (closure upon the regular light-to-dark transition) was explored in the classic system of leaflets with tertiary pulvini of *Albizzia lophanta* floating on various experimental solution combined with systematic pharmacological treatment based on the animal system paradigm (Bergareche et al. 2014). NO levels were manipulated by applying, in the experimental solutions, NO donors, NO scavengers and an inhibitor of NO synthase, and an inhibitor of a plant-specific pathway of NO production via nitrate reductase (*ibid.*). NO partially inhibited leaflet closing. Endogenous NO production in the leaflets (though not necessarily in the pulvini) was demonstrated as the appearance of nitrate + nitrite in the solution. These experiments suggested that the unperturbed nyctinastic leaflet closure achieves a smaller angle due to endogenous NO (*ibid.*). In addition, cGMP levels (in leaflets, not necessarily in pulvini) were manipulated (also as in an animal paradigm) by applying exogenously cGMP (in a form of its membrane-permeating derivative, 8-bromo-cGMP), or increasing endogenous cGMP by inhibition of phosphodiesterase-5, or decreasing endogenous cGMP by inhibiting guanylate cyclase. This led to the conclusion that cGMP also inhibits leaflet closure (*ibid.*). Inhibiting leaflet closure means inhibiting

extensor shrinking (or promoting extensor swelling) and /or inhibiting flexor swelling (or promoting flexor shrinking). Which of these were effected by NO and/or cGMP? How were NO and cGMP linked?

In guard cells, which, by their light responses, are likened to extensors (Moran 2007a), adding 8-bromo-cGMP (an equivalent of cGMP) initiated swelling (and stomata opening), indeed, like in the extensors of *Albizzia lophanta*. In contrast to Albizzia, in guard cells, the effect of NO (normally induced by ABA stimulation) is to produce 8-nitro-cGMP and further to elevate cytosolic Ca^{2+}, thereby causing cell shrinking (i.e. stomata closure, Joudoi et al. 2013). In *Albizzia lophanta* leaflets, Ca^{2+} also enhanced leaflet closure, i.e. enhanced extensor shrinking, which suggests that Ca^{2+} does *not* participate in NO *inhibition* of leaflet closure (Bergareche et al. 2014). Could NO inhibit leaflet closure by a two-prong effect: causing swelling of the extensor via cGMP without Ca^{2+} and causing flexor shrinking (concomitant with extensor swelling) via 8-nitro-cGMP and elevation of cytosolic Ca^{2+} levels in flexors?

4.3.4.2 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 *M. pudica*, and in a couple of plants with thigmonastic movements (as reviewed by Schildknecht and Meier-Augenstein 1990). Furthermore, since only one out of two PLMF 1 enantiomers was active, a reaction with a specific receptor has been proposed (Kallas et al. 1990). The colocalization 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 in support of the hypothesis that PLMF-1 may be acting as a chemical signal during the seismonastic response of *Mimosa* (Varin et al. 1997).

4.3.4.3 A New Jasmonate Cell-Shrinking Signalling Pathway?

In *Albizzia julibrissin* leaves, Ueda's group identified a naturally occurring bioactive metabolite of a jasmonic acid, 12-O-β-d-Glucopyranosyljasmonic acid (12-O-Glc-JA), which, when applied in a transpiration stream to detached *Albizzia* or *Samanea saman* leaves at a concentration of about 10 μM, induced nyctinastic leaf folding and induced also shrinking of isolated *Samanea* pulvinar extensor protoplasts (Nakamura et al. 2011, and references therein). Interestingly, the non-glycosylated 12-hydroxyjasmonic acid (12-OH-JA, isolated initially from potato leaves, *ibid.*) did not evoke any of the typical jasmonate responses, such as tendril coiling, or activation of a variety of genes in several plants (*ibid.*), but both the aglucon 12-OH-JA and its glucoside 12-O-Glc-JA acted in the same manner as the tuber-inducing factor in potato. Enantiomeric probes synthesized from the

glucoside helped discover in the *Samanea* extensor a 38 kD unidentified membrane protein, which bound selectively only one enantiomer of one of the probes and which the authors named "a membrane target protein of jasmonate glucoside (MTJG)". None of the proteins known to participate in the "typical" jasmonic acid signalling in plants bound to these probes. Based on this, and, in particular, on the specificity for one enantiomer, the authors suggested that (12-O-Glc-JA) is a signalling molecule, not just a jasmonate degradation metabolite, and that the MTJG is a receptor mediating the extensor shrinking and leaf closure within a hitherto undescribed jasmonate signalling pathway (Nakamura et al. 2011, and references therein).

4.3.4.4 A "Leaf-Opening Factor"

Isolespedezate (named after its initial source, the "Chinese bushclover", *Lespedeza cuneate*), has been identified in *Cassia obtusifolia*, another leaf-moving legume. When fed via the petiole, the "leaf-opening factor" was also effective at concentrations 1–10 μM, but only in plants within the genus *Cassia*. A specific binding of its bioactive derivative identified a cytosolic target protein in Cassia, MetE (a 5-methyltetrahydropteroyl-triglutamate-homocysteinemethyltransferase, a cobalamine-independent methyltransferase participating in the final step of methionine biosynthesis; Ueda et al. 2011). The relevance of these findings to the in situ signalling pathways regulating the osmotic machinery in leaves awaits further corroboration.

4.3.4.5 Temperature

One of the hallmarks of the circadian clock is "temperature compensation", i.e. the period and phase remain constant over a rather broad range of temperatures (McClung and Davis 2010; Franklin et al. 2014, and references therein; even if the extent of compensation varies somewhat in different accessions of *Arabidopsis* Kusakina et al. 2014). This compensation is manifested also in the overt leaf rhythm (Kusakina et al. 2014). The underlying mechanism for this stability versus temperature operates in spite of the strong dependence of the circadian periods on the turnover of the clock mRNA or clock protein, which, by themselves, are strongly temperature dependent; without compensation, a more rapid turnover of clock mRNAs or clock proteins would result in short periods, and a slower turnover—in longer period lengths (Ruoff et al. 1997; Kusakina et al. 2014).

The clock may be entrained separately by temperature pulses, just as by light, since their input pathways to the clock are different. For example, a mutation rendering the plant irresponsive to light entrainment preserved its responsiveness to temperature entrainment (Salome et al. 2002). How are the temperature pulses perceived? The enigma of the plant receptor for temperature entrainment is beginning to unravel: in resemblance to the heat- or cold-sensing TRP channels in mammals (Voets et al. 2004), cyclic nucleotide-gated channels (CNGCs which

conduct calcium) and Ca^{2+} signalling have been implicated in higher temperature sensing in *Arabidopsis* (CNGC2) and in the moss *Physcomitrella patens* (CNGCb and CNGCd) (Finka et al. 2012; Finka and Goloubinoff 2013).

The mechanism of the circadian clock temperature compensation includes counterbalance between the "morning loop" and the "evening loop" of the clock, converging with inputs from red- and blue-light receptors (reviewed by Franklin et al. 2014). This convergence (could it be at the level of Ca^{2+} signalling? see Sect. 4.3.3.1 above) may underlie the already known phenomenon, whereby elevated temperature is "interpreted by the clock" as daylight signals and cold temperature—as darkness signals (Franklin et al. 2014, and references therein).

4.4 Unanswered Questions

The light-signalling transduction steps converging on the "osmotic motor" are outlined schematically in Fig. 4.8. The information about pulvini is scarce. In fact, most of the questions below have not yet been answered, or answered adequately, in any plant system.

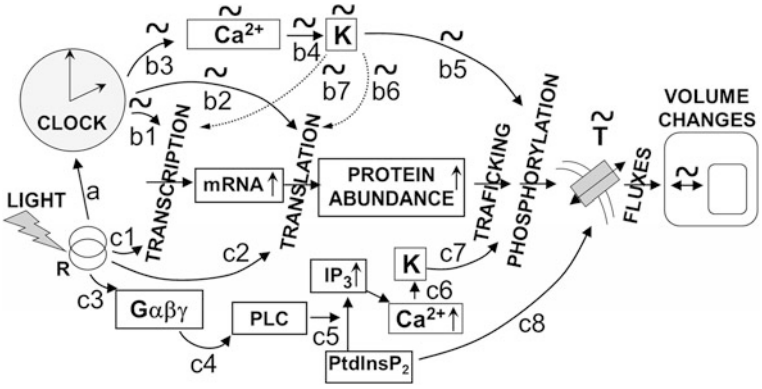

Fig. 4.8 Ca^{2+} involvement in the volume changes (a [partial] schematic model). *bn* are clock output signalling pathways, and *cn* are the "acute" signalling pathways from the light-activated receptor, *R*. The acute "shrinking signalling" includes the PtdIns pathway, starting with the activation (*c*3) of a trimeric G protein (Gαβγ), followed by the activation (*c*4) of Phospholipase C (PLC) and formation of 2nd messengers by breakdown (*c*5) of $PtdIns_2$. Kinase (*K*) can be activated (*c*6) by the increased (↑) concentration of free cytosolic Ca^{2+} and phosphorylate (*c*7) transporters (*T*) or their modifying protein(s), including those involved in trafficking. $PtdInsP_2$ may affect some of the transporters directly (*c*8). Some of these *elements* and *links* have been demonstrated in the pulvinar cells. The clock may originate Ca^{2+} oscillations (*b*3), via the $PtdInsP_2$ pathway or via a Ryanodine receptor cADP-ribose pathway. Ca^{2+} effects (*b*4–*b*5) may resemble those of the acute pathway (*c*6–*c*7). The clock effects on the transporters may be "enabling", modulating 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. 4.4 for unexplained signs

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

4.4.1 Acute, Fast Signalling

Signalling pathways are still not well understood, and therefore, there is a long list of questions to be answered.

Do ABA or jasmonate or NO (or their combination) act as *physiological* mediators of ***shrinking*** in pulvinar cells? Are they 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 $InsP_3$ or $InsP_6$ the actual messenger mobilizing Ca^{2+} from internal stores? 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 in the pulvini? Is a G protein a mediator of light signaling?

The following questions are only a partial list. The acute (i.e., the clock-bypassing) effects on transcription or translation, or membrane trafficking, indicated in the model, all invite, obviously, 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 the lysis of the membranal phosphatidylcholine (PtdCh) by PLA_2 and 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, but PLA_2 inactivated by a short preincubation 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 also be 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 are likely a 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 like sphingosine? The "guard cell paradigm" (see for example Chaps. 9 and 12) is indeed an inspiring framework for studying the fast, acute pulvinar signal transduction.

4.4.2 The Clock Input and Output

There is also no end to questions which may be addressed with regard to the regulation of pulvinar movements through the clock and by the clock (see Chaps. 8

and 10 for updated descriptions of the circadian plant clockworks). An intriguing question still lingers, posed already several decades ago, and apparently forgotten during the flurry of recent discoveries of the clock molecular components: do membrane ion channels feed back into the oscillator (especially in the light of increasing evidence that rhythmic *metabolism contributes* to the circadian network, Haydon et al. 2013)?

The future. Given the abundance of basic scientific questions they encrypt, pulvini currently attract insufficient attention as model systems. Large, reversibly moving pulvini, with oppositely functioning flexors and extensors (i.e., with "built-in" "controls"), amenable to easy protoplasts isolation, such as those of *Samanea*, can afford extensive physiological and biochemical assays of the interactions of the osmotic motor with the clock (roughly suggested in the model, Fig. 4.8). These would be greatly aided by combination with genetic tools, for example via transient protoplast transformation. Studies at the protoplast level could be easily combined with studies on the whole (isolated) pulvinus level and intact leaf level. These could be especially enhanced by automation of measurements of cycling phenomena, which would be of paramount importance to the generation of acceptable quantitative data for meaningful conclusions!

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Chapter 5
Spatial and Temporal Responses in Stomatal Behaviour, Photosynthesis and Implications for Water-Use Efficiency

L. McAusland, S.R.M. Vialet-Chabrand, J.S.A. Matthews and T. Lawson

Abstract Stomatal conductance (g_s) determines CO_2 uptake for photosynthesis (A) and controls the amount of water loss via transpiration, promoting evaporative cooling and nutrient uptake. It is therefore clear that stomatal behaviour has major implications for plant productivity which can be assessed by water-use efficiency (WUE) ($W_i = A/g_s$). However, stomatal responses to changes in environmental stimuli or intra-cellular cues are rarely synchronised under the continually fluctuating environmental conditions experienced in the field, leading to unnecessary water loss or limiting A required for promoting high yields. In addition, stomatal responses are intra- and inter-specific, varying both temporally and spatially over a single leaf and at the whole-plant level with further implications for canopy and ecosystem fluxes of CO_2 and water vapour. The effect of this variation in g_s responses on both A and W_i is discussed, along with the underlying anatomical characteristics which determine the potential of these physiological responses. Recent advances in both imaging techniques and modelling of guard cell membrane transporters have provided the means to more accurately assess the sensitivity, speed and magnitude of dynamic stomatal behaviours to environmental stimuli and the further impact on A and W_i at a range of scales. Use of these technologies provides a route to targeted manipulation of stomatal function to promote responses, which will improve whole-plant W_i without compromising CO_2 uptake for productivity. Here, we discuss how scaling processes are currently used to predict g_s, and the importance in considering the heterogeneous nature of stomatal dynamics, to improve the representation of how stomata influence the local and global fluxes of CO_2 and water along the soil–plant–atmosphere continuum.

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5.1 Introduction

Over 400 million years ago, the evolution of the leaf cuticle inter-spaced with stomata played a pivotal role in land plant colonisation through homoiohydry. The combination of these features enabled the control and conservation of water evaporation from the inner cell surfaces to the external environment by forming a water-proof barrier and adjustable pores for gas exchange (Hetherington and Woodward 2003; Raven 1977, 2002). Stomatal aperture is controlled by a pair of specialised cells, the guard cells that surround the pore. Guard cells increase or decrease in volume, driven by changes in solutes and water movement in and out of the cell, which in turn alters cell turgor pressure and aperture. This regulatory feature enables guard cells to modify pore aperture controlling the exchange of CO_2 between the external and internal environment for photosynthesis, water loss (in the opposite direction), evaporative cooling and nutrient uptake through transpiration (Farquhar and Sharkey 1982). Typically, stomata occupy a small proportion (between 0.3 and 5 %) of the leaf surface, yet the majority of all gas exchange between the external environment and leaf interior occurs through stomata (Morison 2003). Stomatal conductance (g_s) is a measure of the diffusion of water vapour and CO_2 through the pores, and an indication of stomatal opening. Stomatal behaviour and g_s have significant control on rates of photosynthetic CO_2 assimilation (A) through the regulation of CO_2 diffusion into the inter-cellular air spaces. High g_s has been shown to correlate with high A (Fischer and Edmeades 2010; Wong et al. 1979) and growth rates (e.g. Hosey et al. 2003), whilst low g_s can limit CO_2 uptake and therefore A which, when integrated over a growing season, could have negative implications for biomass accumulation and crop yield (e.g. Fischer et al. 1998). Additionally, high g_s has been shown to improve plant fitness, fruit prolificacy and seed yield through increased rates of evaporation and leaf cooling when plants have been exposed to high temperatures (Crawford et al. 2012; Lu et al. 1994; Radin et al. 1994).

The ratio of carbon gain to water loss is termed water-use efficiency (WUE) (W_i) and is a breeding trait currently subject to intensive investigation (Condon et al. 2002, 2004). As the rate of water loss from stomata is orders of magnitude higher than the rate of CO_2 uptake, high g_s not only promotes greater CO_2 diffusion but can also increase the likelihood of inducing drought stress. Stomata are therefore continually adjusting to maximise mesophyll photosynthetic demands for CO_2 whilst maintaining an appropriate balance with water loss and the hydraulic status of the plant. With the global population predicted to stabilise at around 9 billion by 2050 (Godfray et al. 2010), the allocation of fresh water resources has become an important issue not only for food and fuel security but also for supporting existing ecosystems and sanitation (Davies 2014). The abstraction of water for crop irrigation has already led to severe drought in particular areas of China (Piao et al. 2010) and the south-west of the USA (Ward 2013). It has been predicted that agricultural productivity will increase food production by 36 % by 2030; however, this estimate has only been reached with an addition of 13 % increase in water for

crops (UNESCO 2009). Furthermore, the predicted changes in the climate including an increase in mean atmospheric temperature by 1–3 °C per year (IPPC 2007) and the more erratic patterns of annual precipitation will result in increased evapotranspiration, putting greater pressure on irrigation practices to sustain crop yields (Ciais et al. 2005; Tubiello et al. 2007). Despite the allocation of up to 70–90 % of global fresh water to agriculture (Morison et al. 2008), drought is regarded as one of the major abiotic constraints on crop yield and has been attributed to yield losses of up to 40 % in maize (Boyer 1982; Harrison et al. 2014) and ca. 15 % in wheat (Foulkes et al. 2002, 2007). It has been estimated that 98 % of water taken up by the roots evaporates from the plant through the stomata with only 2 % remaining for the biochemical processes (Morison 2003), which translates to an estimated 60 % of all land precipitation (Katul et al. 2012). Moreover, 95 % of all gaseous flux including H_2O and CO_2 uptake in terrestrial vegetation occurs through the stomata (Lawson et al. 2012); therefore, stomatal behaviour has major implications for global carbon and hydrological cycles (Hetherington and Woodward 2003). The impact of stomatal behaviour and stomatal response to changes in climate at the ecosystem level has been illustrated recently by Keenan et al. (2013), who reported that yearly reductions in stomatal conductance, driven by increasing atmospheric [CO_2], are improving WUE (ratio of CO_2 assimilation to water loss through transpiration) in northern hemisphere temperate and boreal forests (Keenan et al. 2011, 2013). These authors concluded that impact of these shifts in the carbon and water economics and the consequences for coupled vegetation–climate models may need to be re-evaluated.

The regulatory role of stomata on photosynthetic processes and water loss makes guard cells an obvious target for improving plant WUE. However, before such an approach is possible, we must first fully understand stomatal responses in the field environment, including the spatial and temporal behaviour both within and between plants. Taking into account the intra- and inter-specific variation is essential for scaling through the soil–plant–atmosphere continuum.

5.2 Spatial Variation in g_s

Stomatal conductance is determined by both aperture and density of stomata, and it is well established that these and other stomatal characteristics vary considerably over the leaf surface. Heterogeneity in stomatal characters including size, density, behaviour and sensitivity has been reported within leaves (Weyers et al. 1997; Weyers and Lawson 1997), between leaves (Poole et al. 1996), and between plants and species (Solárová and Pospisilova 1983; Tichá 1982). Adding to this complexity, spatio-temporal variability has also been reported at all of these scales (Lawson and Weyers 1998; Pospíšilová and Šantrůček 1994; Weyers et al. 1997). A full understanding of the role of stomata in plant–water relations and photosynthetic carbon capture at the crop level, and the scaling implications for global carbon and hydrological cycles is not possible without considering the

spatio-temporal variability in stomatal behaviour. An understanding of the nature of stomatal heterogeneity and its origins is also important as it may provide plants with some functional advantage or disadvantage. Additionally, knowledge of such variation is needed when designing experimental procedures and developing sampling protocols.

Although spatial heterogeneity in stomatal activity has been known since 1898 (Darwin 1898), it was neglected until the late 1980s when Downton et al. (1988) and Terashima et al. (1988) reported that the assumption of uniform stomatal aperture and therefore g_s resulted in the erroneous conclusion that abscisic acid (ABA) affected photosynthesis directly and not solely via stomatal closure (Downton et al. 1988; Terashima et al. 1988). This 'patchy' stomatal closure causes errors in the calculation of internal CO_2 concentration (C_i) from gas exchange techniques (Laisk et al. 1984) and therefore has implications for gas exchange measurements under conditions that may induce non-uniform stomatal behaviour, such as reduced water availability (e.g. Grassi and Magnani 2005). The development and subsequent advancement of imaging techniques such as thermography to measure leaf temperature and indirectly infer stomatal conductance have enabled spatial and temporal behaviour in stomatal responses to be assessed at a range of levels from the leaf to the canopy (Guilioni et al. 2008; Jones 1999). Figure 5.1 provides an example of whole-plant g_s derived from thermography and illustrates the large spatial heterogeneity that is apparent between different leaves of the same plant, and different plants (Fig. 5.1a). The derived g_s from thermal imagery shows an approximate five-fold variation across a single plant and also highlights the extent of intra-specific variation.

The capacity of the stomata to exchange both CO_2 and water vapour and the ability to respond rapidly to changing environment conditions are determined from the anatomical features of the guard cells and the potential maximum conductance ($g_{s\max}$), which is predominantly determined from the anatomical characteristics of stomatal density (D) and size (S) (Franks and Beerling 2009; Körner et al. 1979; Parlange and Waggoner 1970). As these parameters vary over the leaf surface (Wilmer and Fricker 1996), it may be expected that large spatial differences in $g_{s\max}$ exist. Körner et al. 1979 demonstrated that crop species often exhibit higher values of $g_{s\max}$ when compared to species grown in the wild or from ecologically comparable groups, providing a possible insight into how traditional breeding may have inadvertently or actively favoured increases in g_s as a means to improve CO_2 diffusion (and/or decrease leaf temperature), photosynthesis and yield (e.g. Condon et al. 1987; Fischer et al. 1998). Additionally, it would be predicted that large spatial differences in $g_{s\max}$ would translate to large differences in g_s in response to environmental perturbations (Jones 1992). Imaging techniques such as thermography described above provide an unrivalled opportunity to examine spatial g_s behaviour and the impact of changing environmental conditions on stomatal dynamics, and will be discussed in further detail in Sect. 5.4.

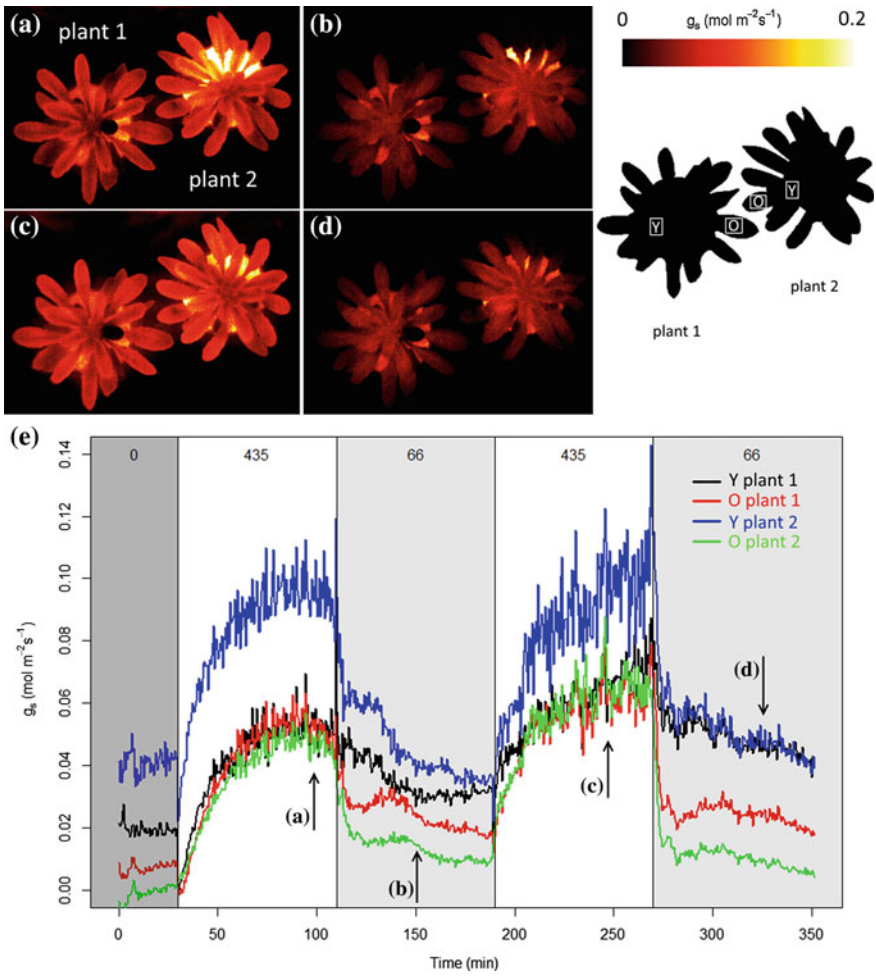

Fig. 5.1 Spatial and temporal heterogeneity in stomatal conductance to water vapour (g_s). Thermography was used to derive spatial patterns of g_s in two plants of *A. thaliana* (**a**–**d**) following the methods of Jones (1999) and Guilioni et al. (2008). To assess spatio-temporal responses in g_s, plants were subjected to a succession of step changes in light between 0 and 435 µmol m^{-2} s^{-1} (light intensities are displayed at the top of each phase of Fig **e**). A representative section from young (Y) and older (O) leaves was chosen from each plant (locations shown in the plant schematic diagram on the right) and the respective g_s followed over the measurements period (**e**). The *arrows* represent the time at which each picture (**a**–**d**) were captured. All measurements were taken at 60 % of relative humidity and a temperature of 20 °C

5.3 Coupling Stomatal Behaviour with Mesophyll Demands for CO_2

In order to function effectively (maximising CO_2 uptake and minimising water loss), guard cells must be able to sense and respond to changes in environmental conditions and internal signals (Raschke 1976). In most plants, with the exception of those exhibiting CAM metabolism, stomatal opening is stimulated by increasing photosynthetic photon flux density (PPFD), low vapour pressure deficit (VPD), low CO_2 concentrations [CO_2] and high temperatures, whilst closure is promoted by high VPD, high [CO_2] and low PPFD (reviewed by Outlaw 2003).

A close correlation between g_s and A has been observed for a wide range of species (Buckley et al. 2003; Wong et al. 1978, 1979) under different irradiances and CO_2 concentrations (Hetherington and Woodward 2003; Radin et al. 1988). It is generally accepted that the stomatal responses to the concentration of CO_2 in the intra-cellular spaces of the leaf (C_i) have led to this close relationship. Stomata close when C_i increases above ca. 2/3 atmospheric [CO_2] and open when the concentration drops below this level. The concentration of C_i within the leaf depends upon g_s and the consumption of CO_2 by A (Mott et al. 2008). Therefore, changes in C_i are dependent not only on the rate of CO_2 diffusion through the stomatal pore, but also the capacity of the mesophyll to assimilate CO_2, which differs greatly between species (Lawson et al. 2012). It is believed that this mechanism acts to coordinate g_s responses with A under dynamic fluctuating environmental conditions such as those observed in the field (see Lawson and Blatt 2014). It should however be noted that although the relationship between A and g_s is conserved, it is not always constant. Stomatal responses to a change in the surrounding environment are an order of magnitude slower than changes in A, where A is limited due to slow pore opening, or where lags in stomatal closure lead to unnecessary water loss relative to A achieved (Lawson and Blatt 2014; Lawson et al. 2003, 2010, 2012). However, generally over the long-term (hourly) changes in A and g_s will be coupled. Figure 5.2 illustrates the paralleled although uncoupled behaviour in A and g_s using proxy measurements, in the model plant Arabidopsis following application of DCMU (inhibitor of photosynthetic electron transport). In this example, chlorophyll fluorescence imaging was used to determine the operating efficiency of PSII photochemistry (which is directly related to the rate of electron transport), whilst thermal imaging was used to assess leaf temperature, indicating a change in g_s. Application of DCMU resulted in a steady decrease in F_q'/F_m', to 50 % of the initial value 1.5 h after application. Over this period, leaf temperature increased by ca. 3 °C which corresponds to a decrease in g_s of ca. 170 mmol m^{-2} s^{-1}. However, although the degree of change in A and g_s was of a similar magnitude (ca. 50 %) after 1.5 h following the application of DCMU, the response and coordination between the two parameters were not linear. F_q'/F_m' showed a steady decline over the entire measurement period (Fig. 5.2m), whilst the greatest increase in leaf temperature was observed during the initial 40–60 min, after which little change in temperature, and therefore change in g_s were observed (Fig. 5.2n).

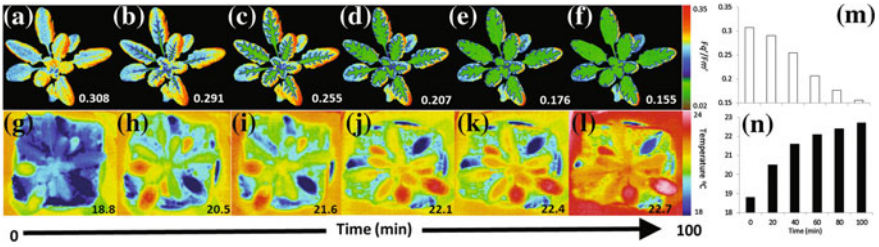

Fig. 5.2 Coordination between A and g_s responses following application of DCMU. Chlorophyll fluorescence measurements of the operating efficiency of PSII photochemistry ($F_q/F_{m'}$) were used to provide an indication of A (**a**–**f**). Changes in leaf temperature measured by thermography (**g**–**l**) provided an indirect measure of g_s. Images were captured every 20 min following application of 0.1 mM DCMU at time zero. Mean whole-plant values of A(**m**) and g_s(**n**) at each time point illustrate the uncoupling of the stomatal and photosynthesis response

Whilst it has long been thought that stomata respond to a constant ratio of atmospheric CO_2 (C_a) to C_i of about 2/3 atmospheric CO_2 (Mott 1988), some also argue that changes in C_i are often too small to account for the changes in stomatal aperture, with reports of stomatal responses to PPFD occur even when C_i is held constant (Lawson et al. 2008; Messinger et al. 2006; Wang et al. 2008). These observations have led to the hypothesis of an additional, diffusible signal between the guard cells and the mesophyll which act as a link between stomatal responses and photosynthesis (Lee and Bowling 1992; Mott et al. 2008; Wong et al. 1979). Due to the close correlation between the mesophyll photosynthetic capacity and g_s observed under a wide range of conditions, it has been suggested that this molecule may be a photosynthetically derived metabolite (Grantz and Schwartz 1988; Heath 1947; Wong et al. 1979). As starch is derived from the Calvin cycle, sucrose has long been implicated as a potential signalling molecule, coordinating the stomatal and photosynthetic responses (Kang et al. 2007; Lawson et al. 2014; Lu et al. 1997, 1995; Outlaw and De Vlieghere-He 2001). Malate^{2-} has also been implicated as a potential signal, derived both from its production in the guard cell cytosol and its potential import from neighbouring cells (Araújo et al. 2011; Fernie and Martinoia 2009; Lee et al. 2008). Identification of this signal molecule is a key to providing a potential target for genetic manipulation to improve the sensitivity of the stomatal response to rapid environmental fluctuations experienced in the field. For a more comprehensive recent review on this subject, please refer to Lawson et al. (2014).

5.4 Dynamic Stomatal Behaviour

In the previous sections, we have focused on inter- and intra-specific spatial variation; however, in the field environment, stomata experience continual perturbations in environmental factors and must therefore respond to multiple stimuli in an integrated and hierarchical manner, not only to maintain growth and development but

also avoid stress (Kaiser et al. 2014; Lawson and Blatt 2014; Merilo et al. 2014; Sharkey and Raschke 1981; Wang et al. 2008). Heterogeneity in dynamic stomatal responses is superimposed on to spatial variation in g_s. For example, in Fig. 5.1, we explored the spatial variation in stomatal conductance, both between plants and between individual leaves of the same plant. Following a step change in irradiance stomatal responses is unlikely to be identical across a leaf or plant. Heterogeneous temporal g_s behaviour is illustrated in Fig. 5.1e, which shows asymmetric increases and decreases in g_s in different leaves. The temporal response of g_s in older leaves shows a similar dynamic response to that observed in young leaves however with a lower g_s value (even in darkness). In contrast, the young leaves displayed different temporal responses of g_s with differences in the speed of response when light is manipulated. For example under low light, the young leaf in plant 1 shows a slower rate of closure (and therefore absolute g_s value) when the PPFD was decreased to 66 μmol m^{-2} s^{-1} and a slower rate of opening when light was returned to 435 μmol m^{-2} s^{-1}. What is immediately apparent from these data is that although in this case the general stomatal responses were similar, both the speed of the responses and magnitude of change are not identical between leaves of the same plant.

5.5 Temporal Stomatal Behaviour Influences *A* and W_i

Past research into stomatal behaviour has mainly focused on dissecting measurements of steady-state stomatal responses to PPFD, VPD and CO_2, and less work has focused on the effects of dynamic stomatal behaviour and the impacts on *A* and therefore intrinsic WUE ($W_i = A/g_s$). This can vary on a scale of seconds to minutes under naturally fluctuating environmental conditions (Lawson and Blatt 2014). In the field, the response of *A* and g_s is largely dominated by fluctuations in PPFD (Way and Pearcy 2012). Therefore in a crop canopy, leaves may be exposed to a wide range of irradiances, varying from deep shade to full sun as well as varying in spectral quality. During the day, stomata constantly adjust their response to fluctuations in PPFD, rarely reaching steady state in the field (Lawson and Morison 2004). Stomatal responses to changes in PPFD are one of the most well-studied approaches for determining differences in the speed of opening and closing between species (e.g. Assmann and Grantz 1990; Assmann et al. 1992; Kirschbaum et al. 1988; Pearcy 1990; Tinoco-Ojanguren and Pearcy 1993). These studies are particularly important as lags in stomatal response time not only constrain CO_2 diffusion and limit the more rapid response of photosynthesis, but also lead to water loss when *A* is PPFD limited. Furthermore, g_s has also been shown to 'overshoot', increasing after *A* is PPFD-saturated, increasing water loss with no further carbon gain (Kirschbaum et al. 1988; Lawson et al. 2010; Tinoco-Ojanguren and Pearcy 1993), highlighting the temporal uncoupling of *A* and g_s responses. Although such response may have negative implications for W_i, it should be noted that g_s is an adaptive mechanism which is also essential for maintaining plant growth and

development, with roles in leaf cooling (Crawford et al. 2012; Leakey et al. 2003), nutrient translocation, and protection against xylem embolism (Jones 1998).

Differences in the speed of stomatal opening and closing and magnitudes of g_s are known to exist both between species and between individuals of the same species (Assmann and Grantz 1990; Drake et al. 2013; Franks and Farquhar 2007; Ooba and Takahashi 2003; Vialet-Chabrand et al. 2013; Vico et al. 2011). These differences in response time are also compounded by leaf age (Urban et al. 2008), water status (Lawson and Blatt 2014), humidity (Nejad and van Meeteren 2008), history of stress (Ackerson 1980; Davies and Kozlowski 1975) and the magnitude and duration of changes in PPFD (Weyers and Lawson 1997).

Combining thermal images with images of chlorophyll fluorescence (which provides an estimate of photosynthetic electron transport and therefore an indirect measure of carbon assimilation) has provided the opportunity to examine 'real-time' stomatal responses relative to the underlying mesophyll photosynthetic rates. Using a combination of thermal and chlorophyll fluorescence imaging approaches has also led to the development of new technologies to screen for plant WUE (McAusland et al. 2013). Furthermore, the use of dynamic imaging also enables the spatial variation in measured parameters to be evaluated. Figure 5.3 provides an example of thermography and chlorophyll fluorescence imaging on an Arabidopsis plant following a step change in PPFD from 200 to 800 µmol m^{-2} s^{-1}. The images of A and g_s revealed a spatial pattern and a large diversity in A and g_s that translates into significant spatial variation in intrinsic plant WUE, W_i, over the whole plant. When illuminated with 200 µmol m^{-2} s^{-1} PPFD, whole-plant assimilation rates ranged from 2.7 to 7.3 µmol m^{-2} s^{-1} with a median of 5.8 µmol m^{-2} s^{-1}. When PPFD was increased to 800 µmol m^{-2} s^{-1}, the median value rapidly increased by ~20 % to 7.2 µmol m^{-2} s^{-1} with a much broader range of values observed (1.6–10.4 µmol m^{-2} s^{-1}). The frequency distribution graphs (Fig. 5.3g–i) show the pixel distribution of measured parameters corresponding to a whole-plant image selected from the low- and high-light environment. When subjected to an increase in light, A displayed an increase in the median from 6.5 to 8.5 µmol m^{-2} s^{-1}, with an increase in maximum value from 11 to 19.6 µmol m^{-2} s^{-1}. This large increase in maximum A value was as a consequence of the spatial heterogeneity of the A response, with some leaves or part of the leaves displaying a step increase in A (blue area without overlapping red in Fig. 5.3h). A similar shift in g_s was also observed when light was increased by 600 µmol m^{-2} s^{-1} although the range of values remained similar. This response could signify that despite the non-uniformity in g_s over the leaf, stomatal responses in this example remained coordinated. The uncoupled responses of A and g_s to an increase in PPFD resulted in shifts in distribution of W_i over the plant, however the lack of shift in the median of the distribution, suggested a general coupled response in A and g_s at the leaf level. Analysis of spatial and temporal heterogeneity of individual leaves supports these conclusions. Selected young (Y) and older (O) leaves in Fig. 5.3a illustrate variation between and within leaves, by a change in the shape of the distribution

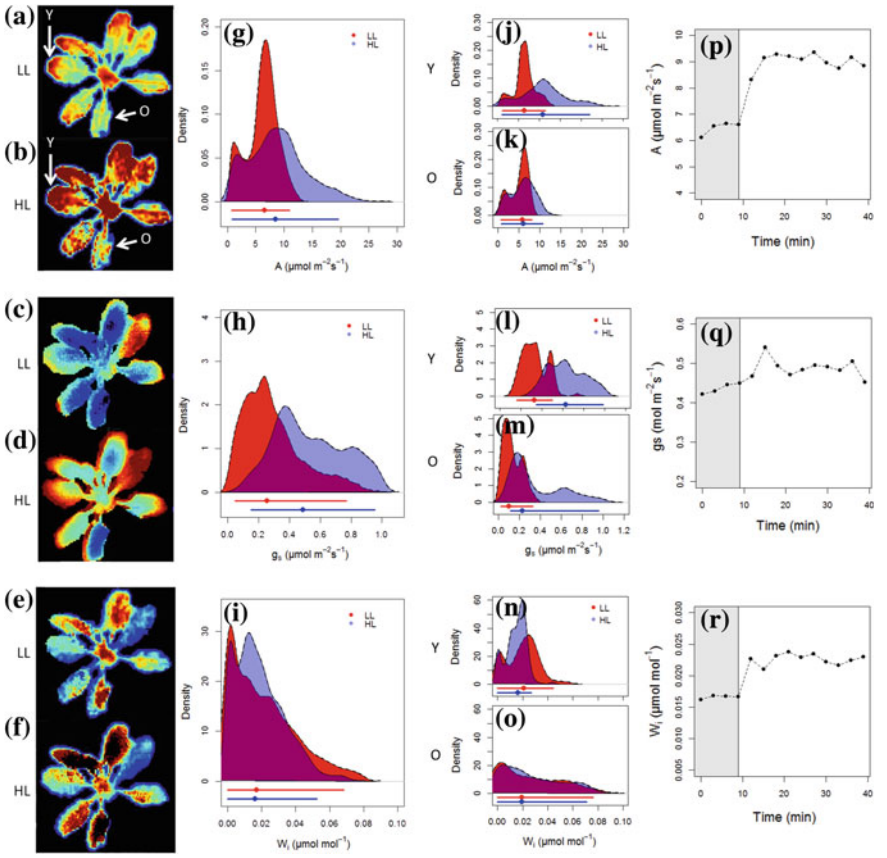

Fig. 5.3 Heterogeneity in whole-plant net assimilation (A) (**a**–**b**), stomatal conductance to water vapour (g_s) (**c**–**d**) and intrinsic water-use efficiency (W_i) (**e**–**f**) in response to changes in light from low light (LL 200 μmol m^{-2} s^{-1}, *red*) to high light (HL 800 μmol m^{-2} s^{-1}, *blue*). Low-light images were captured following 10 min stabilization to the light and high-light images were captured after 40 min at high light. Frequencies distribution plots (**g**–**i**) illustrate the spatial variation in each of the representative images. The area under the curves represent the distribution of the values assessed using kernel density estimation. Frequency plots from a represented young (Y) and older (O) leaf illustrate the spatial and temporal variation exhibited in different individual leaves (**j**–**o**). The segments below the frequency plots represented the quantiles at 2.5 and 97.5 %, whilst the point shows the median value. Mean whole-plant values for A(**p**), g_s(**q**) and W_i(**r**) over the entire time course show the general plant dynamic responses without taking into account the intra-specific variation

following a temporal response to increased PPFD. In general, there was a trend of increasing A and g_s, with the median shifting to the right with increasing PPFD. On average, the younger leaf demonstrated higher rates of assimilation under both low (5.7 ± 3.0 μmol m^{-2} s^{-1}) and high light (9.6 ± 5.4 μmol m^{-2} s^{-1}) when compared to the older leaf (4.3 ± 2.6 and 5.1 ± 3.2 μmol m^{-2} s^{-1}, respectively), achieving

68.8 % increases in carbon assimilation compared to 21.2 % achieved by the older leaf. In older leaves, this manifested in a conserved W_i between the two light levels, illustrating similar spatial and temporal adjustments of A and g_s in these leaves; however, in younger leaves, a small decrease in median W_i value was observed, due mostly to a reduction in the variation of W_i within the younger leaves. It is worth noting that when whole-plant mean values are used to examine the dynamic responses of the A, g_s and W_i (Fig. 5.3p–r), the response to a step change in light appears greatly dampened relative to the individual leaf-level responses, this is due to averaging the intra-specific variation when using whole-plant measurements, and particularly important when determining g_s responses.

5.6 Influence of Stomatal Dynamics

Improving the rate of the g_s response to changing environmental conditions or mesophyll demands for CO_2 is an obvious approach to maximise A when conditions permit and minimise water loss when assimilation rates are reduced (Lawson and Blatt 2014), therefore improving W_i without compromising photosynthetic productivity. In a recent review by Lawson and Blatt (2014), it was estimated that if stomata responded instantaneously to changes in PPFD, and therefore were in complete synchrony with A, the cumulative W_i over the defined timescale could be improved by up to 22 %.

Most species also demonstrate an asymmetry in the time taken to open and close in response to changes in PPFD (Ooba and Takahashi 2003; Vico et al. 2011). If there is no delay in opening or closing optimal leaf, gas exchange is achieved (Cowan and Farquhar 1977), with any delays in the response time, indicating evolutionary pressures to maximise A or minimise transpiration (Manzoni et al. 2011; Ooba and Takahashi 2003; Vico et al. 2011). The behaviour of g_s is thought to reflect the priorities of the plant; for example, under water-limited conditions, it has been shown that stomata will respond to conserve water at the expense of carbon gain (Knapp and Smith 1988), whilst when measured under non-water-limited conditions, stomatal behaviour will maximise CO_2 diffusion even at the expense of water loss (Allen and Pearcy 2000).

In general, the rate of the opening response of stomata to increases in PPFD is thought to reflect the necessity of the plant to utilise the irradiance for photosynthesis. First observed by Tino-Ojanguren and Pearcy (1992), species grown under shaded conditions demonstrated rapid increases in g_s in response to increasing PPFD. Conversely, the closing g_s response to decreasing PPFD has also been correlated with the water status of the environment in which the plant is growing, with the fastest response times observed in plants growing in high PPFD and warm ambient temperatures (Ooba and Takahashi 2003) and the longest response times observed for shaded species (Allen and Pearcy 2000). Similar dynamic variation in the dynamic nature of stomata is also seen at the leaf level using traditional gas exchange measurement techniques. For example, Fig. 5.4 shows gas exchange

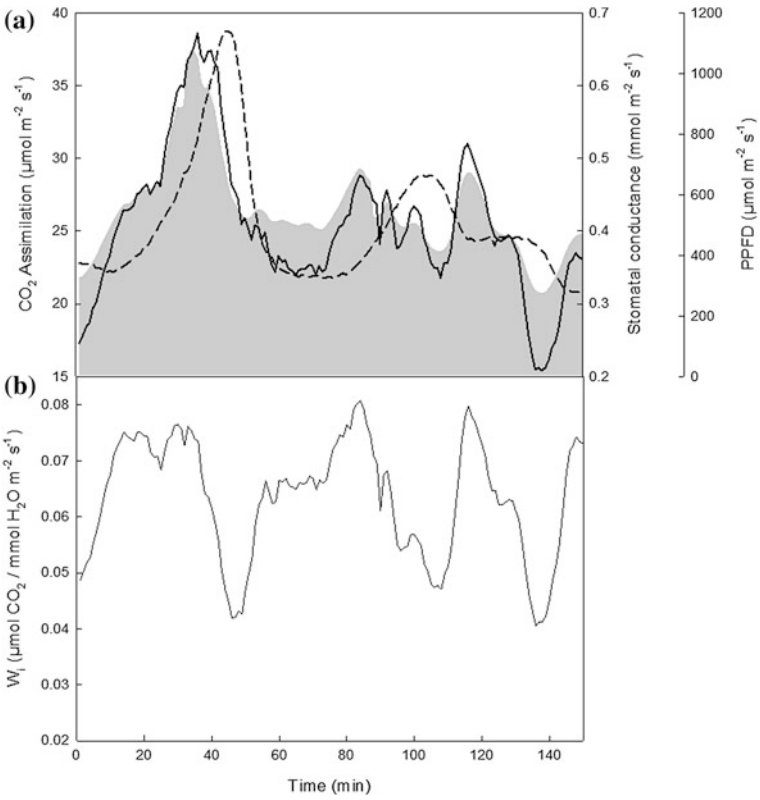

Fig. 5.4 (**a**) Variation in PPFD, net CO_2 assimilation (A) and stomatal conductance (g_s) over a 150 min period for broad bean (*Vicia faba*) experiencing natural PPFD fluctuations in a greenhouse at the University of Essex. Fluctuations in PPFD are represented by the *grey shaded* area, whilst A is indicated by the solid line and g_s by a *dashed line*. Average temperature in the greenhouse was 25 °C (±2 °C) and the humidity was 50 %. Measurements were taken every 60 s. (**b**) W_i calculated from the data collected in A using the following equation $W_i = A/g_s$

measurements of A and g_s over a 150 min period for *Vicia faba* experiencing natural fluctuations of PPFD in the greenhouse at the University of Essex, and the impact on A and W_i is illustrated. It is immediately apparent from these data that the dynamic light environment has different impacts A and g_s responses with an often-uncoupled A and g_s response, similar to those reported above (Fig. 5.4a). The lags in stomatal responses to a change in PPFD are obvious within the first 30–50 min in which A increases with light up to a maximum of about 0.38 mmol m^{-2} s^{-1} at a PPFD of 1100 µmol m^{-2} s^{-1}, whilst g_s does not reach a maximum for a further 10–15 min, reaching a value of 0.65 mmol m^{-2} s^{-1} when PPFD has already returned to 500 µmol m^{-2} s^{-1}. The lack of synchrony between stomatal behaviour and A is noticeable here, but also at ca. 100 min when g_s is increasing, whilst A is decreasing due to a decrease in PPFD. These uncoupled responses in A and g_s mean

that W_i shows significant variation in temporal responses over the course of the day (Fig. 5.4b), with major implications for the carbon and water status of the plant. Measuring the stomatal response of leaves of glasshouse grown species, such as *V. faba*, may not be indicative of the responses measured in canopy conditions. Whilst PPFD to the top of the canopy is mainly limited by cloud cover, PPFD to the understory can be significantly reduced by shading from neighbouring leaves, sun angle and the architecture of the developing crop plant (Chazdon and Pearcy 1991; Drewry et al. 2014; Pearcy 1990; Way and Pearcy 2012). In monoculture, whilst the youngest top leaves of crops such as Tobacco receive and assimilate full sunlight, the vast majority of the canopy is acclimatised to the cooler, shaded understory. Stomatal responses observed on shaded leaves are reported to be highly sensitive to rapid changes in light intensity (Aasamaa and Aphalo 2015) but also maintain a consistently high conductance to maximise CO_2 assimilation (e.g. Chazdon and Pearcy 1991; Tinoco-Ojanguren and Pearcy 1993; Woods and Turner 1971). In leaves exposed to high PPFD, a rapid opening response may be advantageous to minimise limitations on CO_2 diffusion but a high steady-state conductance increasing the likelihood of drought stress and possible embolism of the xylem. Additionally, upper canopy sites also often experience higher temperatures and greater fluctuations in humidity making rapid stomatal opening and closing advantageous for these leaves as an adaptive mechanism to conserve water (Ooba and Takahashi 2003). These behaviours suggest that the speed and magnitude of the stomatal response to changes in PPFD are adaptive to the spatial leaf positioning and the progressively changing PPFD conditions within the canopy. Although we have focused this chapter on leaf- or plant-level spatial and dynamic aspects of stomatal behaviour, there is the added complexity of such spatio-temporal responses of g_s for scaling stomatal behaviour and the implication for A and W_i, to the canopy or ecosystem level.

5.7 Scaling Up Leaf-Level Dynamics to Canopy Responses

Stomata are at the interface of the soil–plant–atmosphere continuum and their dynamic behaviour play a key role in ecosystem carbon and water fluxes by regulating the amount of carbon fixed and the amount of water lost via transpiration. The dynamics of stomata are also an important determinant of W_i and photosynthetic capacity at the leaf and canopy level. Investigating the role of stomata is essential for a mechanistic understanding of carbon and water fluxes at the leaf–canopy–ecosystem scale, and can be predicted with the use of scaling and mechanistic models. Furthermore, when attempting to model or scale-up, it is important to consider the complexities in temporal and spatial variations of stomatal behaviour at smaller scales, to gain a better understanding of how stomatal dynamics affect ecosystem-level fluxes of CO_2 and water vapour.

Current opinion recognises the importance of integrating stomatal conductance to water vapour (g_s) into scaling and modelling processes (Bernacchi et al. 2007;

Lawson et al. 2010; Bonan et al. 2014; De Kauwe et al. 2015); however, spatial and temporal variations in stomatal behaviour are somewhat overlooked. The intricacies of steady-state models and processes currently used in scaling g_s and A are well documented in the literature (Farquhar and Sharkey 1982; Jarvis and McNaughton 1986; Ball et al. 1987; Jarvis 1995; De Pury and Farquhar 1997; Farquhar et al. 2001; Bernacchi et al. 2003; Ainsworth and Rogers 2007; Bernacchi et al. 2007, 2013) and therefore will not be covered here. Nevertheless, this section will briefly cover some of the problems and difficulties associated with these scaling processes.

The objective of scaling is to establish a relationship between fluxes measured at one scale to those observed at another, so that the rate of CO_2 and water vapour exchange at larger spatial and temporal scales is equal to the sum of all the individual components within a system, including the microenvironments surrounding leaves (Jarvis 1993, 1995). Despite this seeming like an easy objective, in reality, it represents a great challenge, as processes affecting the flux of CO_2 and water vapour are subject to multiple interactions of environmental factors and the significant heterogeneity in leaf traits that influence the rate of these fluxes (Jarvis 1995; Lawson et al. 1998).

Current models of stomatal conductance predict g_s in steady state (a hypothetical equilibrium state under stable environmental conditions). This has made them successful tools for modelling stomatal response to changes in atmospheric conditions at larger scales (spatially and temporally). However, they are limited as tools for understanding the dynamic behavioural response of stomata, as they do not take into account stomatal physiological dynamics, and therefore limit the confidence with which they can predict response to future climatic change (Dewar 2002; Buckley et al. 2003; Dewar et al. 2009; Baldocchi 2014). Recently, new models focusing on the dynamic response of stomatal conductance have been developed, which integrate stomatal response to rapid variations in the environment, known to be a limitation in steady-state models (Vico et al. 2011; Vialet-Chabrand et al. 2013).

The ‘bottom–up’ approach of leaf to canopy scaling integrates individual leaf-level responses to controlled steady-state environmental factors into a canopy level (Jarvis and McNaughton 1986; Baldocchi et al. 1991; Baldocchi 2014), often using physiological characteristics such as leaf area index to quantify leaf to canopy scaling processes. This form of scaling can be described as multi-layer or dual source as they often utilise data from many spatial and temporal sources (Baldocchi et al. 1991; Baldocchi and Harley 1995; Jarvis 1995; De Pury and Farquhar 1997; Wang and Leuning 1998; Leuning et al. 1998; Wang 2000; Xu and Baldocchi 2003), although these approaches could be improved by incorporating the intra- and inter-leaf diversity of g_s response. Alternatively, ‘top–down’ or ‘big-leaf’ approaches can be utilised, which use reductionist explanations by breaking down a defined system, and mapping those properties onto a single leaf (Jarvis 1995). For example, the ‘top–down’ system enables a simplistic, but also useful approach for estimating ecosystem health and response to future environmental change. This approach uses data collected from remote sensing systems, such as eddy covariance, and assumes that the net carbon canopy flux has the same relative response at

the canopy and leaf level, and is therefore fundamentally linear (Friend 2001). Using the 'big-leaf' method can lead to error within the system as leaves in a canopy respond differently as they are subjected to rapid changes in environmental conditions and are dependent upon the vertical structure of the canopy itself (Dai et al. 2004; Porcar-Castell and Palmroth 2012). As a result, the accuracy of 'big-leaf' models can be impacted by the continuous fluctuation in input energy, associated with spatial and temporal dynamic fluctuations 'seen' by canopies during the diel period (Buckley et al. 2003), which can be taken into account using dynamic models of g_s. Such fluctuations include brief periods of increased incident light, known as sunflecks, which represent a partial illumination of the leaves increasing the heterogeneous response of A and g_s within the canopy. Studies have shown that the sunflecks represent a critical supply of energy to lower parts of dense canopies (Porcar-Castell and Palmroth 2012) and can be the dominant source of energy for driving photosynthesis (Roden and Pearcy 1993a, b), significantly improving carbon gain in shaded understories and lower canopy levels of deciduous forests by 10–20 %, and in tropical understories by 30–60 % (Way and Pearcy 2012).

These fluctuations represent a new source of error when considering different levels of scale, as spatial heterogeneity in stomatal conductance and the rate of photosynthesis impact the accuracy of scaling models, and can lead to overestimation of canopy-level photosynthesis. De Pury and Farquhar (1997) managed to avoid such errors by using an empirical coefficient, which separates sections of the canopy into leaves that can be described as sunlit or shade, integrating them as sections of a single model. Following increased consideration in the importance of stomatal dynamics on canopy photosynthetic and transpiration rates, scaling research is beginning to incorporate stomatal dynamics into new scaling models, specifically looking at the spatial and temporal heterogeneity in stomatal conductance and carbon assimilation associated with the different layers of a canopy (Wang and Leuning 1998; Leuning et al. 1998; Tuzet et al. 2003; Drake et al. 2013; Ding et al. 2014; De Kauwe et al. 2015). These developments improve the accuracy of 'top–down' scaling processes, by integrating the effect of stomatal conductance on canopy-level parameters, whilst still enabling the evaluation of ecosystem-level CO_2 and water fluxes without the major errors associated with 'big-leaf' models.

When scaling from leaf- to the canopy-level, the disproportionate relationship between g_s and A can result in significant decreases in ecosystem-level W_i. The next step for modelling efforts scaling from leaf to canopy or ecosystem level is the integration of dynamic responses of stomata to real-time fluctuations in environmental factors, and the effect large-scale heterogeneity of leaf traits have on the flux of CO_2 and water at the canopy and ecosystem level. In a natural environment, plants and stomata are exposed to these constant fluctuations, to which they must respond. It is often the speed of stomatal response that is critical in assessing carbon uptake and transpiration dynamics (Raschke 1975; Kirschbaum et al. 1988; Lawson and Morison 2004; Lawson and Blatt 2014).

5.8 Conclusions and Future Work

In this chapter, we have focused on heterogeneity in both spatial and temporal dynamics of stomatal responses, the effect on A and W_i at the leaf/plant level, and the impact and importance of variation in these responses for scaling g_s to the canopy and ecosystem levels. Variation is apparent in the speed in which stomata respond to environmental stimuli as well as the magnitude of the response and can be visualised using both traditional gas exchange leaf-level measurements and through advancing imaging technologies at the whole-plant scale. Different sensitivities and responses in guard cell adjustments in conjunction with underlying anatomical features (and therefore capacity e.g. g_{smax}) are all possible mechanisms for intra- and inter-specific spatio-temporal variation in g_s responses. The regulatory role of stomata on photosynthetic processes and water loss makes guard cells an obvious target for manipulation to improving plant W_i and therefore productivity. However, before such an approach is possible, we must first fully understand stomatal responses in the fluctuating field environment, including the spatial and temporal behaviour both within and between plants. Moreover, taking into account intra- and inter-specific variation is also essential for scaling through the soil–plant–atmosphere continuum, to provide greater accuracy in predicting gaseous exchange between plant and atmosphere at different scales, enables high frequency responses of g_s to the rapid variation in the environment to be incorporated into the ecosystem water and carbon budget.

In addition to identifying the means to improve coordination between CO_2 assimilation in the mesophyll and stomatal behaviour, it is also necessary to identify guard cell-specific features which promote increased sensitivity to environmental change and the ability to rapidly alter pore aperture in response. The rate of the g_s response to any given environmental or internal stimuli is determined by the ability of the guard cells to sense changes in the environment, and/or respond to downstream signals following such a sensory pathway, to alter the accumulation or release of osmotically active molecules to increase or decrease water potential and turgor pressure of the guard cells (Blatt 2000; Kollist et al. 2014; Lawson 2009; Lawson et al. 2014; Pandey et al. 2007). There are several solutes that play a role in guard cell osmotic relations, with both active and passive transporters responsible for the movement of such solutes across membranes. This leads to a huge complexity in both the hierarchy and combination of activities of these different transporters and the effect on metabolic processes within guard cells and subsequent responses at the individual cell level. Genetic manipulation of stomatal function may need to take into account a combination of transport processes and integrated approach in order to manipulate solute transport in guard cells. To address these issues, Blatt and colleagues have developed a cellular transport model that enables the integrated responses of various transporters to be assessed in terms of dynamic stomatal behaviour (see Hills et al. 2012; Wang et al. 2014; Blatt this book). Such models provide significant predictive power for targeting cellular processes for genetic manipulation of stomatal function in order to enhance plant WUE (Lawson

and Blatt 2014), but this chapter has illustrated the importance of taking into account spatial and temporal variation in stomatal responses at both the leaf and whole-plant scale.

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Chapter 6
The Pollen Tube Oscillator: Integrating Biophysics and Biochemistry into Cellular Growth and Morphogenesis

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Abstract Individual cells generate ultradian rhythms in different systems and levels of organisation. A cell biology approach is necessary to better understand the intrinsic nature of these biological 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 structural, biochemical as well as biophysical oscillations. As commonly seen in complex systems, these oscillations involve almost all cellular components but, in this case, their causal relations have not yet been fully identified. Most studies consider growth as a reference to establish the relation with other oscillating variables, interpreted to be a cause if its peak occurs before and as a consequence if it occurs after that of the variable in question. 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 trafficking and cell wall synthesis. Despite the progress made in this domain, however, a central core-controlling mechanism is still missing, and even less is known about how all components interact to produce the macroscopic outcome, i.e. structurally and temporally 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,

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

6.1 Finding Stability on Instability

For a long time, the view of a biological system as organised structures in a state of equilibrium has led researchers to observe snapshots and to treat the averages as a close reflection of reality. 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 has been changing as increasing importance is given to the dynamics and non-equilibrium states of biological systems. Progress in this field can be attained only by the use of new methodology enabling in vivo monitoring on a nearly continuous timescale, as well as different mathematical tools of analysis and modelling developed to this effect (Feijó et al. 2001; Kroeger and Geitmann 2012).

The existence of specific nonlinear behaviours, such as oscillations or pseudo-chaotic fluctuations, has been observed in a wide range of biological processes. These include calcium waves in plant and animal cells, oscillations in metabolite production in yeast, waves of cell aggregation in *Dictyostelium* or lamellipodial contractions in spreading and migrating animal cells, as well as robust oscillations and a myriad of circadian clock-dependent behaviours (Goldbeter 1997; Novák and Tyson 2008).

The generalised view is that, in terms of biological function, these complex behaviours exist in order to allow organisms to attain homeostasis. Therefore, they would be characterised by tending to a stable equilibrium state, with the property that any deviation from that state would have a tendency to diminish and converge back to it. This is often called an "attractor", in the sense that there would be a set of conditions under which the regulatory properties of a system would draw or "attract" the system's state into a given dynamical status. This also introduces a certain degree of robustness to the system, which can be advantageous when dealing with unpredictable natural conditions. Many attractors have, in fact, the property to generate some sort of rhythmicity, often as defined oscillations as in the case of limit-cycle oscillators. The maintenance of rhythmic behaviours, however, implies the existence of a tight control through feed-forward and/or feedback regulatory loops, intertwined into a self-organising mechanism.

To understand the viability of this putative "instability" in a living cell, one needs to consider the inherent complexity of such a biological system, both structural and functional, where several complex pathways work simultaneously and in parallel, regulating—as well as being regulated by—numerous feedback

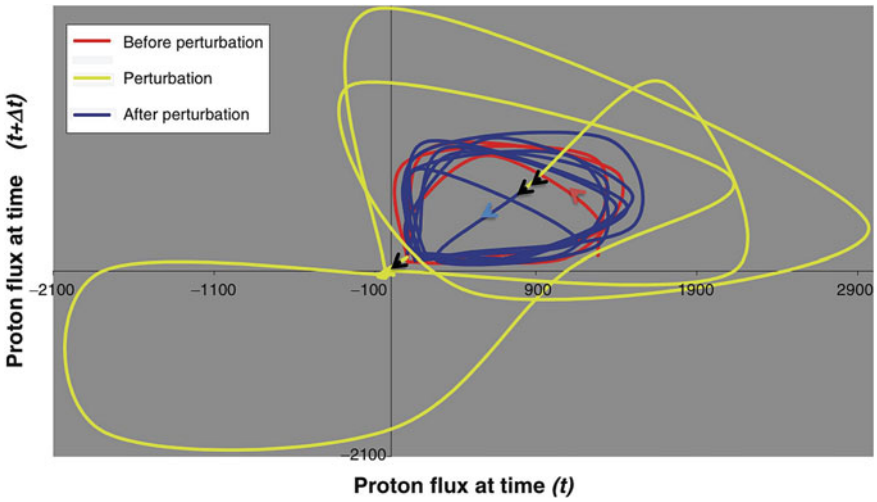


Fig. 6.1 Phase-space reconstruction using a time series of proton influx at the tip of a growing pollen tube. In a simplified definition, the "phase-space" represents the possible states of a system, being a graphic tool for analysing the dynamics of a complex nonlinear system. It is aimed at representing 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 (dt), 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 that, in this specific example, is a limit-cycle attractor. The convergence of trajectories in time represents a regulatory behaviour of this system, representing its dynamic characteristics. The regulatory 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 black arrowhead), likely corresponding to membrane de/hyperpolarisation, transiently resulting in reverse, mixed or null fluxes. After three cycles, however, the system re-entered the limit-cycle area (double black arrowhead) and became engaged in the original oscillatory regime (blue line; the blue and red arrows represent time-course direction; adapted from Feijó et al. 2001)

loops. Due to this inherent complexity, living cells appear as a perfect stage for oscillatory/chaotic behaviour to occur. At a theoretical level, it seems fairly simple to achieve this based on nonlinear dynamics: essentially, one needs only two variables and a manner to regulate their interactions in a sufficiently nonlinear manner, as expressed by delayed negative feedback and other regulatory loops in differential equation systems (Goldbeter 1991; Novák and Tyson 2008).

Oscillatory behaviours, such as the ones focused on this chapter, are often under the influence of cyclic attractors, or "limit-cycle" oscillators. In a phase-space plot, as shown in Fig. 6.1, oscillations correspond to the evolution of the system towards a closed curve. This periodic orbit is attained independently of initial conditions, given that they fall within the regulatory regime of the limit cycle—also called the

attractor basin. When at this stable dynamic condition, the key variables of the system should oscillate with a characteristic period, corresponding to the time to complete a full cycle. This period and the phase relationships between oscillations in different components then become the most easily accessible variables 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 also dealing with its importance at the level of cellular growth and morphogenesis.

6.2 Why Pollen Tubes?

The pollen tube is a unicellular structure appearing in the life cycle of higher plants to deliver the male gametes to the ovule, thereby enabling fertilisation 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 of several centimetres, 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 characterised 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. 6.2a; Boavida et al. 2005b).

Underlying this polarity, pollen tubes show a conspicuous organisation of ion dynamics, expressed in the polarisation/activation of specific channels/pumps, which results in well-defined membrane domains with specific ion pumping characteristics (Fig. 6.2b; e.g. Feijó et al. 1999, 2001, 2004; Zonia et al. 2001, 2002; Michard et al. 2008). These are presumably involved in maintaining cellular homeostasis in specific sub-cellular localisations and may give rise to different ionic gradients along the pollen tube, which seem to be necessary for growth (reviewed by Holdaway-Clarke and Hepler 2003). Somehow interrelated with these, membrane flow and the actin cytoskeleton are also highly dynamic (Fig. 6.2c). What makes pollen tubes an interesting and unique system is that all these components appear to oscillate with the same period. Not surprisingly, their growth rate, which corresponds to the macroscopic outcome of many 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 allows us to reason about causal relationships on a temporal sequence basis, aimed at developing a mechanistic model contributing to a novel, complex and fascinating paradigm of cell growth and morphogenesis.

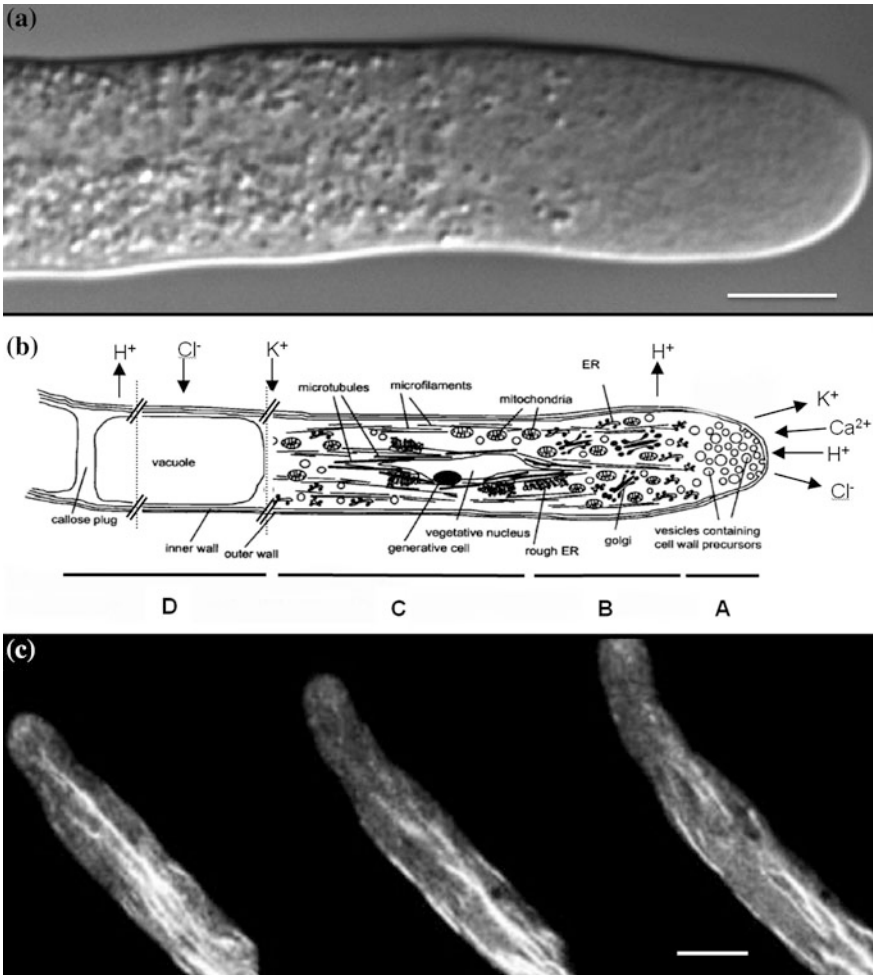


Fig. 6.2 **a** DIC image of a *Lilium longiflorum* pollen tube (diameter ~15 μm). The polarisation of the growing tip is striking; the growing apex is devoid of large organelles and presents no clearly organised intracellular movement (the "clear zone"). Behind, all the larger organelles are sorted out and move backwards in a fast, 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 of *Nicotiana tabacum* pollen tube growth transiently expressing a fusion protein construct of LAT52 (pollen-specific promoter), ADF (actin-depolymerising factor) and GFP, imaged with two-photon microscopy. The three frames are separated by 30 s each. Growth 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, the apical fringe, but this sequence was contrast-optimised to reveal the long actin cables promoting the vigorous streaming that is strongly characteristic of these cells (adapted from Boavida et al. 2005b) (bar in **a** and **c** represents 10 μm)

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

6.3 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 fertilisation proceeds on a first-come, first-served basis, and in order to compete successfully, individual pollen tubes must grow as fast as possible, in some species reaching rates of several centimetres per hour (Barnabas and Fridvalszky 1984).

One of the easiest observable oscillatory features using imaging analysis of 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, threefold 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; Michard et al. 2008). In fact, a closer examination of the fine growth characteristics of tobacco pollen tubes reveals more than only one discernible oscillatory component (Fig. 6.3). 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 red line). In addition, a moving average with a window of 3 s applied to these data (thick blue 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

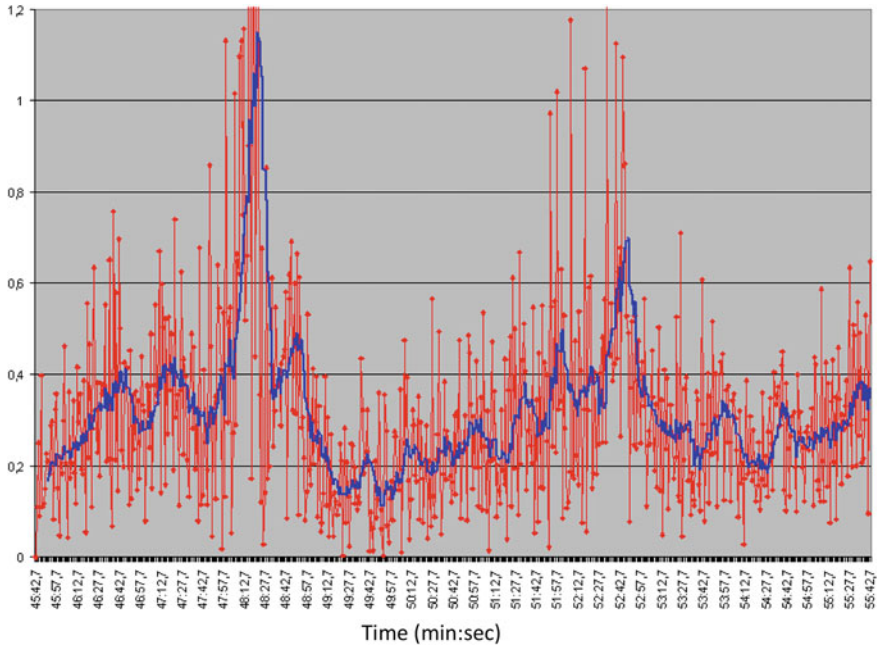


Fig. 6.3 Oscillatory growth characteristics of *Nicotiana tabacum* pollen tubes. A time-course movie was acquired using a PlanApo NA1.4, 60× Nikon lens with a Princeton Micromax 5 MHz camera, run by MetaMorph at full acquisition speed. Frame rate was higher than five frames per second. The sequence was then analysed 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, localisation 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. Raw data (*thin red line*) show an underlying oscillation with a pronounced peak in the Fourier spectrum with period of about 2.5 s. A moving average with a 3 s window was applied to the data (*thick blue line*) highlighting 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 characterised 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

correspond to the intermediate period (20–90 s), unless otherwise specified, we here consider these as characterising the oscillatory behaviour of pollen tubes and corresponding to the output of 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 observe 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. In fact, oscillating pollen tubes in vitro were reported to grow faster than others without clear oscillations in *Lilium longiflorum* (Messerli and Robinson 1997), although oscillations in vivo have never been observed (Iwano et al. 2009) and interspecific variation of this pattern has not been fully addressed. Furthermore, non-growing tubes can also show oscillations in other cellular components, suggesting the existence of an oscillatory system that is independent of growth (Iwano et al. 2009).

In terms of regulation, apical growth has been linked to reactive oxygen species (ROS) produced by NAD(P)H oxidases in root hairs and pollen tubes (Foreman et al. 2003; Potocky et al. 2007) mainly via modulation of calcium channels (Wudick and Feijó 2014). In addition, NO is able to regulate and reorient pollen tube growth in a Ca^{2+}-dependent manner (Prado et al. 2008). Lassig et al. (2014) observed remarkable changes in the amplitude and frequency of oscillations in growth rate on NAD(P)H oxidase mutants (RBOHH-1 and RBOHJ-2) in Arabidopsis. Pollen tubes from the double-mutant *rbohh-1 rbohj-2* showed elevated growth rates but also early tube collapse, which was correlated with high frequency and low amplitude of growth oscillations. These authors suggest that ROS would be involved on the attenuation of growth rate variations, promoting coordination between cell wall deposition and elongation rate and, consequently, not compromising growth. Intriguingly, the reduced ROS production also affected K^+ homeostasis, which could affect the turgor maintenance and cause the early pollen tube collapse. However, the mechanism by which ROS control the amplitude and frequency of growth oscillations and disturb K^+ homeostasis is not known.

The ultimate cause of growth is considered to be vesicle fusion with maintained turgor, which will be separately addressed below.

6.4 Under Pressure

Chloride has often been associated to turgor pressure, being involved in salt extrusion and controlling the system osmolarity (White and Broadley 2001). In pollen tubes, chloride fluxes are in phase with growth oscillation and can exceed 1000 pmol cm^{-2} s^{-1} (Zonia et al. 2001, 2002). This makes it very tempting to assume that turgor pressure is ultimately controlled by the 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; Winship et al. 2010). 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 of the magnitudes above that value were not reflected in any change in the 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 somewhat different physical properties. This could lead to localised changes in turgor that, due to some compensation mechanism, could be imperceptible elsewhere. Ultimately, such small variations in volume would lead to an infinitesimal decrease in magnitude that 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 groups (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 circumstantial arguments, nor have they produced any meaningful alternative data on this aspect of turgor in pollen tubes.

Following this same discussion, according to Winship et al. (2010), the time required to change the water potential in order to alter the internal hydrostatic pressure cannot account for the rapid changes in oscillatory growth rate observed in pollen tubes. In contrast, a hydrodynamic model has been proposed to explain the mechanism for stochastic and oscillatory growth, which predicts that turgor pressure oscillates during pollen tube growth (Zonia and Munnik 2007; Zonia 2010; Zonia and Munnik 2011). According to this model, turgor pressure is more elevated at the tip compared to the shank, being able to control exocytosis, cell wall mechanical properties and growth (Zonia and Munnik 2011). However, in a reply to Zonia and Munnik (2011), Winship et al. (2011) points out the impossibility of such assumptions, demonstrating that turgor pressure and water fluxes are "passive" parameters in plant cells that cannot be regulated directly. Even in oscillating pollen tubes, small pressure changes can be expected, but they would be the result of relaxation-driven cell wall expansion, and not the cause. In fact, in a model where oscillations in turgor were predicted, they were below the resolution of available experimental methods and the consequence of changes in ion concentrations (Liu and Hussey 2014). Furthermore, Kroeger et al. (2011) predicts that turgor and growth rate become uncorrelated for high turgor pressure, thus unlikely to be the driver of growth oscillations.

Despite of arguing for the lack of causal connection between turgor pressure and growth oscillations, it is important to stress that turgor is fundamental to growth, having a role not only as a spatial constraint for tip growth morphogenesis, but also on the penetration of the stigma and non-homogenous cellular structure within the female organs, probably by providing the necessary rigidity for penetration and growth on differential pressure conditions (Money 2001).

6.5 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 while also being 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). Although the absence of both callose and cellulose from the tip has been reported (Li et al. 1994; Ferguson et al. 1998), more recent studies have shown pectin enrichment at the tip, being the major cell wall component, as well as the presence of typical cell wall polymers in its lower proportion (Cai et al. 2011; Chebli et al. 2012). The amount of new cell wall material delivered at the tip by exocytic vesicles was found to predict to a high degree the magnitude of the subsequent growth pulse, in both lily and tobacco pollen tubes (McKenna et al. 2009).

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. Pectin methylesterase (PME), which are present in pollen tubes (Li et al. 2002), progressively de-esterify pectins generating carboxyl groups that will be cross-linked by calcium or borate, stiffening the apical wall (McKenna et al. 2009). Thus, according to its importance as a cross-linker of acidic residues, the concentration and availability of calcium also affect tip growth (Palin and Geitmann 2012; Wolf and Greiner 2012). Protons are also a product of the de-esterification by PMEs due to methoxyl to carboxyl conversion (Willats et al. 2001), being another possible regulatory candidate. They may act as regulator of cell wall since 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 proposed (Bosch and Hepler 2005).

Independently or not of ionic interaction, in some species, like 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, an extra deposition of the cell wall precursors can be measured. These could be explained by a partial uncoupling between growth rate and cell wall deposition, i.e. growth rate changes, but cell wall deposition or maturation occurs at a uniform rate. In other species, such as *Lilium*, these events do not occur naturally although they can be induced experimentally (Li et al. 1996).

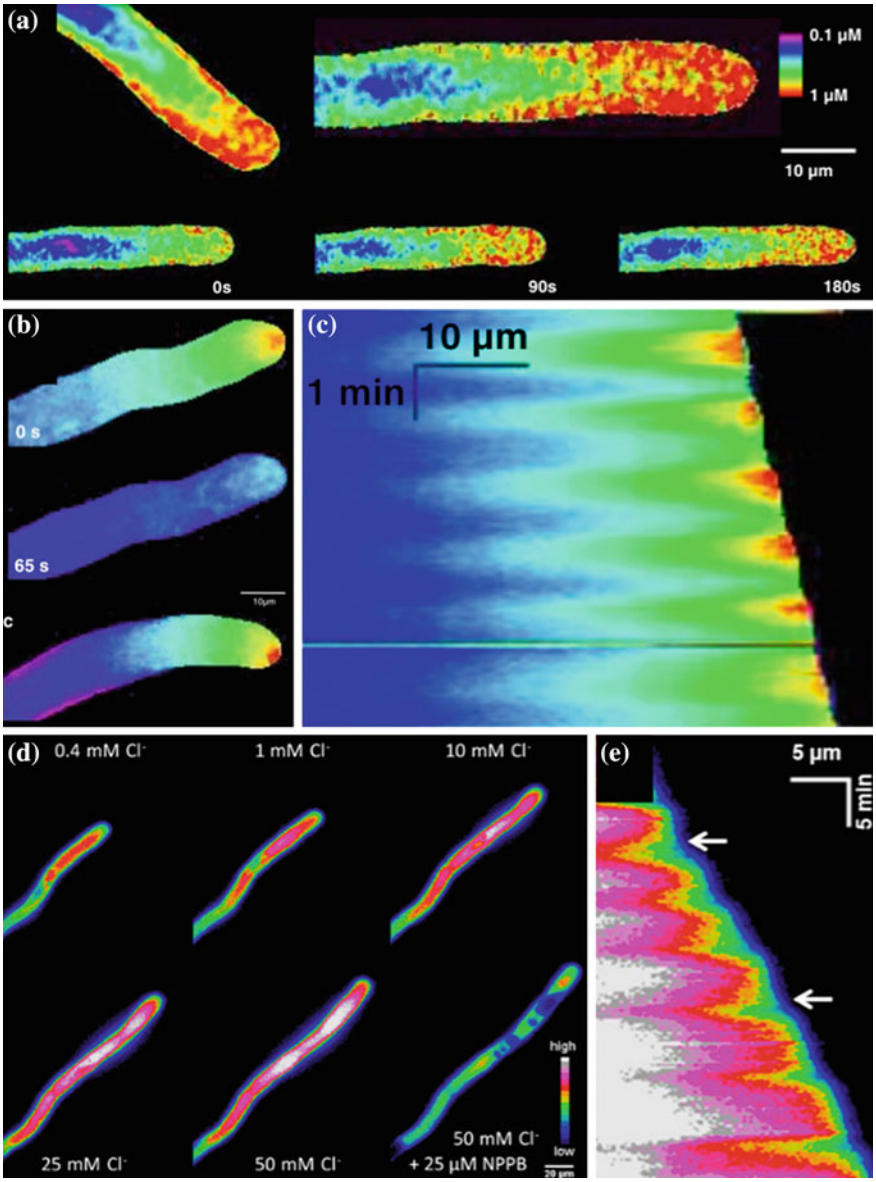
Rhamnogalacturonan II (RG II) and its cross-link with borate esters seems to be important for the formation of a pectic network present in the plant cell wall (Matoh and Kobayashi 1998) since it is needed to dimerize this polysaccharide. Hence, it has been suggested that the boric acid present in most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

Finally, at a broader scale, pollen tube growth polarity and cell shape could be maintained by a gradient in wall viscoelasticity across the growing tip, as described in root hairs (Dumais et al. 2006) and proposed for pollen tubes (Geitmann and Steer 2006; Geitmann and Parre 2004). Such mechanic gradients in stiffness were, however, recently challenged (Vogler et al. 2013). While the differential chemical nature of the cell wall at the tip is not under dispute, this apparent homogeneity in wall stiffness remains an intriguing point.

6.6 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 cells whereby calcium, protons and chloride are considered to be of major importance (Feijó et al. 1995, 2001; Gutermuth et al. 2013; see Fig. 6.4). Cytosolic free calcium ($[Ca^{2+}]_i$) is a highly versatile intracellular signalling molecule (Berridge et al. 2003; Konrad et al. 2011) which is known to be present in pollen tubes in a non-ubiquitous fashion, forming a tip-focused gradient (Miller et al. 1992) and also in pollen tubes growing in vivo (Iwano et al. 2009). Because the $[Ca^{2+}]_i$ pool at the apex oscillates (Pierson et al. 1996; Evans et al. 2001; Michard et al. 2008) and might participate in the vesicle fusion mechanism with the plasma membrane (Roy et al. 1999; Augustine 2001), it has also been associated with delivering cell wall precursors and, consequently, considered to support growth (Messerli and Robinson 1997). Furthermore, it has been experimentally verified that pollen tubes cease growth when the $[Ca^{2+}]_i$ gradient is dissipated and, conversely, growth interruption can cause $[Ca^{2+}]_i$ dissipation (Pierson et al. 1994). Another interesting result is that the highest $[Ca^{2+}]_i$ in specific spots at the tip seems to be predictive of the turning direction of pollen tubes, suggesting a role of tip focused gradients in polarity maintenance and guidance, (Malhó and Trewavas 1996).

Establishment of intracellular ion gradients is dependent on the uptake from the extracellular medium or release from intracellular stores. 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 vicinity of sub-membranar of the tube apex (Messerli et al. 2000). Despite a phase


(a)
0.1 µM
1 µM
10 µm
0s
90s
180s
(b)
0 s
65 s
c
(c)
10 µm
1 min
(d)
0.4 mM Cl-
1 mM Cl-
10 mM Cl-
25 mM Cl-
50 mM Cl-
50 mM Cl-
+ 25 µM NPPB
high
low
(e)
5 µm
5 min

◀ **Fig. 6.4** $[Ca^{2+}]_i$, pH_i and Cl^- oscillations imaged in *Nicotiana tabacum* pollen tubes using genetically encoded probes. **a** $[Ca^{2+}]_i$ imaged with yellow cameleon 3.3 under confocal microscopy. The concentration on the tip saturates the probe and is likely to reach up to 10 µmol, oscillating almost in phase with growth with a lag of a few seconds. The peak-to-trough ratio exceeds one order of magnitude. **b** Cytosolic pH imaged with ratiometric pHluorin and widefield microscopy. The acidic tip is conspicuous and can reach differential concentrations to the shank of almost 1 pH unit. A sub-membranar alkaline stripe at the shank is also visible. **c** Kymograph of an oscillatory sequence in proton concentration producing a pH variation of 0.3–0.5 over 6 consecutive cycles (a–c from Michard et al. 2008). **d** Oscillations of cytosolic Cl^- visualised with a Cl^--sensor. There is a negative gradient of Cl^- concentration from the tip, and evident oscillations of the cytosolic concentration especially on rising concentrations of extracellular Cl^-, as evidenced in the kymograph in **e**. (d–e from Gutermuth et al. 2013)

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, ranging from apparent synchronisation 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 (Holdaway-Clarke et al. 1997; Messerli and Robinson 1997) presumably measured the extension of the cytosol (affected by the Poisson decay of fluorescence in the extreme), whereas the latter (Messerli et al. 2000) measured the cell wall refringence properties used by DIC. As depicted by a processed DIC sequence for a lily pollen tube shown in Fig. 6.5, 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 showing oscillations, 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 counterintuitive, 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 most channels present in pollen tubes. Although Dutta and Robinson (2004) have described a putative stretch-activated calcium channel in pollen tube protoplasts, this data has not yet been confirmed by the other groups. Given the contentious nature of this type of channel and doubts about channel characterisation, this study is not the final word on calcium transport into pollen tubes. Adding to this the 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,

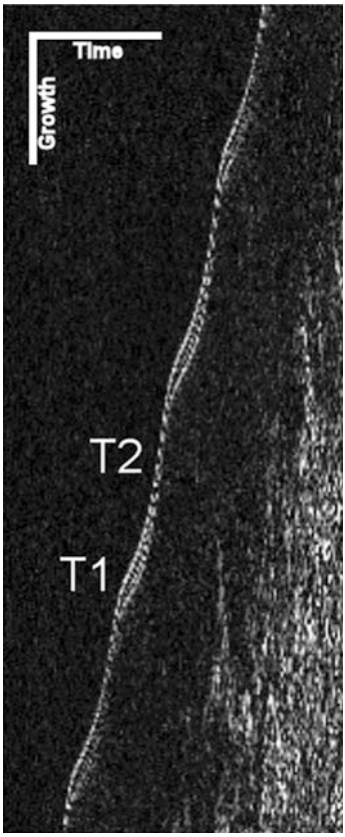


Fig. 6.5 Kymograph showing membrane and cell wall interfaces from a DIC time-lapse sequence of a growing pollen tube after a Prewitt detect edges filter—an image processing algorithm which highlights the limits of defined optical objects. The kymograph depicts the edges of the plasma membrane and cell 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 tube, 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. The plasma membrane (inner curve relative to the tube content) is the first to reduce and to increase speed. The cell wall presents a 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 maximising vesicle fusion but thereafter is independent of concentration (*horizontal bar*, 10 min; *vertical bar*, 10 μm)

image processing of sequences acquired with a lower NA, dry DIC lens (e.g. 20x) produces different phase shifts than when using a higher-quality oil immersion lens also with DIC (e.g. NA = 1.4, 40× oil immersion), which has revealed that chloride effluxes are essentially in phase with growth rate (Zonia et al. 2002).

Due to the complex interdependence between the processes involved in tip growth, causal relations cannot be immediately inferred solely from the description of phase relations, requiring knowledge about the role of other ions. The development of new imaging tools and biosensors, as genetically encoded probes, such as yellow cameleons, R-GECO1, pHluorin, and chloride sensor (Nagai et al. 2004; Zhao et al. 2011; Miesenbock et al. 1998; Markova et al. 2008, respectively) allowed significant advances on the field, providing a robust approach for monitoring spatio-temporal changes of the intracellular ion dynamics. Considerable advances, especially related to Ca^{2+} signalling, were possible due the extensive use of the yellow cameleon sensor, which became of significant ease through the use of stable transgenic lines.

Iwano et al. (2009) attempted to identify regular oscillations in vivo, monitored Arabidopsis and tobacco pollen tubes growing through the style but could only observe irregular oscillations. Although a regular frequency and amplitude of $[Ca^{2+}]_i$ oscillations were observed under in vitro conditions, they were mostly irregular under in vivo and semi-in vivo conditions.

Expressing calcium sensors of non-overlapping emission spectra, different studies showed qualitative changes of the $[Ca^{2+}]_i$ signature in the synergid cells during pollen tube reception in Arabidopsis (Iwano et al. 2012; Ngo et al. 2014). Transition to an oscillatory regime of $[Ca^{2+}]_i$ in the receptive synergid is triggered after pollen tube arrival at the micropylar region, indicating a putative involvement of $[Ca^{2+}]_i$ oscillations in cell–cell communication (Iwano et al. 2012; Ngo et al. 2014; Denninger et al. 2014). Presumably, the coordination of calcium crosstalk between female and male gametophytes is fundamental for sperm delivery and consequently fertilisation, which is disrupted in *fer*, *lre, and nta* mutants showing abnormal calcium signatures during synergid–pollen tube interaction. The FERONIA receptor-like kinase (FER) and the glycosylphosphatidylinositol-anchored LORELEI protein (LRE) seem to be necessary to activate the $[Ca^{2+}]_i$ oscillatory regime in the synergid cells, while NORTIA (NTA), a member of the plant-specific MILDEW RESISTANCE LOCUS O (MLO) family protein, seems to modulate the amplitude of such $[Ca^{2+}]_i$ oscillations (Ngo et al. 2014).

Furthermore, changes in the amplitude of calcium influx oscillations were described in the presence of glutamate receptor agonists and antagonists and, interestingly, with the agonist D-serine, which triggered calcium influx oscillations in previously non-oscillatory pollen tubes (Michard et al. 2011). Additionally, other sorts of oscillatory behaviour were described in semi-vivo systems, not only on the pollen tubes, but also on the different cells of the embryo sac (Denninger et al. 2014; Hamamura et al. 2014). Taken together, these results suggest that changes in frequency and/or amplitude of oscillations elicit different cellular responses.

Although calcium dynamics have received much emphasis on deciphering the mechanism of pollen tube growth, protons also have been shown to have a crucial role. 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 known to be involved in modulating enzymatic activity (Guern et al. 1991), phosphorylation (Blowers and Trewavas 1989),

cytoskeleton activity (Beaulieu et al. 2005; Yonezawa et al. 1985), endo/exocytosis (Cosson et al. 1989; Smith et al. 2002), energetic cycles (Sanders and Slayman 1982) and even as a *bona fide* neurotransmitter (Beg et al. 2008). 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 set-up, it was possible to demonstrate that the tube shows an acidic domain appressed to the membrane apex, which exists only when the tube is growing, and a sub-apical alkaline area of magnitude apparently oscillating in anti-phase with the growth rate (Feijó et al. 1999). The exact role of this gradient is still far from clear but possible links lie in the control of calcium channels (Chen et al. 1996), or the localisation and rates of endocytosis and/or exocytosis (Davoust et al. 1987; Smith et al. 2002) or, yet, actin cytoskeleton modulation via pH-dependent actin proteins such as the actin-depolymerising factor (ADF; Chen et al. 2003). The localisation of a pollen-specific H^+-ATPase (Nt AHA), which is excluded from the tip in tobacco pollen tubes, provides evidences that correlate with the H^+ extracellular fluxes and the existence of an acidic domain at the extreme apex (Certal et al. 2008). Lefebvre et al. (2005), using immunocytolocalisation, also identified a plasma membrane H^+-ATPase (NpPMA5) in *Nicotiana plumbaginifolia* that shows the same localisation. Given these results, it is tempting to interpret that proton dynamics is involved on mechanisms for polarity maintenance that ensure pollen tube growth.

A tip-focused Cytosolic chloride was described upon the transient expression of chloride sensor in tobacco pollen tubes (Gutermuth et al. 2013; see Fig. 6.4d, e). Oscillatory variations of pollen tube growth rate are in anti-phase with oscillations in the cytosolic anionic concentration, supporting Zonia et al. (2001, 2002) results. The existence of a negative gradient of cytosolic anion concentration focused on the tip in negative correlation with the $[Ca^{2+}]_i$ suggests a possible mechanism for controlling the anionic fluxes at the tip. After a molecular screening, Gutermuth et al. (2013) performed the identification and characterisation of the anion channel SLAH3 and its interaction with both Ca^{2+}-dependent protein kinases CPK2 and CPK20. Variations on $[pH]_i$ and $[Ca^{2+}]_i$ were also correlated with changes in extracellular chloride concentration, indicating a putative temporal coupling of anion and calcium/pH homeostasis. This Ca^{2+}-dependent regulation of anionic channels is a good example of the expected interconnected relationship among ions at the tip, and of how intricate is the regulation of ion dynamics in pollen tubes. Identifying sensors that could act as cytosolic controllers capable of ensuring ion homeostasis would contribute to the elucidation of the regulatory repertoire and localise missing links of downstream pathways, enabling inferences about the ion signalling cascades.

6.7 On the Outside: Ions and Fluxes

Four decades ago, Weisenseel et al. (1975) demonstrated that pollen tubes are living electric dipoles, 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 steady state to a pulsating behaviour as pollen tubes reached a certain length, with the latter 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 artifacts 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 and, on the other hand, are operated at much lower frequencies, thus being less 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; 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 calcium 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; Michard et al. 2008; reviewed by Michard et al. 2009). Representative time series of extracellular proton and calcium influx at the tip of pollen tubes of Arabidopsis, lily, tobacco and Agapanthus recorded using ion-selective vibrating probe are shown in Fig. 6.6.

The study of calcium influx in the tip has revealed a clearly oscillatory behaviour (Fig. 6.6; Pierson et al. 1996). These fluxes exhibited the same period as growth rate 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 calcium at the tip 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 calcium into the cytosol, due to the binding of this ion to carboxyl groups that appear unbound in the newly formed cell wall (Li et al. 1994). The specific kinetics of this binding would be an explanation for the reported delay between the peaks of intra- and extracellular ionic oscillations relative to growth. This theory does not take into consideration another possibility set forth by Carpita and Gibeaut (1993), who proposed that the reaction between Ca^{2+} and the acidic residues in

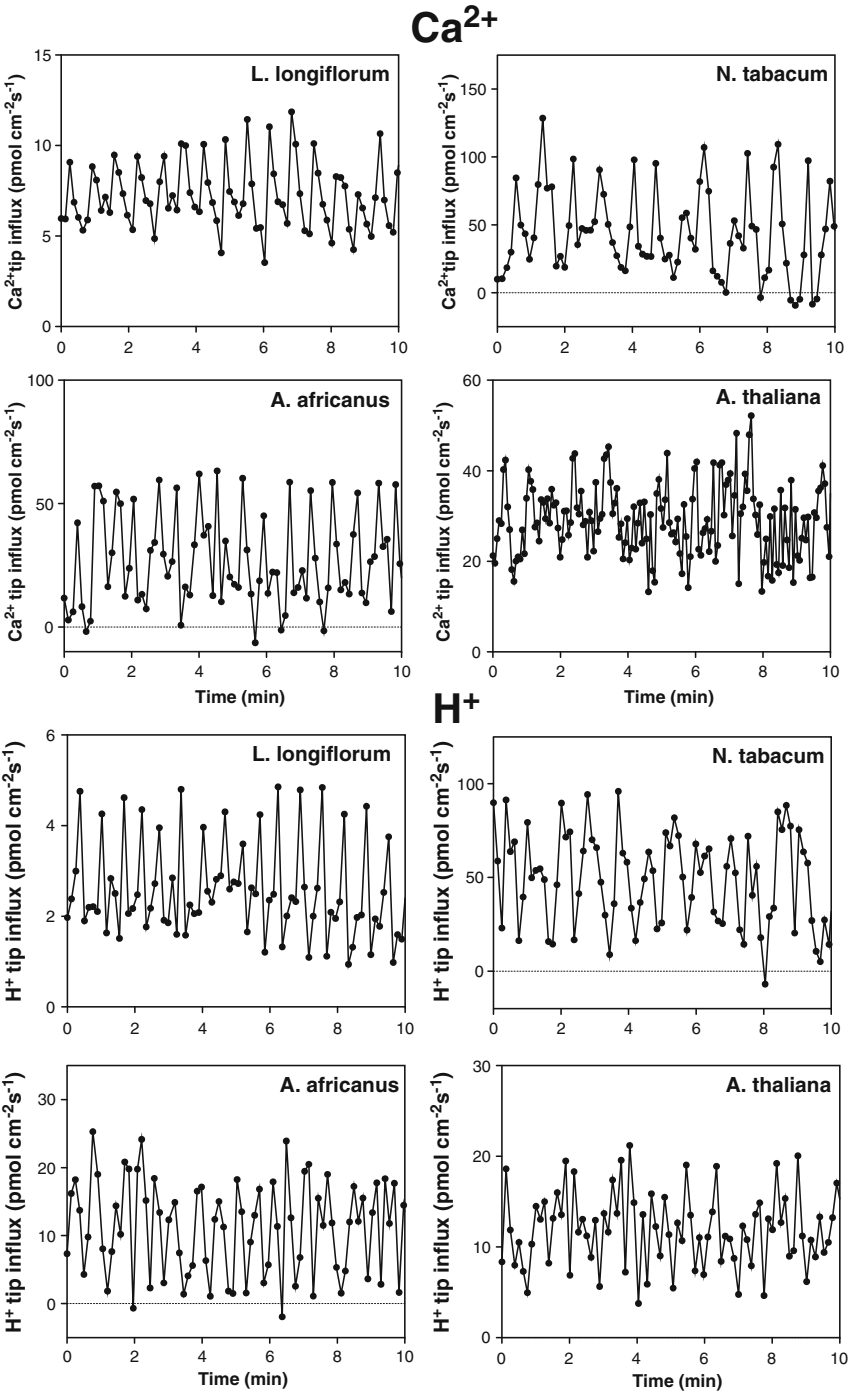


Fig. 6.6 Representative time series of oscillatory traces of extracellular Ca^{2+} and H^+ influx at the tip of *Lilium longiflorum, Nicotiana tabacum, Agapanthus africanus* and *Arabidopsis thaliana* pollen tubes (raw data). Tubes germinated in vitro with at least 200 μm of length were measured using the ion-selective vibrating probe technique

homogalacturonan pectin and cross-link adjacent chains in the cell wall would confer rigidity to it. This appears not to occur since growth rate does not seem to be affected as it would be expected under such hypothesis, remaining 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.

Michard et al. (2008) characterised the intra- and extracellular dynamics of the calcium and protons in tobacco pollen tubes and reported, using Fourier and wavelet analysis, the presence of oscillations with one or two major components for both ions. This result could suggest the existence of at least two molecular oscillators controlling such behaviour in pollen tubes. However, to dissect the mechanism behind this process, the identification of the key players responsible for generating and sustaining oscillations in pollen tubes is necessary. The molecular identification of the underlying ion transporters located at the tip and the intracellular sensors is fundamental to make assumptions about the cellular mechanism/regulation. Frietsch et al. (2007) identified the presence of the cyclic nucleotide-gated channel (CNGC) 18 at the plasma membrane of growing tip in *Arabidopsis* pollen tubes, related to Ca^{2+} transport. Additionally, Michard et al. (2011) reported the identification of glutamate receptor-like channels (GLRs) linked to Ca^{2+}-transport, more specifically, promoting the Ca^{2+} influx and consequently modulating apical $[Ca^{2+}]_i$ gradient in pollen tubes. The identification and localisation of other putative ion channels is still incomplete and would be of fundamental importance to better characterise the ion dynamics.

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 studies, an important point because the ionophore cocktails usually utilised 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 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 oscillations on both

growth rate and chloride efflux occur in phase, a relationship between chloride efflux and vesicle trafficking towards the apex was also suggested (Zonia et al. 2002).

Messerli et al. (2004) have reproduced these results both in terms of spatial distribution and chloride-equivalent fluxes, but questioned the reliability of the 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. The argument was further extended by the alleged absence of chloride channels in pollen tubes, as detected by patch-clamp (Dutta and Robinson 2004). This would be a remarkable finding, since it would turn pollen tubes into the only plant cell that would 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) indicated various putative pollen-specific expressed chloride channels. Later analysis by properly controlled patch-clamp experiments showed the clear existence of these anionic currents (Tavares et al. 2011), and a Ca^{2+} regulatory feedback loop of one such channel SLAH3 (Gutermuth et al. 2013) that may be part of the molecular basis of the oscillator.

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

6.8 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 as well.

Inhibition of actin polymerisation by means of cytochalasin D “freezes” the cytoskeleton; thus, 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 latrunculin B (latB), which also blocks actin polymerisation, oscillations are abolished (Vidali et al. 2001) and growth rate decreases slightly, suggesting that actin polymerisation has an active role in controlling pollen tube oscillations, which is consistent with vesicle oscillations at the tube apex (Parton et al. 2001). The actin-stabilising drug Jasplakinolide (JASP) was also

shown to have this effect (Cárdenas 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 depolymerisation (latB) or stabilisation/polymerisation (JASP).

The tip-localised F-actin microfilaments oscillate with phase differences of 15–30 s preceding growth in tobacco pollen (Fu et al. 2001), suggesting a strong involvement of actin in the growth process. Networks of actin microfilaments and microtubules (MTs) are both prominent in pollen tubes. The actin cytoskeleton is involved in cytoplasmic streaming and in delivering secretory vesicles to the apical plasma membrane (Hepler et al. 2001; Lovy-Wheeler et al. 2007; Cheung and Wu 2008; Chebli et al. 2013; Rounds et al. 2014), while MTs were implicated in actin-independent internalisation and exocytic events taking place in the apex (Idilli et al. 2013; Onelli et al. 2015), although cytoplasmic streaming is not dependent of them.

The apical actin filaments are organised as a fringe, a palisade of longitudinally oriented microfilaments, positioned in the cortical cytoplasm close to the plasma membrane (Lovy-Wheeler et al. 2005; Vidali et al. 2009). Pharmacological evidences demonstrate that latB causes the degradation of the actin fringe and inhibits pollen tube growth, thus indicating that actin is required for such process (Cárdenas et al. 2008). Apical actin is considered to be critical for exocytosis, given that the fringe is involved in targeting vesicles to preferred sites at the tip, thereby contributing to changes in growth direction and polarity control (Dong et al. 2012; Rounds et al. 2014), indicating a close coupling between actin fringe and polarised pectin deposition.

Actin-binding proteins, including actin depolymerisation factor—cofilin, formin, profilin, and villin; and signalling proteins, such as Rho-of-plants (ROP) GTPases; as well as their effectors (ROP interacting crib-containing proteins [RICS] such as Ric3, Ric4 and ICR1), also have been shown to play critical roles in growth, calcium, actin dynamics and membrane trafficking (Fukata et al. 2003; Hwang et al. 2005). Different actin-binding proteins can interact with calcium and promote the fragmentation of actin microfilaments, as shown for villin/gelsolin, which compromise the stabilising effects of an actin-binding LIM domain protein (Wang et al. 2008) and suppress actin polymerisation (Kovar et al. 2000). Thus, both the structure of the motile apparatus as well as the activity of the motor protein may be inhibited by Ca^{2+} at the apex of the tube.

Actin is controlled by Rho GTPases, among other factors (Fukata et al. 2003). Reports suggest 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). In pollen tubes, ROP1 is located in the cytosol but appear 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 localised differentially in the cell, the

antagonistic action of RIC3 and RIC4 can be explained by the interaction with their downstream targets, $[Ca^{2+}]_i$ and actin, suggesting the existence of a self-regulatory system. ROP1 activity, described through the fusion protein RIC4::GFP, has been shown to oscillate with peaks of activity occurring before growth rate peaks, in phase with F-actin tip assemblies. ROP1 is present at the tip in the plasma membrane, in an asymmetric distribution "anticipating" direction changes in tubes, which suggests some kind of regulatory activity (Hwang et al. 2005). RIC3 activity, involved in $[Ca^{2+}]_i$ 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. (2005) suggest that the RIC3 pathway is longer and more complex than the RIC4 pathway, consistent with the delay between responses. Furthermore, 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).

Models explicitly proposing the existence of ROP1-based oscillators will be discussed in the modelling section below.

6.9 Membrane Trafficking and Signalling on the Road

In nature, pollen tube development is not an independent cellular process but rather a result of pollen–stigma interactions, which direct pollen tube growth through physical and chemical cues. The membrane plays a very important role in this respect because it is in contact with both 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.

Given that the membrane, as an interface with the exterior, is critical for this behaviour, the way it is deposited and recycled should underlie growth oscillations, as vesicles have to interact with cell wall precursors as to generate nonlinear 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 precedes growth peaks. Even more relevant, in a second set of experiments, these authors provided the first published evidence that oscillations may continue even when pollen tube growth has stopped, since membrane trafficking oscillates as long as intracellular streaming continues, independently of growth (Parton et al. 2003). According to these authors, by inhibiting vesicle trafficking with brefeldin A (BFA), 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, suggests the existence of 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 still occurring.

The other major role of the membrane involves phospholipid signals (Meijer and Munnik 2003). 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 IP_3, 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. (2006) 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 localisation pattern. In non-growing pollen tubes, this substrate was localised homogeneously in the membrane while, 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). Studies in tomato pistils reported that the STIGMA-SPECIFIC PROTEIN1 (STIG1) acts as a peptide signalling molecule, having a binding site for pollen receptor kinase (LePRK2) and also a phosphatidylinositol 3-phosphate (PI(3)P) binding motif. Both interactions are required for pollen tube growth, since they affect ROS production (Huang et al. 2014). This study exemplifies the complexity of signalling cascades, especially the ones mediating pollen–pistil interactions. The development of new sensors (Potocky et al. 2014), combined with the localisation of different signalling phospholipids, would contribute to elucidate its dynamics and correlation with oscillatory growth.

The role of the endomembranar 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 organisation (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 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. The role of membrane trafficking and specially of ROP1 in generating oscillations will be further discussed in the section below.

6.10 Modelling Oscillations: A Long and Winding Road

6.10.1 What Is the Pollen Tube Oscillator Anyway?

Although pollen tube growth has been modelled in many ways, featuring diverse mathematical formalisms, numerical methods and working hypotheses, there is no integrated view on the oscillatory system underlying the observed rhythms (reviewed in Kroeger and Geitmann 2012 and including Kroeger and Geitmann 2013; Pietruszka 2013; Liu and Hussey 2014). A fundamental challenge is to determine the essential regulatory interactions capable of generating oscillations. Simplistically speaking, biological oscillators are built around a core negative feedback loop, which generates the oscillations, with additional positive feedback loops, which enable the tuning of oscillatory characteristics and/or confers robustness (Novák and Tyson 2008; Tsai et al. 2008). Models of pollen tube oscillations proposed so far differ on the nature and organisation of the core negative feedback loop, with some involving growth directly or indirectly and yet others based on alternative systems.

6.10.2 Growth-Based Oscillators

The main negative feedback loop in the growth-based models of Kroeger et al. (2008), (2011) and Kroeger and Geitmann (2013) involve Ca^{2+} entry through stretch-activated channels, promoted by elevated growth rates, which inhibits vesicle exocytosis through decreased actin polymerisation (Kroeger and Geitmann 2013), thereby inhibiting growth. The predicted phase relationship between Ca^{2+} influx and growth (Kroeger et al. 2008) and changes in amplitude and frequency of oscillations upon changes in turgor or cell wall viscosity (Kroeger et al. 2011; Kroeger and Geitmann 2013) are coherent with part of the available experimental data, with important caveats. In an elegant mechanical model, the negative feedback promoting oscillations was based on the cell wall expansion inhibiting the delivery of exocytic vesicles, also assuming the opening of calcium stretch-activated channels that inhibits actin polymerisation, being compatible with the phase relationship between growth rate and exocytosis, among other variables (Rojas et al. 2011). However, oscillations in these and other (Pietruszka 2013) growth-based models rely on mechanical force caused by growth, being unable to explain the regular $[Ca^{2+}]_i$ oscillations observed in non-growing tubes (Iwano et al. 2009). Multiple interacting self-sustained oscillators were argued to coexist in pollen tubes (Kroeger and Geitmann 2013), however, there is still little supporting evidence as discussed below. Furthermore, assuming the existence of tip-localised Ca^{2+} stretch-activated channels is still controversial, until further confirmation of their existence and characterization of their properties is provided.

6.10.3 The ROP1 Oscillators: Growth-Related

Models where growth is only indirectly involved include oscillators based on the regulation of the trafficking protein ROP1, responsible for promoting F-actin polymerisation and the fusion of exocytic vesicles at the tip (Hwang et al. 2008; Yan et al. 2009). Hwang et al. (2008) proposed a negative feedback of exocytosis on ROP1 both by a dilution effect, since exocytic vesicles do not contain ROP1, and by direct inactivation by REN1 (ROP1 ENHANCER 1), which is transported by the vesicles. The negative feedback would constrain ROP1 activation at the tip, assisting in polarity maintenance, but the model was not explored mathematically and the biological pathways involved must be further elucidated (Hwang et al. 2008; Wu and Lew 2013). In a mathematical model of a ROP1 oscillator, the negative feedback was based on Ca^{2+}-induced F-actin depolymerisation or activation of negative ROP1 regulators (Yan et al. 2009). ROP1 activation would lead to a delayed increase in $[Ca^{2+}]_i$ concentrations by the effector RIC3, which would trigger Ca^{2+} influx and calcium-induced calcium release from intracellular stores. The model predicts a correct phase delay of $[Ca^{2+}]_i$ and F-actin, but the Ca^{2+} currents have to be validated and put into perspective with other ions. Although not including growth directly, both of these models would imply in elongation of the plasma membrane due to vesicle fusion, unless compensated by tight coupling with endocytosis, sharing the problem of growth-based models of not addressing the maintenance of the oscillations in the absence of growth.

6.10.4 Growth-Independent Oscillators

Ion dynamics is a natural candidate for a mechanism that could oscillate even without the participation of growth. Oscillations were reported in a model based on membrane voltage and its effects in ion conductance, including Ca^{2+}, H^+, Cl^-, K^+ in a Hodgkin–Huxley-like formalism and featuring Ca^{2+} entry through voltage-activated channels (Michard et al. 2009; Liu et al. 2010). In the later, the tip and shank were modelled as two different compartments, allowing the reproduction of some basic phenomena observed experimentally, as the occurrence of oscillations at the tip but not in the shank, and the possibility of having Ca^{2+} oscillations without growth (Liu et al. 2010). A further extension also incorporates cell wall expansion, turgor pressure and other hydrodynamic variables, proposing a context-dependent role of turgor by characterisation of different regimes (Liu and Hussey 2014). The model also predicts the possibility of small amplitude oscillations in turgor, but which would be well below the resolution of available experimental methods. However, the complexity of these models does not allow understanding the origins and properties of the oscillations, so the core oscillator and its dynamic characteristics remain unclear. Furthermore, although the effort of developing an integrated model is commendable (Liu and Hussey 2011), the magnitudes and direction of the fluxes are disputable and many parameter values

lack validation, rendering the model premature. It should be noted that growth is indeed part of these models and influences oscillations (Liu et al. 2010; Liu and Hussey 2014), however, does not compose the core oscillator, which is based on membrane voltage and ion dynamics in the pollen tube tip and shank.

6.10.5 Multiple Oscillators?

Robust periodic behaviours of numerous cellular processes showing defined phase relationships have been reported, favouring the existence of a core pollen tube oscillator, or at least a synchronised oscillatory system, that would induce rhythmicity in downstream pathways. However, determining the identity of this oscillator is challenging since it may be composed of multiple regulatory loops or even of several distinct self-sustained oscillators. Favouring the multiplicity of oscillators hypothesis, Rounds et al. (2010) reported that oscillations in growth rate persist—although with longer period—after inhibition of ATP production, which abolished oscillations in NAD(P)H fluorescence, a proxy for electron transport chain (ETC) activity. Normally, oscillations in the ETC are synchronised and precede growth rate, interpreted as not being the cause of growth oscillations but, instead, a distinct limit-cycle oscillator. However, concluding the existence of independent self-sustained oscillators requires rigorous experimental design and data analysis, evidencing distinct free-running period and the effects of putative interoscillator coupling. In fact, different frequencies were proposed to compose oscillations in pollen tube growth (as in Fig. 6.3), ion fluxes (Michard et al. 2008) and vesicle trafficking (Parton et al. 2003), but further investigation is required. Many lessons can be learned by the long quest of dissecting oscillatory systems in other fields, such as the circadian oscillators (Bell-Pedersen et al. 2005) and polarity establishment systems (Mogilner et al. 2012).

6.10.6 Modelling Oscillators: Unwinding Structure and Function

Despite of numerous experimental and theoretical efforts, both the biological functions and the origin of the oscillations observed in pollen tubes remain to be determined. The absence of reported in vivo oscillations (Iwano et al. 2009) further raised the question of functionality, which has an interesting parallel in other systems involved in polarity establishment. In many different eukaryotic organisms, oscillations were proposed to derive from an unpolarised state, where an excitable system enters the oscillatory regime due to the lack of a stabilising directional cue (Wu and Lew 2013; McClure and Lew 2014). Thus, rather than being functional in themselves, oscillations could reflect a particular dynamical regime of a polarity system. Clearly, this does not discard other potential functions, including sensing and guidance, but constitute a possible reason for not observing oscillations in vivo.

A major difficulty of finding the origin of the oscillations is that virtually any behaviour can be expected from a model with sufficient parameters, as epitomised by John von Neumann's quote: "with four parameters I can fit an elephant and, with five I can make him wiggle his trunk" (Dyson 2004). This is not to invalidate the strive for detailed models, which are necessary to understand the role of specific components of interest, however, they require a compatible amount of data. In contrast, more general models help to unveil the fundamental principles driving a phenomenon of interest, helping to identify the key regulatory interactions, albeit lacking details. Thus, there is no inherently correct level of modelling (Mogilner et al. 2012), instead models vary on how informative they are according to the available body of knowledge. Ultimately, the oscillatory phenomenon has to be better characterised by establishing the underlying frequencies, phase relationships and synchronisation state in different pollen tube growth regimes, with rigorous experiments and data analysis. The long and winding road that leads to the heart of the pollen tube oscillator can only be travelled in the company of multiple modelling approaches and data with the appropriate spatio-temporal resolution.

6.11 Conclusion and Future Prospects

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 and with appropriate temporal resolution, several independent variables relevant to the description of the system should be assessed continuously and, finally, the synchronisation of these variables in time should be evaluated (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 dynamical 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 noise or chaotic behaviour. It is crucial to know which factors are involved in maintaining the synchronised behaviour, which would give some insights about its onset and transitions between dynamical regimes.

Using differential interference contrast images or fluorescence imaging of appropriate genetically encoded probes, it is easy to estimate growth through pattern recognition softwares, which traces the location of the pollen tube tip. Hence, it is possible to collect precise data and attempt to establish which cellular 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 cannot be identified by Fourier analysis due to changes in oscillatory regimes in time or to the number of points, which are usually impeditive

for the traditional approaches. Nevertheless, it is still unknown if pollen tubes have one or more oscillatory sub-systems, and how each regulatory point may influence the whole system. Measurements will have to be done as to minimise the influence of external factors to avoid triggering of any kind of periodic response, as well as 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. To date, the strongest candidates seem to be the membrane trafficking machinery and ion dynamics, since they are the only candidate mechanisms that may oscillate independently of tube extension (Parton et al. 2003). However, one cannot ignore the fact that blocking actin polymerisation 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 characterising different cellular states. The fact that concentration peaks do not seem to anticipate growth spurts suggests that it could work rather as a switch. After a certain concentration threshold, maximum exocytosis would occur but the levels of calcium at the apex would continue to rise, possibly having other roles as well. This threshold explains why the growth phase peak can occur only seconds before calcium peaks, with no subsequent influence of the latter in tube growth rate. Importantly, rigorous studies allowing proper inference of causality must be performed, since as "correlation does not imply causation", temporality also does not necessarily imply in causality.

Despite the fact that several oscillator components are known, the interactions between these remain obscure, leaving us far from the whole picture of the biophysical and biochemical components allowing this specialised cell to achieve such high growth rates and complex dynamics. Given that pollen tubes oscillate in vitro independently of other cells or tissues, it may be too simplistic to consider these oscillations as a way to control cell growth alone. The ionic currents generated by oscillating pollen tubes have peaks of higher magnitude than the steady-state growth currents and could be used as signalling cues to the surrounding medium. To date, evidences that oscillations are involved in the communication with the female gametophyte have been raised, although the challenges of imaging pollen tubes in vivo do not usually allow an accurate measurement of growth and to determine under which precise 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 of explaining not only the oscillatory properties of the system but also providing a link to its relevance in growth and morphogenesis. The fact that the observed rhythms are generated in vitro by the cell itself suggests that oscillations could be the natural way for these cells to develop and to achieve their goals, while if it lacks a direct biological function in vivo, having measurable rhythms can help to understand other mechanisms regulating intracellular physiology.

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Chapter 7
Oscillations in Plant Transpiration

Anders Johnsson

Abstract Plants take up water via the root system and transpire water vapor through stomatal openings in the leaves. 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 some hours). 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. Modeling has been strongly extended in later years. The chapter concentrates on experimental findings of overt transpiration rhythms as well as on oscillatory events in the guard cells. Results on the occurrence of rhythms, their period, amplitude, and modulation are discussed. The impact of external environmental parameters on the rhythms is dealt with, e.g., humidity, light, osmotic changes, and 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 be chaotic and the advantages of such a system are shortly discussed. The behavior of transpiration rhythms reveals many dynamic features of the stomatal control system. Some comments and a short discussion on water-use efficiency (WUE) of plants and vegetation end the chapter.

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7.1 Introduction and Overview

Plants transpire water vapor through *stomatal* pores in the surfaces of their leaves or needles. The *stomata* have a typical dimension of about 10 μm. The width of the stomatal opening is regulated by an oppositely placed pair of *guard* cells, and the volumes of these cells are fundamental in controlling the size of the pore opening. Despite the small pore dimensions, the global transpiration through stomata is estimated to account for up to 80–90 % of the terrestrial evapotranspiration, thus responsible for the cycling of about 60,000 km^3 water per year (or about 60×10^{15} kg per year; Jasechko et al. 2013, who also cite earlier estimates of the order of 14,000–41,000 km^3 per year).

Thus, the stomatal pores in the terrestrial biomass play an outstanding role in the global water flux. But they also control CO_2 transport from the atmosphere to the stomatal cavities (estimated to represent 125×10^{12} kg carbon per year; Beer et al. 2010) to be used in the photosynthetic processes of the plants. The stomata and the guard cells are thus the site of delicate and fundamental regulatory mechanisms that control the pore openings under varying external conditions.

The water transpiration of many plants (including bushes and trees) can be *rhythmic,* or oscillatory, under a wide variety of environmental and experimental conditions (Kaiser and Paoletti 2014; Barrs 1971; Hopmans 1971; Cowan 1972; Raschke 1979; Sack and Holbrook 2006; Yang et al. 2005; further references at end of chapter). The pore size and its characteristics then change rhythmically, and the guard cell volumes will change rhythmically and so will other variables in the water system of the plant.

Oscillations with a period of about 1–2 h or less have been studied both in monocots and in dicots, in whole plants/trees and in young plants as well as in old ones. Longer periods, e.g., circadian, also exist (see Sect. 7.4.2). Plants showing transpiration rhythms have quite different stomata 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). Altogether, oscillatory transpiration is a general phenomenon, mirroring basic mechanisms in quite different species under quite different conditions.

The water transpired by a plant has to be replaced by water taken up from the soil/medium. It then enters the root system and moves via the *xylem* structures into storage elements and the leaves, underway experiencing different flow resistances. Storage tissue can be part of the trunk in trees. In the leaves, it reaches three important dynamic elements in the water regulation of the plant: the *guard cells* mentioned above with their neighboring *subsidiary* cells (or *supporting tissue)* and *mesophyll cells*. The water then evaporates through the stomatal pores via the

stomatal cavities. A rhythmic transpiration will become accompanied by a rhythmic water transport as well as rhythmic changes in volume, in concentration of ions, in transport of ions, in membrane potentials, etc. of the cells involved. In Sect. 7.2, this description of water transport will be discussed.

Section 7.2 will also focus on the fact that an overall transpiration rhythm from an individual 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 (e.g., Kaiser and Kappen 2001; Mott and Buckley 2000; Kamakura et al. 2012a, b). However, a sufficiently large number of synchronized stomata must be present when rhythmic transpiration is recorded.

During the last decades, much interest has been focussed on oscillations in the *calcium concentration* in guard cells(see reviews by Yang et al. 2004, 2005). $[Ca^{2+}]_i$ oscillations form a signaling pathway and $[Ca^{2+}]_i$ in the guard cells may act as a second messenger (McAinish et al. 1995; Blatt 2000b; Yang et al. 2003; Chap. 9 of the present volume). Water channels, *aquaporins*, might be downstream elements of $[Ca^{2+}]_i$ oscillation signaling (Kaldenhoff and Fischer 2006; Yang et al. 2005; Lopez et al. 2004; Nielsen et al. 2002). In Sect. 7.3, we will discuss some aspects of these guard cell oscillations. In Sects. 7.4 and 7.5, we will focus on experimental findings in studies of oscillatory transpiration under different experimental and environmental conditions.

When the whole plant organism—for example, a bush or a tree—or big parts of it show a rhythmic overall transpiration, its different parts must also be synchronized. This means that *coupling* between the different parts (and their stomata) will exist and they constitute a network. Examples of such overall transpiration oscillations have been found in several species.

A complete description of the oscillations in a network 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 1999). The network of cells can produce effects which are not found in studies of reactions and mechanisms in individual cells (or isolated, e.g. as protoplasts). One has, therefore, 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 1999).

With a network concept in mind, results from measurements on oscillatory transpiration of plants are discussed in this chapter and in Sect. 7.5, some remarks and comments will be given.

7.2 Hydraulic Models for Rhythmic Water Transpiration

7.2.1 *Overall Description—"Lumped," Hydraulic Model of Leaf*

Water will be transported between two points in the plant if they have different *water potentials*. The concept of water potential, ψ_w, is derived from the thermodynamic definition of electrochemical potential of water (see Nobel 1999). 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 1999), but some examples should be given here. Typical values for roots can be -5×10^5 N/m^2, in xylem of roots about -6×10^5 N/m^2, in the xylem of leaf at 10 m height -8×10^5 N/m^2. All values are relative to pure water under standard conditions. The water (vapor) potential in the air outside the leaf, ψ_{air}, is dependent on the water content of the air and is lower the dryer is the air. ψ_{air} at 95 % relative humidity is about -70×10^5 N/m^2 (= −70 bars), at 60 % about -700×10^5 N/m^2, and at 50 % about -950×10^5 N/m^2.

The transpiration through the guard cell pores depends on the water potential difference between the outside air and the air in the stomatal cavity, $\Delta\psi_w = (\psi_{air} - \psi_{stoma})$, as well as on the conductance of the stomatal pore, g_s. An open pore has a high g_s and a totally closed pore a g_s equal to zero. Since the pore opening in this way is crucial for the transpiration, the volumes of the guard cells and their supporting epidermal tissue (often denoted subsidiary cells) are important in regulating the transpiration. The volumes of these cells in turn depend on their water content. 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 through the pores. $\Delta\psi_w$ could easily be of the order of -900×10^5 N/m^2, as exemplified by the data given above.

Transpiration through the *cuticula* of the leaves adds to the stomatal transpiration. However, the conductivity for this pathway is often much lower and is in many cases neglected. Typical values of the cuticular conductance are $g_{cuticular}$ = 0.004−0.02 mol m^{-2}s^{-1} (Caird et al. 2007 list useful data) to be compared with about g_s = 0.1−0.35 mol m^{-2}s^{-1} for the stomatal conductance g_s (Marenco et al. 2006; cotton daytime) and 0.01−0.25 mol m^{-2}s^{-1} (Marenco et al. 2006; cotton nighttime). Stomatal conductance values for trees can be found in Wullschleger et al. 1998.

An overall, very simplified picture of the hydraulic water regulation is given in Fig. 7.1. 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, the cells will shrink. The decrease in *guard cell* volume leads to a closing of the stomatal pore, thus decreasing g_s, and a counteracting decrease in water vapor transport through the transpiration. On the other hand, with abundant water present, the guard cell volumes will increase, and due to their structural features, the stomatal opening and

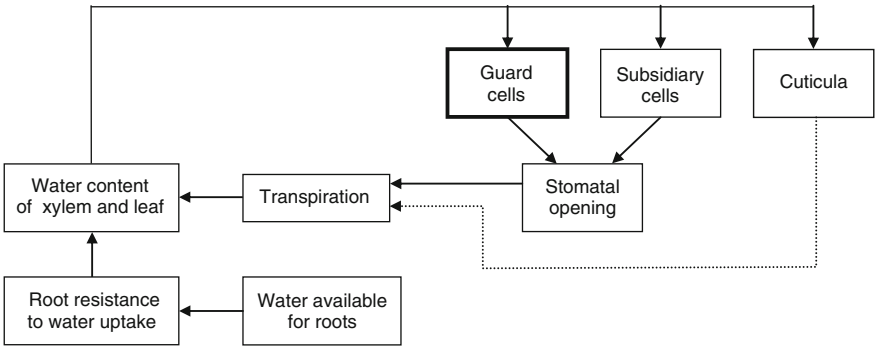

Fig. 7.1 *Simplified feedback scheme for water regulation in plant.* The water uptake of the plant occurs via the roots, lowermost two boxes. The water is transported via the xylem into the leaves (where mesophyll cells form the majority of cells). The stomatal openings in the leaf are the keys to the transpiration, being affected by the *guard cells* and *subsidiary cells*, to the right of figure. The stomatal opening is increased if guard cell volumes are increased, while it decreases when the subsidiary cells increase their volume (thus a positive and negative regulation). The stomatal openings are decisive for the plant transpiration, the most important regulation is via the guard cells—*thick box*. The transpiration in turn affects the water status of the plant (feedback connection, *full line*). Transpiration through the cuticle of the leaf is much less than through the stomata (feedback connection, *broken line*). The scheme emphasizes the feedback nature of the regulatory system

g_s will increase, thus increasing transpiration. The guard cell control of the stomatal opening thus functions as a control system and is an example of a *feedback* system.

The guard cells are supported by neighboring subsidiary/epidermal cells. They exert a counteracting pressure on the guard cells when all the cells increase their volumes. In this way, they counteract the increase in pore opening initiated by the guard cell control. In a corresponding manner, the subsidiary cells counteract the guard cell's closing of the pore when the water supply decreases. The main regulation of the stomatal pore occurs via the guard cells (therefore the more heavy line surrounding the symbol in Fig. 7.1), but the water transport into the subsidiary cells might be faster—thus causing a temporary regulation in the "wrong" direction.

The picture outlined is an overall description of the water transport system of, e.g., a typical leaf. It forms a frame for models of the overall transpiration and describes a *feedback control system* of the water regulation in a leaf. The model can be denoted as "lumped" model, in which 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. Since it is basic and descriptive, it is also a "frame" model to be taken into consideration when extensions and details will be added.

It is well known from control theory that oscillations often arise in feedback systems under certain conditions (amplification and phase change in the feedback 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. Such time delays can be simple transport delays (of, e.g., xylem water) but also delays in

biochemical reaction sequences and signaling in the guard cells. *Hysteresis* in reactions can likewise work as time delays (Ramos and Prado 2013) and increase oscillatory tendencies. Furthermore, the time constants of the volume changes are important for oscillations to arise. Since guard cells and the subsidiary cells counteract each other in their effects on the stomatal pores, there can be an increased tendency for regulatory "overshoots" and oscillations.

The hydraulic feedback model roughly outlined above has been studied in more elaborated forms with physiological concepts, parameter values, and stability criteria identified. Cowan (1972) showed that *such a system can describe an oscillatory water regulation and thus lead to transpiration rhythms.* The description forms the basis for several other approaches to rhythmic transpiration (e.g., Delwiche and Cooke (1977); review by Sack and Holbrook (2006) and Kaiser and Paoletti (2014) with references). It has been successful in describing an astonishingly large number of experimentally found features of transpiration oscillations (e.g., in series of studies on transpiration rhythms in excised *Avena* leaves, see the reference list). The model is also nonlinear, allowing complicated oscillatory patterns in transpiration. Of course, it does not go into descriptions of the important cellular mechanisms in the guard cells that respond to the water status. An overview of models for stomatal conductance is given by Damour et al. (2010).

However, there are several experimentally found features of the oscillatory transpiration which are *not* explicitly described in this hydraulic feedback model. Pro's and con's 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. Remembering the task of the stomata—to allow atmospheric CO_2 to pass into the plant for optimal photosynthesis and, simultaneously, control the water vapor transpiration out from the plant—it is not astonishing that the modeling will be more and more complicated. Our information about how the water potentials in the guard cells are changed by cellular ion mechanisms like Ca^{2+} oscillations, and by the presence of ion pumps in guard cells, aquaporins at several levels in the plant, abscisic acid, etc. have increased substantially since the hydraulic models were first published.

This leads to a remark: Essential extensions of the essentially hydraulic model must take care of the questions of

- how *carbon dioxide*, CO_2, participates in the stomatal regulation and accompanies the rhythmic transpiration,
- how the *abscisic acid* molecule, ABA, exerts its action in the cell regulation and affects the transpiration rhythms,
- how *water* is transported across membranes (e.g., those of the guard cells) and thus how ψ_{stoma}, $\psi_{guard\ cell}$, and ψ_{xylem}, etc. are controlled and the role of *aquaporins*
- how *molecular ion pumps* and transport channels of the guard cells affect the osmotic conditions

Detailed cellular approaches have thus to be included, and values of model parameters (e.g., for water conductance in membranes) have to be elaborated and new concepts introduced. Details of the guard cell regulation and mechanisms can be found in the present volume (see Chaps. 8 and 9 of the present volume).

7.2.2 Overall Description—"Composed" Hydraulic Models of Leaf

Composed models have been constructed to represent a higher level of complexity and to describe how individual stomata can be *coupled* to better represent the behavior of the overall, whole leaf (e.g., Rand et al. 1982). The nature of the coupling between stomata is a central issue (Mott et al. 1999; Gradmann and Buschmann 1996; Gradmann and Hoffstadt 1998; Sack and Holbrook 2006), but one mode is via the water potential of the mesophyll of the leaf. The volume change in guard cells of one stomatal unit can be visualized to affect the ψ_w in the neighboring stomatal units via this "hydraulic coupling." Affecting transpiration in one part of the leaf will then affect the transpiration in neighboring parts. Coupling can of course also consist of transmissions of biochemical or electrical signals between cells and stomatal subunits (Gradmann and Buschmann 1996; Gradmann and Hoffstadt 1998).

The water system can now be modeled in a one-dimensional way ("stomata on a line") so that *waves of rhythmic transpiration* could arise, waves moving from subunit to subunit along the line (could be illustrated, for example, by adding several stomatal units to the right in Fig. 7.1). Rand et al. (1982) found theoretically that a one-dimensional system of coupled stomatal oscillators could show spatially uniform behavior, i.e., the different surface sections were synchronized across the leaf surface. But under *non-uniform* light conditions 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 ("stomata on a surface") which then simulates a leaf with its stomata subunits. 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 (see Sect. 7.2.3) of subunits with different amplitudes and periods emerged under conditions where stomatal parameters were spatially randomized. Thus, partly uncoordinated stomatal behavior 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 alternative coupling modes can certainly be suggested as electric coupling (e.g., Gradmann and Buschmann 1996; Gradmann and Hoffstadt 1998), Ca^{2+} signaling (see below), or plant hormone signaling (Hetherington 2001). The hydraulic architecture of leaves was discussed by Cochard et al. (2004) concluding that in some species, at least the

flow resistance has a low-resistance, vein component as well as a high-resistance, mesophyll component. Models for different types of oscillations in plants have also been presented, based on coupling between ion transporters (Gradmann 2001).

Also *whole plants* can show synchronized transpiration: In several cases, rhythmic transpiration has been recorded from trees in the field or in controlled laboratories, as well as from bushes and whole plants like orange trees (Dzikiti et al. 2007; Steppe et al. 2006a), rose bushes (Rose et al. 1994), and tomato trees (Wallach et al. 2010). The existence of oscillations requires coupling between leaves and/or parts of the plant or the tree. Figure 7.1 can be used to illustrate the overall situation—the hydraulic coupling may exist via water potential in root, xylem, trunk, etc. If one regards the water "pool" in the plant to represent a hydraulic coupling between leaves (or likewise between branches/twigs in trees and bushes), it is evident that oscillations in one part of the plant can affect other parts and possibly extend the oscillatory tendency.

Models similar to the one in Fig. 7.1 have also been used to discuss transpiration oscillations in trees, then including water flux resistances etc. in trunks and branches (Zweifel et al. 2007). Cavitation or *embolism* in xylem vessels and refilling of cavities are relevant phenomena in estimates of flux resistances. The wood water content is important to measure and model, and methods for non-destructive measurements are developed (Hao et al. 2013). Tuzet et al. (2003) designed a model for plant canopies in comprising coupled stomatal conductance, photosynthesis, leaf energy balance, and transport of water through the soil–plant–atmosphere continuum. Stomatal conductance in the model depends on light, temperature, leaf water potentials, and intercellular CO_2 concentration via photosynthesis.

It can be noticed that the period of the transpiration oscillations in whole plants seems to be roughly the same as in single leaf oscillations, possibly indicating that stomata parameters are more important for the period of oscillations than other time constants in the overall hydraulic systems.

7.2.3 Models with "Patchy" Stomatal Areas. Distributed Stomata Parameters

In the models discussed up to now, one has by and large regarded the stomata as individual but identical units which can be coupled in different ways. A further description can take into consideration that the network of stomata of a leaf can be "patchy," a property denoting that small "patches" of stomata respond differently from those in adjacent regions of the leaf, for example, due to "patchy stomatal conductance" (Ferraz et al. 2009; Mott and Buckley 2000; Haefner et al. 1997; Cardon et al. 1994). The patchy behavior can also be found in patchy photosynthesis as demonstrated in recording of leaves (e.g., in *Quercus crispula,* Kamakura et al. 2012b; Siebke and Weis 1995)

Basically, it is possible to introduce patchiness in the models by using statistical models for the stomatal parameters. For example, distributions of conductance parameters can lead to patchy transpiration behavior, as demonstrated by, e.g., Takanashi et al. (2006), who test both bell-shaped and bimodal stomatal distributions, and Haefner et al. (1997). A general discussion on stomatal patchiness and network operation is presented by Mott and Peak (2007).

7.3 Rhythmic Cellular Processes in Guard Cells

7.3.1 Self-Sustained Cell Oscillations

Oscillatory patterns are ubiquitous in organisms and cells and cover a broad frequency range. In most cases, they also exemplify so-called *endogenous,* self-sustained, or inherent oscillations. This might not be a very surprising finding, since in every cell the many biochemical processes and control chains are often of a feedback type—apt to be oscillating under certain conditions.

In the present context, it is pertinent to mention some examples of oscillatory, cellular phenomena which are relevant for water transport into and out from cells—as is the case for the guard cells. In Fig. 7.1, such oscillatory reactions will be situated in the box denoted "guard cell." They might thus control the volume of the guard cells and can, therefore, subsequently contribute or even cause rhythmic transpiration.

Oscillatory water transport in plant cells is fairly common, and one example is provided from the pulvinus tissue of some leaf stalks, e.g., in the plant *Desmodium (Codariocalyx)*, but several others (*Mimosa,* etc.) show a corresponding reaction pattern. In the cylindrical structure of the *Desmodium* (review Johnsson et al. 2012), water of leaflet pulvini is pumped into and out of the cells in a regular manner, giving rise to short-period movements of small leaflets. They have an up and down or elliptical movement with a period of about 4 min (at roughly 25 °C) and can be observed easily by eye inspection. The rhythmic water transport is concomitant with K^+ transport out from or into the cells, of H^+ transport, of membrane potential changes, etc. (Engelmann and Antkowiak 1998; Johnsson et al. 2012).

Guard cells show many similar mechanisms and some oscillatory processes will be mentioned below (concentrating on Ca^{2+} oscillations). However, we will not go into details (see Chap. 8 of this volume)—in the present chapter, the important point is to emphasize that self-sustained oscillations in the guard cells might be coupled to rhythmic and measurable transpiration from the leaves.

7.3.2 Oscillations in Membrane Transport, Membrane Potentials

Oscillations in several plant membrane transport processes have been recorded and studied, as well as concomitant voltage oscillations (Shabala et al. 1997a, b, 2006). The presence of such cellular oscillations has been documented since long, but the more detailed knowledge has increased substantially in the last decades. This is true also for cell processes where oscillatory water transport into and out of cells plays an important mechanistic role, as, for example, in guard cells.

In an important paper, Shabala et al. (2006) model in detail a range of oscillatory membrane transport processes connected with the H^+ pump of the plant cells (and with the K^+ transport and several other cell transport mechanisms across the cell membranes). The period of the rhythms depends on many parameters but is found in a window ranging from 30 s to 15 min. The existence of slower oscillations (1–2 h) was also reported. The existence of these oscillations shows up in a wide variety of cells of which the presence in guard cells is of particular interest here.

The electric events accompanying ion transport causes voltage oscillations. Shabala (1997) recorded bioelectric potentials from plant leaves in response to white light pulses and discovered maximum voltages when the period of the pulses was 30–40 min. This resonance was interpreted to be associated with stomatal cell reactions in the leaves. Similar resonance experiments on primary *Avena* leaves (but with sine wave white light signals) have revealed maximum *transpiration* response in the same period region (Brogårdh and Johnsson 1974b).

7.3.3 Self-Sustained Guard Cell Calcium Oscillations

Intense work has been focused on the role of cytosolic calcium, $(Ca^{2+})_{cyt}$ in volume changes of guard cells: Relevant for plant transpiration rhythms, Ca^{2+} oscillations have been recorded by different techniques (e.g., fluorescence spectroscopy) and are often found to show up as regularly occurring concentration spikes. The period of these oscillations is about 5–10 min in plants (e.g., Allen et al. 2000; McAinsh et al. 1995; Trewavas and Malhó 1998; and others) but Berridge and Galione (1988) lists periods between ca 0.3 and ca 120 min in animal cells.

$(Ca^{2+})_{cyt}$ oscillations are shown to be an information carrier in plant signal chains and Ca^{2+} is a signaling ion also in guard cells (reviews by Blatt 2000b; Yang et al. 2003, 2004, 2005; Schroeder et al. 2001; see also Chap. 9 of this volume and other references at the end of the present chapter). The oscillations can be an endogenous basis for self-sustained volume oscillations in the guard cells (Blatt 2000a, b; Li et al. 2004) and can, as mentioned above, be coupled to several physiological chains in the water regulatory system, including the overt transpiration oscillations.

In wild-type *Arabidopsis,* guard cells, abscisic acid, oxidative stress, cold, and external calcium create $(Ca^{2+})_{cyt}$ oscillations of differing amplitudes and frequencies and induce stomatal closure. However, in guard cells of the V-ATPase mutant *det3*, external calcium and oxidative stress elicited non-oscillating prolonged calcium increased and stomatal closure was prevented. Conversely, cold and abscisic acid elicited calcium oscillations in *det3*, and stomatal closure occurred normally; furthermore, in *det3* guard cells, external calcium-induced oscillations rescued stomatal closure. The coupling between calcium oscillations and guard cell movements, and implicit possibilities to rhythmic transpiration, is thus strongly indicated (Allen et al. 2000).

In the scheme of Fig. 7.1, the $(Ca^{2+})_{cyt}$ oscillations would represent water potential changes in the block denoted "guard cells." It is understood that such self-sustained oscillations in the water potential of the guard cells could interfere with simultaneous, hydraulically initiated rhythmic tendencies and interact or even *drive* the overall transpiration oscillations in the regulatory feedback loop. This can give rise to interference between two oscillating systems, as was partly explored by Prytz (2001). Complicated entrainment patters could arise from such a coupled two-oscillator system—dependent on relative frequency and strength of the oscillators.

The properties and mechanisms of *aquaporins* are of great interest in the water regulation (Finkelstein 2013; Maurel et al. 2008; Tyerman et al. 1999, 2002; Moshelion et al. 2014; Lopez et al. 2013) and are probably downstream elements of $[Ca^{2+}]_i$ in the oscillation signaling (Chaumont and Tyerman 2014; Kaldenhoff and Fischer 2006, Yang et al. 2005). This involves also ion transports, e.g., the guard cell K^+ transport—which in turn affects the membrane voltages.

7.3.4 Coupling Between Water Stress and Calcium Regulation; Abscisic Acid

The phytohormone *abscisic acid, ABA*, plays a central role in numerous growth and developmental processes in plants (the name originates from its discovery in leaf abscission processes). It is important in the water regulation and control as well and in adaptation to water stress. An overview of ABA action—synthesis and response—is published by Finkelstein (2013), and the field expands rapidly.

In particular, under water stress, the root system of plants produces ABA and the guard cells respond to the ABA transported to the leaves (thus a phytohormone; review Finkelstein 2013; Pantin et al. 2013; Kuromori et al. 2014). It has been shown that ABA added to the transpiration stream accumulates in the guard cell apoplast and causes stomatal closure in *Vicia faba* (Zhang and Outlaw 2001). ABA induced a rapid increase in $(Ca^{2+})_{cyt}$ and this preceded stomatal closure (e.g., in *Commelina communis* McAinsh et al. 1990, 1995). ABA perception and transduction in the guard cells as well as the ABA signaling pathway have been studied

and partly identified (Cutler et al. 2010; Joshi-Saha et al. 2011a, b; Pandey et al. 2007; Finkelstein 2013). Both S-type and R-type anion channels are activated and massive K^+ outflux is a result—sucrose transport is also involved (Talbott and Zeiger 1998). Subsequent changes in ψ_{guard} and guard cell volume occur as well as in other membrane transport processes involved. External Ca^{2+} likewise affects the $(Ca^{2+})_{cyt}$

Features to be discussed in our context are, inter alia, the following ones:

- ABA induces $(Ca^{2+})_{cyt}$ oscillations in the guard cells (involving, e.g., phosphoinositide-specific phospholipase C) (http://www.pnas.org/content/96/4/1779.short)
- ABA sensitizes many transporters in the guard cells (review Kim et al. 2010). H^+-ATPase is one of them and is controlled both by ABA and by light (Assman and Shimazaki 1999), see also Sect. 7.4.3.3.)
- ABA affects anion channels and Ca^{2+} channels and is essential in the stomatal closure (e.g., Zhang and Outlaw 2001)

The pattern of ABA controlled mechanisms in guard cells is complicated and the signaling network in guard cell is intensely studied (for further references, see Finkelstein 2013; Pantin et al. 2013; Hills et al. 2012; Kim et al. 2010; Sirichandra et al. 2009; Li et al. 2006; Pei and Kuchitsu 2005);Hetherington 2001).

7.4 Some Experimental Findings on Transpiration Oscillations

The transpiration rhythms are usually characterized by their period, frequency content, amplitude, curve form, 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.

7.4.1 Basic Experimental Methods to Study Rhythmic Transpiration

Only a short summary of some methods will be given here (for further information, see textbooks on water relations of plants, e.g., Weyers and Meidner 1990).

Measurements of the *water uptake* of a plant mirror the overall transpiration from the plant. The method was adopted already by Stephen Hales (1727), probably the first one to publish scientifically on the water relations of plants and trees. The amount of water taken up can be weighed out automatically; it can also be replenished and recorded automatically (e.g., Brogårdh and Johnsson 1973a), and rhythmic water uptake thus documented.

Rhythmic transport of water to leaves, branches, etc. Rhythmic variation of water flux in stems and trunks has been measured by heat pulse techniques (a heat pulse given to the xylem fluid can be detected at a given distance, and the time difference between sent and picked up signals allows the velocity to be calculated), as well as with other flux techniques, e.g., frequency domain reflectometry (see Hao et al. 2013). Time lags between transpiration and sap flow were recorded in pine by using the heat probe (Phillips et al. 1997). Stem diameter can be useful in recording both rapid and more slow oscillations in the water regulation (Steppe et al. 2006a, b; Barlow et al. 2010).

Rhythmic transpiration has been recorded by enclosing the unit to be investigated (typically a leaf or parts of it, or a whole plant or bush) in a container or a *cuvette* which allows a controlled air flow to pass the unit. Modern integrated sensors or commercial instruments can conveniently be used to record changes in humidity, CO_2, temperature, composition in general of the incoming, and outgoing air. The transpiration, etc. of the object can then be calculated.

Gas analysis has been used to record the patterns of CO_2 assimilation (Lawson and Weyers 1999). Photosynthesis assimilation rate has often been regarded as an indirect measure of the distribution of the stomatal conductance and therefore studied (e.g., Genty and Meyer 1994), but a simple relation between photosynthesis and conductance has been questioned (Jones 1998).

Stomatal conductance has been widely studied (see review by Pospisilova and Santrucek 1994) with direct *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.

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. Excision of root system or leaves has been used to study immediate dynamic effects on transpiration or pulse responses, thus mapping parts of the control system (Powles et al. 2006).

Applying a pressure across the xylem vessels in the stem/stalk can increase the water flow resistance, thus affecting the transpiration oscillations. A device to compress the very base of a leaf and then excise the root system below the water level was used to study the transpiration rhythms of the primary *Avena* leaf (Brogårdh and Johnsson 1973b) without roots. The physical compression can replace the water flux resistance of the root with the consequence that transpiration oscillations can occur while being absent in excised leaves. Since the roots and parts of the transport vessels were removed, ions and molecules in the root medium could now enter the xylem vessels directly and reach the stomatal regions (see Sect. 7.4.4). The method can, therefore, be used to study *solute and ion effects on the ongoing transpiration rhythms* without root influences.

Other indirect methods have also been used to study transpiration rhythms. The fact that water evaporation requires heat (about 540 cal/g water) means that transpiration through stomatal openings can cause a temperature decrease in neighboring parts of the stomata. *Infrared thermography* has been used to study water relations of plants (e.g., Cohen et al. 2005; Kümmerlen et al. 1999; Jones 1999;

Fuchs 1990) and temperature rhythms of leaf surfaces simultaneously with transpiration rhythms (see, e.g., Prytz et al. 2003a).

Detection of fluorescence is a powerful technique once suitable probes have been found. Variations in Ca^{2+} concentration can be recorded using the fluorophore *fura*-2. Fluorescence techniques allow also investigations of patchy behavior in leaves (e.g., Kamakura et al. 2012b).

An interesting method to record non-invasively water stress and its effects on leaf parameters is based on a *magnetic patch clamp pressure* technique (review Zimmermann et al. 2013) and allows light effects on leaves and turgor oscillations to be recorded in real time with high precision (period about 12 min sugarcane; see also Ache et al. 2010 reporting period about 30 min for *Arabidopsis*).

The term *water use efficiency*, WUE, is used to characterize the plants' uptake of water relative to the biomass generated. Methods based on imaging techniques are used to record WUE continuously (e.g., McAusland et al. 2013). Then, the water transpiration can be estimated from thermography, simultaneously with optical detection of chlorophyll fluorescence as a measure of biomass production. The data achieved allow calculating and mapping of the assimilation rate, the stomatal conductance, and the WUE.

7.4.2 Occurrence of Transpiration Rhythms. Period of Rhythms

As mentioned earlier, transpiration oscillations are widely found in outdoor and laboratory experiments on a variety of species. The period varies with the experimental conditions. Reports often present data for just a few transpiration cycles, but in order to properly specify the period of the rhythm, recordings of a sufficient amount of cycles should be analyzed. Examples can be found where the oscillations have continued for several days without appreciable variation in duration of individual cycles (see, e.g., recordings in Johnsson 1973, showing transpiration oscillations between typically 30 and 10 $mm^3\ h^{-1}$ in the primary leaf of young *Avena* plants). With these reservations in mind, the period values published for oscillations in light cluster around a few minutes, around 30–50 min, and around 2–3 h (see Barrs and Klepper 1968; Barrs 1971, etc.). Also in older plants, bushes, and trees, etc., the period seems to cluster around 1–3 h (e.g., tomato plant Wallach et al. 2010). The periods mentioned above qualify the oscillations to be termed *ultradian*.

Circadian transpiration rhythms have been recorded (see Sect. 7.5.1), i.e., rhythmic endogenous transpiration with a period close to but not exactly 24 h. An example is the transpiration rhythm in the tree *Tamarix aphylla* (L.) Karst. with a mean period of around 22 h in light (Hagemeyer and Waisel 1987). The rhythmic transpiration can be *entrained* to—"locked to" or "in phase with"—external rhythmic signals, e.g., light signals. Entrainment can occur in a so-called entrainment region in which the period of the transpiration rhythm is equal to the period of the external signal.

7.4.3 Some Environmental Parameters Influencing Oscillations

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

The transpiration rate must be sufficiently high for oscillations to arise, i.e., the transpiration stream, indicatively given by g_s multiplied by the difference in water potential ($\psi_{stomata} - \psi_{air}$), should not be too low (e.g., with g_s close to zero). Obviously, if the stomata stay fully opened or fully closed, oscillations would cease.

Oscillations have often been recorded under low environmental relative humidity (e.g., Hopmans 1971; Cowan 1972; Johnsson 1973; Caldeira et al. 2014). The nonlinearities of the stomatal conductivity play an important role for the existence and the properties of the rhythms. Period is lengthened by increased xylem resistance—as shown in experiments where the xylem has been physically compressed (Brogårdh and Johnsson 1973b). This is in agreement with the feedback models (the loop gain can be said to be increased).

A recent model for the stomatal responses to humidity has been proposed by Mott and Peak (2013) based on the water potential equilibrium between the guard cells and the air at the bottom of the stomatal pore. The situation is complicated since temperature (and thus irradiation) affects the relative humidity of the air (see below, Sect. 7.4.3.2.)

A change of the water potential of the root medium around an intact root system also changes the ψ_{xylem} and thus the transpiration pattern. Oscillations have been studied both by varying the water potential of the root medium and keeping it constant or by short-term changes of the root medium, so-called pulse treatments (Brogårdh et al. 1974; *Avena*). Resulting phase shifts of the transpiration oscillations were studied—only slight adjustments of the nonlinearities in Cowan's model were necessary to describe the results successfully. A non-oscillating state could be induced by a suitable pulse treatment (see Sect. 7.5).

The way in which water potential changes and water deficit of the root/xylem system is communicated to the shoot and the guard cells is discussed in the literature (e.g., Christmann et al. 2007). It has been demonstrated that abscisic acid, ABA, is produced in response to water stress and mediates stomatal closure (see Chaps. 8 and 9 in the present volume). Even under non-stress conditions, ABA is produced in the leaves and is necessary for the guard cell regulation.

7.4.3.2 Ambient Temperature

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

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

The coupling between temperature effects in the leaves and the direct temperature effects on the water potential in the air makes it difficult to carry out decisive experiments. Mott and Peak (2010) chose to study stomatal response to temperature and humidity in darkness. They conclude that stomatal response to humidity is the same in darkness and in light and thus independent of the processes that cause stomatal opening in light.

Pieruschka et al. (2010) have suggested that plant transpiration is due to the total radiation (including infrared radiation) and not to reactions only in the visible light regions (see section below). This would then include the temperature effects. The hypothesis is discussed by Mott and Peak (2011, 2013), concluding that the published findings can be due to mismeasurements of leaf temperature using thermocouples and that blue and red light reactions are not consistent with the hypothesis (see section on light below).

7.4.3.3 Light

Most studies of transpiration rhythms have been carried out in continuous "white" light. Review articles on the light action on the guard cells have been published (e.g., Shimazaki et al. 2007, focusing on the differences between blue and red light action on the stomata and their interaction in stomatal regulation; Chen et al. 2012a).

White light. In continuous white light, the period of the transpiration oscillations 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 stepwise lowered, a major change in period occurred in *Avena* transpiration rhythms at about 0.2 mW cm^{-2} where the period changed from roughly 30 min to about 110 min (Klockare et al. 1978). This longer period was also encountered in *darkness*. The authors indicate that two different approaches have to be used to describe the oscillations with period about 30–40 min (irradiances about 5–10 Wm^{-2}) and oscillations with period around 100–120 min (irradiances below about 1 Wm^{-2}). Complicated transpiration patterns occurred in the intermediate irradiance region. Possibly, the complicated transpiration pattern and the non-constant period could then be the result of two separate, but interacting, oscillatory mechanisms.

Single, short white 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, single, light pulse treatment (Sect. 7.5.2). The transpiration response to *repeated* light pulses demonstrated an underlying circadian control component, period being around 27 h (Brogårdh 1975). The general pulse responses to "broadband red" and "broadband blue" light pulses were different and complex (op.cit.)

Time varying light signals have been administered to leaves to study the resonance frequencies of the transpiration oscillations. For analysis purposes, sine signals are preferred and Brogårdh and Johnsson (1974b) found for *Avena* that resonance and maximum amplitude occurred for light periods that were equal to the period reported for the self-sustained transpiration rhythms. Responses to repeated *pulse* sequences of white light were used in corresponding transpiration studies Brogårdh and Johnsson (1975a). Shabla (1997) and Shabala et al. (1997a, b) recorded electrical activity from leaf surfaces also using white light pulses with different periods, an amplitude maximum in the electrical potentials occurred at a light period of 30–40 min. A wide variety of species all showed roughly the same resonance period. This correspondence in resonance curves of course points to simultaneous electrical (guard cell) events and transpiration oscillations.

Blue light response. Hopmans (1973) found that the transpiration rhythm period in *Phaseolus* was longer in broadband blue light than in red light and he suggested that blue light decreased the water conductivity of guard cells. The response of guard cells and the stomatal apparatus to blue light is ascribed to the photoreceptor carotenoid (see, e.g., Paolicchi et al. 2005; Kinoshita et al. 2001; Assmann and Shimazaki 1999; Karlsson 1986a, b; Shimazaki et al. 2007), zeaxanthin, and to phot1 and phot2 proteins (Kinoshita et al. 2001). The phot1 protein acts on $(Ca^{2+})_{cyt}$. The blue light pathway as well as the ABA signaling pathway are known both to affect H + -ATPase (Assman and Shimazaki 1999). 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).

Red light. The red light response in the transpiration rhythm is mediated via guard cell photosynthesis by chlorophyll and does thus also depend on the CO_2 regulation of stomata (Shimazaki et al. 2007). Data of Olsen et al. (2002) suggest that the guard cell chloroplasts react to reduced CO_2 and activate stomatal opening through ion uptake, depending on photosynthetic electron transport with downstream components of the blue light transduction cascade.

As emphasized in several publications, the stomata opening and thus transpiration increase is more rapid in response to blue light than after red light administration (Shimazaki et al. 2007; see also Karlsson 1986a; Hopmans 1973)

Darkness. It is interesting that also in darkness, rhythmic transpiration occurs, so-called nocturnal oscillations both in C3 and C4 plants (see, e.g., Caird et al. 2007 and Sect. 7.4.3.4 below). Photosynthesis is absent in darkness but respiration changes in stomatal CO_2 could provide an explanation as to the change in g_s. However, Klockare and Falk (1981) provide data for the rhythm in *Avena* leaves (in darkness) at different O_2 and CO_2 concentrations in air and conclude that lowering the oxygen concentration to 5 % did not affect the existing oscillations. When the CO2 concentration was raised to 0.3 or 3 % in darkness, the transpiration rate temporarily decreased, but the period of the oscillations was not changed.

7.4.3.4 CO_2

The CO_2 concentration in the ambient air influences the transpiration rhythms (see, e.g., experimental results by Hopmans 1971). Not all hydraulic models do include this regulation loop explicitly but allow the water potential of the guard cell to be influenced by CO_2 much in the same way as by light (a review of guard cell signal transduction network, including CO_2 mechanisms, is given by Kim et al. 2010). Upadhyaya et al. (1983) developed a mathematical model for the CO_2 effects on the stomatal dynamics and water-use efficiency. Furthermore, CO_2 increases the guard cell $(Ca^{2+})_{cyt,}$ providing an interesting pathway to calcium oscillations (Webb et al. 1996)

Tuzet et al. (2003) discuss a coupled model where stomatal conductance is fitted empirically to the CO_2 in the intercellular spaces and derive daily transpiration and photosynthesis variations.

In darkness, respiration causes higher CO_2 in the stomata and one would expect them to stay closed. However, as mentioned in Sect. 7.4.3.3, nocturnal transpiration is reported in several species (e.g., Blom-Zandstra et al. 1995; Rawson and Clarke 1988; Kavanagh et al. 2007); and Klockare and Falk (1981) found oscillatory transpiration in darkness.

7.4.4 Ionic and Solution Interference with Transpiration Oscillations

Intact plant roots can represent an efficient barrier toward many ions and molecules and prevent studies of ongoing transpiration rhythms. In *Avena,* no effect on transpiration oscillations was detected when adding, e.g., Mg^{2+}, Ca^{2+} or La^{3+} to the medium of intact plants (Brogårdh and Johnsson 1975b). But when using excised leaves and xylem compression (Sect. 7.3), *solutes and ions could enter the stomatal regions* and the period of the transpiration rhythm was clearly lengthened. In some cases, an effect on period, amplitude, or curve form of the transpiration rhythm could be detected already in the first oscillatory cycle after administration of the ions.

Ca^{2+} (20 mM chloride salt), Mg^{2+} (40 mM), and La^{3+} (2.5 mM) given to the xylem 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 to either ionic effects outside/inside the guard cells, e.g., affecting the K^+ transports, or to changes of the water permeabilities 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 found experimentally. K^+ and Na^+ did not seem to affect the period under the same experimental conditions.

Lithium chloride salt—given permanently to *intact* plants—caused no period changes (control plants had a period of about 40 min). Applying a pulse of 80 mM LiCl to the cut end of excised leaves caused, however, 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. The action of the lithium ions was interpreted to affect the ion-pumping mechanisms and could also be due to interference with calcium oscillations and calcium signaling pathways in guard cells. The experiments should, therefore, be repeated in studies on guard cell oscillations to see whether corresponding period and amplitude changes are experimentally found. 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).

10 mM LiCl given to intact *Tamarix* plants caused no lengthening in the circadian transpiration rhythm (Hagemeyer and Waisel 1987), although lithium slows down circadian rhythms in general, acts on aquaporins in renal functions (Nielsen et al. 2002), and has a broad spectrum of action mechanisms. It might be that the ion could not enter the leaves in the *Tamarix* case (cf. *Avena* experiment mentioned in the previous paragraph).

The short information given above concerns experiments of fairly short duration, mostly of some hours only. The same is true of experiments in which solutions of theophylline, kinetin, or valinomycin have been administered to oscillating, excised *Avena* leaves. In several cases, the substances cause drastic period lengthening (Johnsson 1976), as does ATP. In *Arabidopsis,* it is reported (Tanaka et al. 2010) that extracellular ATP causes an increased cytosolic Ca^{2+} level in plants cells; in root hair cells, one could localize temporal Ca^{2+} oscillations presumably from the nucleotide treatment. Several other ions, e.g., H^+, Cl^-, are important in guard cell reactions (see, e.g., Yang et al. 2005), but the direct action on rhythmic transpiration remains to be studied.

7.5 Further Experimental Findings on Transpiration Oscillations

7.5.1 Modulation of Oscillation. Entrainment

The parameters in the hydraulic control system change in a daily manner under the L/D conditions: root resistance, mean level of g_s, etc. are all varying throughout the day and night (Barrs and Klepper 1968; Hopmans 1971; Cowan 1972, see also Caldeira et al. 2014). These changes might then modulate the ultradian, short-period transpiration oscillations. Their amplitudes are reported to increase toward the end of the day but usually die out in the night.

The daily responses could be direct results of the L/D variations. However, the transpiration of plants also shows a circadian rhythm, due to the endogenous

biological clocks. To be truly circadian, the period should then be close to but not exactly 24 h under constant environmental conditions (see classic review by Bünning 1973). Willmer and Fricker (1996) give some examples showing that also *Arabidopsis* exhibits circadian transpiration (see also Fig. 2 in Webb 1998). The tree *Tamarix aphylla* (L.) showed a circadian transpiration rhythm in light with period about 22 h (Hagemeyer and Waisel 1987). Brinker et al. (2001) indicate that stomatal aperture was under circadian control in the gymnosperm *Ginkgo biloba.*

A circadian *modulation* of many transpiration variables, including the rhythmic ultradian ones, can be carried out by the internal biological clock (also under constant experimental conditions). The transpiration response to light steps has, as an example, been shown to be modulated in a circadian fashion (see Sect. 7.4.3.3) The *range of entrainment* can be studied either by administering light at fixed frequencies in a series of experiments or by using *swept frequency techniques* and sweeping the external frequency slowly over the range of interest. This possibility has partly been explored by using sinusoidal white light signals to entrain the transpiration oscillations in different parts of the primary *Avena* leaf (Brogårdh and Johnsson 1974b) and studying, e.g., the coupling across parts of the leaf.

7.5.2 Singularities of Transpiration Rhythms; Test of Models

A short-term *perturbation* of an oscillating biological system often results in a temporary deviation from its regular amplitude and curve shape. When the perturbation ceases, it might return to its original shape but then often showing changes in *phase* and *amplitude*.

Originally discussed for circadian rhythms, the question has been asked whether biological oscillators in general could be perturbed to reach so-called *singularities* or *fixed points.* An oscillator in such a state is characterized by a halt of the oscillating variables, and it has *stopped oscillating*—temporarily or permanently. This state might be obtained by perturbations. Winfree (1970) explored the possibilities to stop a circadian eclosion rhythm in *Drosophila* by administering blue light pulses of suitable irradiance and duration at a certain phase of the rhythm, and the results could be interpreted to show that 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 given to the *Avena* transpiration rhythm demonstrated, firstly, the phase resetting ability of light and dark pulses. Secondly, it was shown that a suitable pulse or combination of pulses could in fact halt the transpiration rhythm, Fig. 7.2. The oscillatory system was not irreversibly damaged since a subsequent strong light pulse could initiate the transpiration rhythm again (Johnsson et al. 1979).

The nature of this particular singularity seemed to be stable in some cases; in others, it was evidently unstable since the halted rhythm could spontaneously start to oscillate again (see Fig. 7.2). The transpiration oscillations could also be stopped transiently by *osmotic* pulses (−32 bars, 3 or 6 min; Johnsson et al. 1979). This

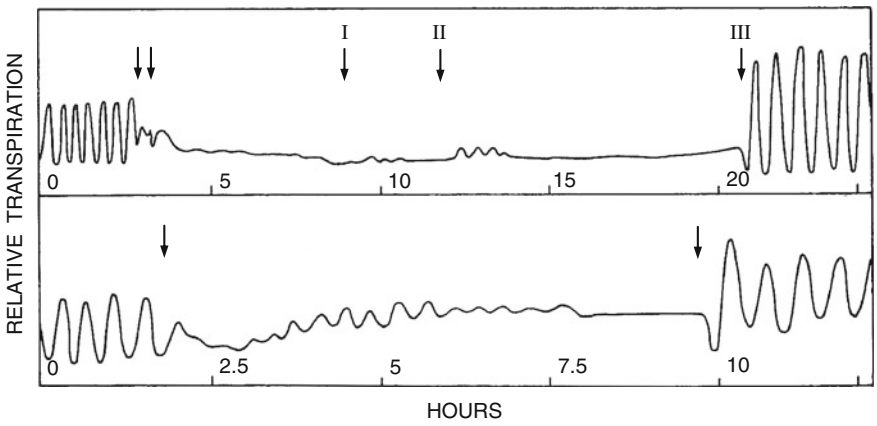

Fig. 7.2 *Light/dark perturbations stopping transpiration rhythms in Avena plants*. In the upper recording, a combination of two light pulses stopped the transpiration rhythm at about 4 h. *Arrows I*, *II*, and *III* indicate white light perturbations with duration of 30 s, 2 min, and 10 min, respectively. The two first perturbations caused small oscillatory responses but only a strong enough perturbation, *III*, could start the rhythm anew. In the lower recording, the damped transpiration rhythm spontaneously showed an oscillatory tendency after about 5 h but returned into a standstill. A stronger light pulse started the oscillations again (redrawn after Johnsson et al. 1979)

indicated that the action of the light perturbations in the singularity experiments may have been mediated via osmotic perturbations of the water control system. Singularity experiments could be critical tests of models for the transpiration rhythms. The results achieved were not possible to simulate with Cowan's model as published, but nonlinear features introduced between the transpiration rate and the stomatal conductance allowed successful simulations.

7.5.3 Period Doubling and Bifurcations in Transpiration—a Way to Chaos?

Several "lumped" models for the water regulatory system include three dynamic variables or elements (chosen as, e.g., water content of the guard cells, the subsidiary cells, and the mesophyll cells; cf. Gumowski 1981, 1983). Coupled models have a correspondingly higher number of dynamic variables. A nonlinear model with three variables can show *period-doubling* and *chaotic behavior* (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 1981, 1983). If time delays are introduced in the reactions, the overall model becomes even more apt to show oscillations.

An example of period doubling in the water transpiration of the primary *Avena* leaf is given in Fig. 7.3, upper curve. One can conveniently introduce the concept of *period-n oscillation* (Strogatz 1994), characterized by a pattern repeating itself every n'th maximum of the transpiration. A period doubling is thus a period-2 oscillatory behavior.

The period doubling is but one of the many complicated oscillatory patterns that has been 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. Period-doubling sequences have been reported in physical and chemical as well as in biological systems (see Lloyd 1997, Cvitanovic 1989 and references therein). However, studies of such oscillating systems are, to our knowledge, very few in plant physiology (a study by Shabala et al. 1997a, b is an exception, see below). Further increase in the bifurcation parameter may 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*. 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.

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. 7.4.3.3). A rather abrupt transition in period from about 30 min to 110 min 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 mentioned could occur simultaneously. Shabala et al. (1997a, b) observed bifurcation and chaos in bioelectric leaf response to rhythmical light (experiments on *Zea Mays*)

Prytz et al. (2003b) demonstrated that the primary *Avena* leaf can show rhythms that are sometimes very complex (examples in Fig. 7.3). Thus, the overall transpiration system approaches under certain conditions chaotic behavior. These transpiration oscillations were recorded when the K^+ concentration in the xylem medium was increased and used as a control parameter. It would be an elucidating step to look for possible *simultaneous* bifurcation patterns in transpiration rhythms and in calcium chain controlled rhythms in guard cells.

7.6 Remarks and Outlooks

This chapter has focussed on direct measurements of rhythmic transpiration of plants. The literature shows that oscillatory transpiration can exist in the regulatory feedback system in a wide variety of leaves of plants, including bushes and trees

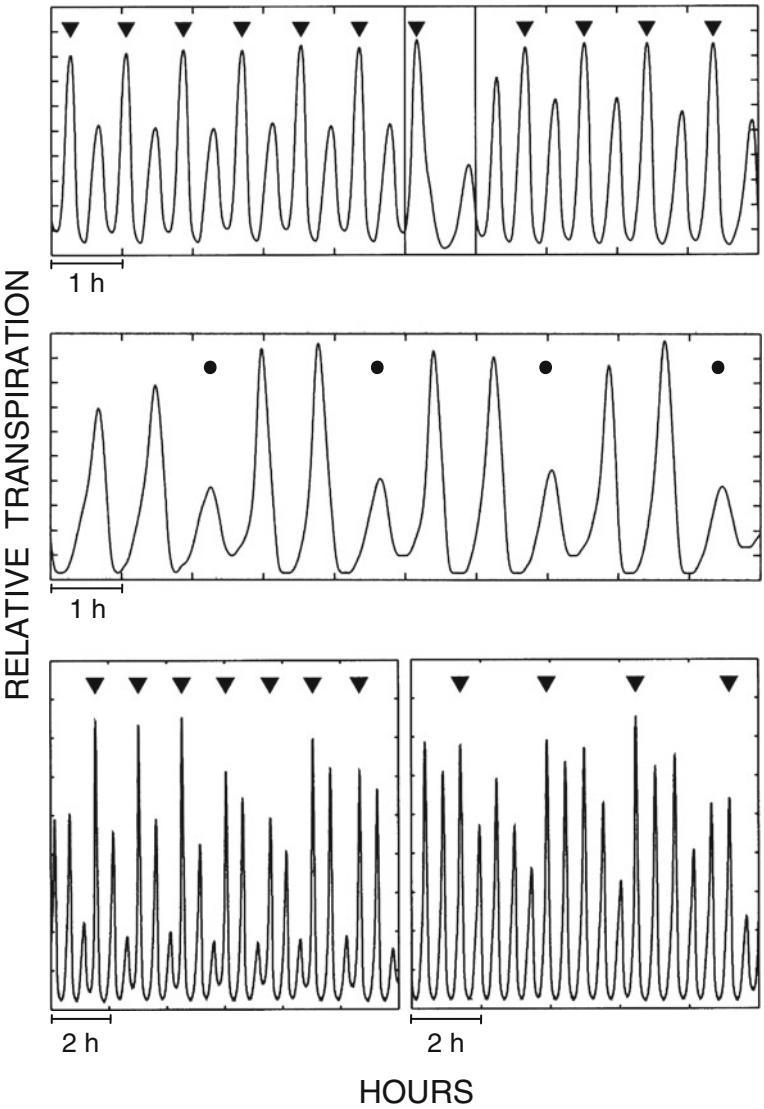

Fig. 7.3 *Complex oscillatory transpiration patterns in Avena leaves* (redrawn after Prytz et al. 2003a, b). *Uppermost curve*: Period-2 oscillations, *triangles* indicate maxima in rhythm (every second peak). A phase shift is induced by a pulse perturbation of 80 mM KCl to standard medium (middle of recording). Root excised, xylem compressed leaf. *Middle curve*: recording showing period-3 oscillations in intact plant. *Filled circles* indicate minima in rhythm (every third peak distinctly smaller than the rest of the peaks). *Lowermost curves—to the left*: period-3 oscillation. *Triangles* indicate maxima (every third peak) and pattern different from period-3 oscillation in the middle curve. 20 mM KCl added to medium. Root excised, xylem compressed leaf. *Lowermost curves—to the right*: recordings of a plant showing period-5 oscillations. *Triangles* indicate maxima. 20 mM KCl added to root medium. Root excised, xylem compressed leaf

and plants with different guard cell anatomy. The hydraulic system comprises guard cells and their self-sustained volume changes and oscillatory reactions, e.g., the $(Ca^{2+})_{cyt}$ reactions, their ABA signaling, and the presence of ion pumps. These facets need to be extended and detailed in any models for the oscillating transpirations and so must the membrane water transport with relevant classes of aquaporins (see Sun et al. 2001; Tyerman et al. 2002). The aquaporins have up to now not been explicitly/quantitatively used in studies of rhythmic transpiration, but are frequent in plants and influence both root, stem, and leaf hydraulic conductivities (cf. Steudle 1997). Complex modeling is needed to approach a more complete picture of the water control system in plants, which also, of course, takes into account, for example, CO_2 transport through stomata (reviews Kim et al. 2010 and Raschke 1979; Hubbard et al. 2012; Upadhyaya et al. 1983). Systems analysis and dynamic modeling already contribute constructively to the guard cell modeling (Chen et al. 2012b).

Details about the time constants involved in the molecular mechanisms to produce the $(Ca^{2+})_{cyt}$ oscillations are important (a simplified reaction scheme is shown by Yang et al. 2004). If ABA is controlling the $(Ca^{2+})_{cyt}$ oscillations and there is then a sequence of events leading to transpiration oscillations with certain characteristic period times—Which cellular constituents and reactions show the same period? And what phase relations exist between the constituents?

Perturbation experiments seem to be a fruitful way of studying the transpiration oscillations. If the transpiration rhythm is sent into a singularity as described in Sect. 7.5.2 and thus suddenly stopped, which other oscillations are stopped? Can, for example, the ABA concentration suddenly be halted by a suitable light pulse in such a way that the Ca^{2+} oscillations are stopped? How do models explain the pulse experiments where the oscillating transpiration is stopped?

Results in these areas will undoubtedly improve our models of the oscillatory transpiration of plants. So will also results from experiments focusing on the nature of the coupling between stomatal regions. And it would be interesting—but not an easy task—to establish whether the transpiration control system is really chaotic, as found, e.g., for the heart rhythm. Chaotic systems can use their sensitivity to stabilize a control by using small perturbations, thus increasing their flexibility and speed in response to varying environmental conditions (see, e.g., Shinbrot et al. 1993, Lloyd and Lloyd 1995 and others). This can be of much biological value in several contexts (Lloyd and Lloyd 1995; Strogatz 1994) and possibly also for the plants' regulation of water transpiration (Souza et al. 2004).

We still have limited amount of data and limited number of oscillatory periods available for the transpiration oscillations—a drawback as compared with, e.g., the amount of data from the rhythmic human heart beat. The complex oscillatory patterns of the transpiration rhythms discussed in Sect. 7.5.3 might, however, be of interest and point in the direction of a possible chaotic underlying system.

The necessity of a rapid guard cell control system is also emphasized when looking at the structure of the irradiation onto leaves and stomata in the field—with rapid changes caused by shadow patterns from moving clouds, leaves, stems, and

twigs. Optimizing the stomata control must certainly also involve a rapid control system.

The electric voltage changes which accompany the ion transports of the guard cells are concomitant variables of the oscillatory transpiration (see, e.g., Sects. 7.3.2 and 7.4.4). They could provide important further aspects on the simultaneous transpiration and guard cell rhythms (e.g., on patchiness). It might also be possible to stimulate the tissue by precise electric signals, precise both temporarily and spatially, to see whether voltage pulses influence the oscillations, for example, by phase shifting them. Corresponding experiments have been carried out on the rapid leaflet movement rhythm of *Desmodium* (review by Johnsson et al. 2012; early experiments by Bose 1913; Johnsson et al. 1993; Fostad et al. 1997; Sharma et al. 2001), where volume changes of pulvini cells are central elements in the movements. *Electric direct current* pulses phase shift these rhythmic leaflet movements, as do pulse exposures to *magnetic* (inhomogeneous) *fields* (Sharma et al. 2000).

We have touched upon some possible advantages that rapid control systems, probably chaotic ones, can show under several circumstances. Efforts to evaluate models on plant transpiration and transpiration rhythms can also imply another aspect, just mentioned in Sect. 7.4.1, namely the water-use efficiency, WUE. This relationship (or, simply, the *ratio*) between water transpiration and the CO_2 absorption of the leaves (e.g., Cowan 1977) can reveal important practical aspects between necessary water loss to the atmosphere in relation to biomass production. A decrease in the stomatal pore size reduces the water transpiration as well as the CO_2 absorption but the water transport reduction is proportionally the most affected one. The WUE (see review by Lawson and Blatt 2014) is therefore changing with the stomatal conductivity. Several approaches to calculate WUE for crops are used (e.g., Buckley et al. 1999), also starting from paradigm equations (see, e.g., Xu and Hsiao 2004), reaching useful predicted photosynthetic WUE (for cotton and sweet corn). The role of aquaporins is discussed in the literature in the frame of crop water-use efficiency (Moshelion et al. 2014).

It has been pointed out that the WUE can in fact increase during limited water supply if oscillatory behavior is present. This points at the beneficial nature of the oscillations (Upadhyaya et al. 1988; Lawson and Blatt 2014; Yang et al. 2004). If so, it is an extremely important work to penetrate the possibilities to increase the WUE of vegetation, thus reducing, for example, the crop need for water, but still with affordable, and hopefully increased, biomass production. The imaging technique by McAusland et al. (2013) to achieve intrinsic WUE has already been mentioned. Wang et al. (2014), using the OnGuard software (Hills et al. 2012), point at experimental modifications of dominant K^+ channels as a favorable mean. However, other ways might also be relevant to follow and it seems reasonable to use a broad research design to achieve more efficient crops. Data must be available for crops and vegetation (e.g., water use of trees—some data in Wullschleger et al. 1998—and drought tolerance in *Arabidopsis*, see Nilson and Assmann 2007).

The oscillatory transpiration of plants reveals much of the dynamics of an important biological control system. As has been pointed out (Cowan 1977), the stomatal system provides more directly than any other aspect 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 provide a way to increased knowledge of some of the most basic and complex sensory and control systems in plants.

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Part II
Circadian Oscillations

Chapter 8
Photoperiodism: The Calendar of Plants

Wolfgang Engelmann

Abstract The daylength varies with the time of the year and can thus be used by plants—and other organisms—to react photoperiodically in developmental steps and morphological features such as cyst formation, germination of zygospores and cell division in certain algae, succulence of stems and leaves, formation of storage organs, and flower induction. The functioning and molecular bases of circadian clocks of plants are mentioned and shown how they are entrained to the day, and whether they are involved in photoperiodic timing. Seasonal aspects for various crops and the evolution of photoperiodism are briefly touched upon.

Abbreviations are in alphabetical order; names of gene products are in capitals; and genes are in italics: *Ac*FT1 (4), *Allium cepa* FT1 (4), see FT; AP1 (2), APETALA 1 (2)*2*, gene: *ap1 (2),* flower meristem identity genes; ATP, adenosine triphosphate; BEL5, homeodomain protein, involved in photoperiodic tuber induction of potato; CAB, chlorophyll A/B-binding protein; *cal,* flower meristem identity gene; CCA1, CIRCADIAN CLOCK ASSOCIATED; *Ch*R-1-2, photoreceptor proteins in eyespot of *Chlamydomonas*; CikA, cyanobacterial kinase; CK2, casein kinase 2; CO, CONSTANS, a B box protein; COL, CONSTANS-like proteins; COP1, constitutively photomorphogenic; CPF1, protein of the cryptochrome/photolyase family; *Cr*CO, Chlamydomonas reinhardtii CO homologue; CRY1 (2), CHRYPTO CHROME 1 (2), gene: *cry1* (*2*); *Cs*FTL1 (2) (3), Chrysanthemum FT-like genes; DD, continuous darkness; DET1, DE-ETIOLATED 1; DTH2, days to heading on chromosome 2; EE, evening element sequence; Ehd1, (4) early heading date 1 (4); EL1, early flowering1 = ELF1; ELF3 (4), EARLY FLOWERING 3 (4); gene: *elf3* (4); E-oscillator, evening-oscillator; FD, FLOWERING LOCUS D, a bZIP transcription factor; bZIP, transcription factor with basic domain and leucine zipper; FKF, FLAVIN-BINDING KELCH REPEAT F-BOX; FLC, FLOWERING LOCUS C, blocks transcriptional activation of SOC1 by CO; FRI, FRIGIDA; FT, FLOWERING LOCUS T; gene: *ft*; GA, gibberellic acid; GBP1, GAMYB-binding

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protein; GF14, 14-3-3 protein; Ghd7/Hd4 grain number, plant height and heading date 7/heading date 4; GI GIGANTEA, *gi*; *Gm*FT2a *Glycine max* FT2a; G-protein (s); HD1, (3,4,5) HEADING DATE 1 (3,4,5); HK, histidine kinase; J, Joule; KaiA, KaiB, and KaiC proteins; LD, light–dark cycle(s), respectively, longday; LFY, LEAFY; gene: *lfy,* a flower meristem identity gene; LHY, LATE ELONGATED HYPOCOTYL, gene: *lhy*; LL, continuous light; LDP, long-day plant(s); LOV, LIGHT OXYGEN VOLTAGE; lx, lux; LUX, LUX ARRHYTHMO, evening-expressed transcription factor; M-oscillator, morning-oscillator; miR172, microRNA involved in regulation of flowering time; N north; *Nt*FT4 floral inducer of *Nicotiana tabacum*, see FT; *Nt*FT1 2 3 floral inhibitor of *Nicotiana tabacum*, see FT; *Os Oryza sativa*; *per1 (2),* period 1 (2) gene(s); PFT1, links photoreceptor and FT; PIF4, PHYTOCHROME-INTERACTING FACTORS 4; PHYA (B,C,D,E), PHYTOCHROME (B,C,D,E); *Pn*CO, *Pharbitis nil* orthologs of CO; *Pn*FL-2, *Pharbitis nil* FLOWERING-2; *Pn*FT1 and PnFT2 *Pharbitis nil* orthologs of FT; *Ppd,* heading date photoperiodism genes; PTFL, post-translational feedback loops; PPR5, (7,9,37) PSEUDO-RESPONSE REGULATOR; RAV family, RELATED TO ABI3 AND VP1; *Rf,* fertility restorer genes; *R*FT1, RICE FLOWERING LOCUS T1, see FT, gene: *RFT1*; SD, shortday; SDP, short-day plant(s); *Se13,* (*OsHY2*) gene involved in phytochrome chromophore biosynthesis; SFT, SINGLE FLOWER TRUSS; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1; SP, SELF PRUNING; SPA, a phyA signaling component; SRR1, heading date; SRR1, SENSITIVITY TO RED LIGHT REDUCED; ST, potato mutant; *St*CO, *Solanum tuberosum* CO; see CO; *St*SP3D and StSP6A, two different FT-like paralogues; T-cycle(s), LD cycles deviating from 24 h; TEM1 and TEM2, TEMPRANILLO 1 and 2, transcription factors of the RAV family; TFL1, a repressor of flower induction; TIC, TIME FOR COFFEE; TOC1, TIMING OF CAB EXPRESSION, gene: *toc1*; TTFL, transcriptional/translational feedback; *Vc*FT, *Vaccinium corymbosum* FT, see FT; *Vrn,* flowering time gene, associated with vernalization independent spring growth habit; wk, week; ZCN8, CENTRORADIALIS 8 CENTRORADIALIS (CEN); Z, Zm *Zea mays*; *Zm*CCT, a homologue of the rice photoperiod response regulator Ghd7; ZTL, ZEITLUPE is a F-box protein, gene: *ztl*

8.1 Introduction and History

Seasonal changes are quite spectacular and obvious especially at higher latitudes. Resulting from the 23° bent axis of the rotating planet Earth on its orbit around the Sun, they affect the daily length of light and temperature. As a consequence, climate, vegetation, environment, and the life of organisms are profoundly affected by it.

Although biologists were aware of these seasonal effects on life, the significance of the changing length of the light period during the course of the year on plants was proposed not before the begin of the twentieth century (Klebs 1913; Tournois

1912) and experimentally tested 10 years later (Garner and Allard 1920), see Evans (1969) for details. The environmental factors such as temperature and daylength correlate with the seasons, but the latter is a more reliable token to determine the time of the year (by the way, Ligr et al. (1995) describe an algorithm to calculate simply and exactly the length of the day for different latitudinal locations). In connection with 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.

Garner and Allard (1920) tried to identify why a giant mutant ('Maryland Mammoth') of tobacco flowered only during the winter and found the shortdays to be responsible. They discovered that flowering in soybean and other plants depends also on daylength and termed this phenomenon as 'photoperiodism.' Went (1959) discovered thermoperiodism, where the length of the high- or low-temperature period of a day leads to a reaction such as flower induction.

Bünning (1969) stated almost 50 years ago that the real problem of photoperiodism as a developmental control mechanism in plants and animals is, *how the responsiveness to light is controlled by the clock* and to *understand the electronics behind the organisms switchboard.* And 35 years later, Locke et al. (2005) sees *'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). An enormous amount of knowledge and insight has accumulated during this period. New experimental methods and technical equipments have allowed scientists to dip into the molecular basis of biological clocks which organisms use 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 (Pajoro et al. 2014; Locke et al. 2005).

An internal *annual* clock could serve as a calendar for an organism to cope with the variations of the seasons. This is indeed realized in quite a number of organisms and also in plants (e.g., seed germination, Bünning (1951), water uptake, Spruyt and De Greef (1987), and stomatal movement of bean seeds, Seidman and Riggan (1968)). However, annual clocks must be synchronized to the exact length of the year. Without synchronization, an annual clock would after a few years not match the physical year any more, because its period can hardly be exactly 12 month. This synchronization occurs in most cases via the photoperiod.

Many studies and experiments on photoperiodism and thermoperiodism have been performed since their discovery. Their biochemical and physiological bases were studied. It was found that photoperiodic tuber formation in potatoes (Razumov 1931) and flowering in *Cosmos* (Whyte and Murneek 1948) is induced in the leaves. Sachs (1880) proposed an organ-forming substance in plants. Knott (1934) extended this idea to the photoperiodic induction of flowering and Chailakhyan (1936) named the responsible substance as 'florigen', see Sect. 8.5. 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 1952). Short- and long-day plants seem to use the same flower hormone (Vince-Prue 1975). The role of

the dark period and the long-day effect of light breaks in the middle of the dark period were the further milestones in studying the photoperiodically controlled reactions and their mechanisms. For a historical essay which includes newer milestones in timing and photoperiodism, see McClung (2006) and a movie produced by Eric Bitman at the University of Massachusetts in Amherst which will be available through the SRBR Chronhistory site at http://srbr.org/.

Some important questions on photoperiodism to be discussed are as follows:

1. How is day length (night length) 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 first look at some examples of photoperiodic reactions in plants. The first one is from unicellular algae. The second example is the succulence of leaves in *Crassulaceae*. The third example is the tuber formation of potatoes. Finally, the flower induction in short-day and long-day plants is described. In the examples chosen, the complexity increases from a photoperiodic event in a single cell, in an organ such as the leaf, in the underground such as stolons with remote perception of the photoperiodic light in the leaves, and in the apex of the stems, again with light perception in the leaves. This chapter finishes with a section on practical aspects by mentioning the importance of photoperiodism in horticulture and floriculture and for crop plants, and finally with a short section on the evolution of circadian rhythms and photoperiodic timing.

8.2 The Circadian Clock and Its Entrainment to the Day

If we are assuming a circadian (latin *circa*: about, dian from latin *dies*: day) clock to be used by organisms to determine the daylength and thus the season, we have to learn a bit about the occurrence and properties of these clocks and get to know some examples. Since further chapters of the book deal with circadian rhythms, only a few ones relevant to the photoperiodic examples given here are mentioned. Circadian clocks

- are widespread among organisms and control many of their biochemical and physiological processes.
- offer adaptive advantages to the organism
- are internal timing devices, since they continue to run after transfer from diurnal conditions to a constant one such as continuous light (LL) or continuous darkness (DD) and constant temperature. However, the rhythm might damp out quickly under LL or DD, depending on the organism.
- periods deviate from 24 h under constant conditions and are therefore termed as circadian.

- period depends only slightly or not at all on the temperature and is therefore said to be temperature compensated.
- are synchronized by environmental time cues such as light–dark (LD) or temperature cycles. For the photoreceptors involved, see Wenden et al. (2011), Millar (2004) and the example sections, and for the mechanism of entrainment by temperature cycles, see Mizuno et al. (2014).
- are based on molecular devices which are heritable and amenable to mutations. Many genes are under circadian control, in *Arabidopsis* for instance at least 6 % of the RNA transcripts, i.e., more than 1000 genes (Harmer et al. 2000).

8.3 Circadian Rhythms and Photoperiodic Reactions in Algae

The mechanisms of the circadian clocks in animals (Hardin and Panda 2013), fungi (Dunlap and Loros 2006), and plants (McClung 2011b) have been studied intensively. They are supposed to consist of transcriptional/translational negative feedback loops, but the clock components of plants differ from those of animals, fungi, and Cyanobacteria. The molecular composition of the *Arabidopsis* clock as proposed first by Alabadi et al. (2001) has turned out to be quite complex (see Chap. 12 and, for review, Hsu and Harmer 2014). As often done in cases where we are dealing with complex and interwoven networks, model building is a way to handle it (e.g., Dalchau (2012) with further references). A similar, but simpler clock as the *Arabidopsis* clock is the one of *Ostreococcus tauri* which will be described in Sect. 8.3.2. The mechanisms of the circadian clocks of the other photoperiodic examples given in the following are mentioned under the corresponding sections.

Circadian rhythms have been found in cyanobacteria (Qin et al. 2010; Brunner et al. 2008), and they are common among eukaryotic algae such as *Euglena* (Mittag 2001), *Chlamydomonas* (Schulze et al. 2010), *Acetabularia* (Yang and de Groot 1992), and *Lingulodinium* (Wagner et al. 2005). Here, we select *Synechococcus elongatus* as a cyanobacterium, the smallest known eukaryote *Ostreococcus tauri,* the more complicated and 30 times larger *Chlamydomonas* (see Sect. 8.3.4), and the unicellular dinoflagellate *Lingulodinium* (see Sect. 8.3.3) as examples exhibiting photoperiodic reactions. Photoperiodic reactions are known also in other algae (Agrawal 2012; *Laminaria:* Lüning 1980).

8.3.1 Synechococcus

Hut and Beersma (2011) discuss why a circadian timing system has emerged in primitive organisms such as cyanobacteria and what enables these bacteria to adapt to seasonal variation in day length in using these clocks. Experiments by Woelfle

et al. (2004) and Ouyang et al. (1998) have shown an adaptive value of a circadian clock for reproductive success in *Synechococcus*.

KaiA, KaiB, and KaiC proteins and ATP form the basis of this temperature compensated circadian clock (Nakajima et al. 2005). Hut and Beersma (2011) propose that this clock is timing the harvesting and storing of sunlight energy when it is available and using up their stores during darkness. KaiC stores ATP during the day and supplies ATP at night. KaiB evolved to suppress the cell division at the end of the day by binding to the KaiC hexamer in a specific phosphorylation state. Since at that time no direct environmental signal is available to trigger this response, a clock was needed. KaiA accelerates KaiC phosphorylation which will change the timing of the blockade of cell division by KaiB. In this way, *Synechococcus* can adjust to the different photoperiodic conditions during the course of the year and allow the cells to expand their geographical range to different latitudes. KaiA would be up-regulated under short photoperiods and down-regulated under long photoperiods.

8.3.2 Ostreococcus tauri

Ostreococcus tauri (Chlorophyta, *Prasinophyceae*) was discovered in 1994 in the Thau Lagoon in Southern France by Courties and Chretiennot-Dinet (1994)—other Ostreococcus species inhabit the Pacific Ocean and the subtropical and tropical North Atlantic (Demir-Hilton et al. 2011). The coccoid cells are haplonts with a rather simple cell structure: Just one chloroplast, one mitochondrion, one Golgi body besides the nucleus, no cell wall. The tiny genome (13 Mb, fewer than 8000 genes with a very low redundancy, see Lozano et al. 2014) has been sequenced and biological information attached to the sequences (see http://www.geneontology.org/GO.evidence.shtml and Corellou et al. 2009). Under LD conditions, almost all genes were rhythmic (Monnier et al. 2010).

Ostreococcus tauri has to cope with fluctuations in light intensities caused by clouds or the different depths in the water. Due to a phase response curve, which possesses a broad dead zone in which light does not shift the phase of the rhythm, the oscillator is only re-entrained by light *outside* its dead zone. While synchronized with the LD cycle, the oscillator is blind to light. Thus, the light intensities might fluctuate considerably without affecting the clock (Pfeuty et al. 2012; Thommen et al. 2012).

Cell division and transcription of the main cell cycle genes such as cyclings and kinases are under circadian and under photoperiodic control (Moulager et al. 2007, 2010). Thommen et al. (2012) analyzed the temporal expression profiles of central clock genes *CCA1* and *TOC1* for different photoperiods. The two protein peaks tracked different times of the day, but all profiles could be reproduced by using the following two-gene transcriptional loop model.

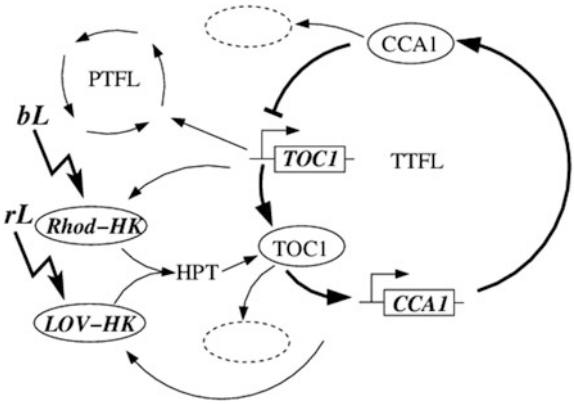

Fig. 8.1 Molecular model of the *Ostreococcus tauri* clock consists of a negative transcriptional/translational feedback loop (TTFL) between TOC1 and CCA1. Transcription and translation of TOC1 (*oval*) activate (*thick arrows*) transcription of the CCA1 protein which represses (*thick lines*) TOC1 expression. CCA1 is degraded (*oval*, *broken border*) by proteasomes with a maximum during the day. Degradation of TOC1 (*oval*, *broken border*) by proteasomes peaks in the dark and is diurnally regulated. This TTFL (*thick arrows/lines*) drives a posttranslational feedback loop PTFL. Normally, the TTFL and PTFL are coupled. However, whereas in DD transcription ceases and the TTFL stops, the PTFL continues to run. Long- (flash and rL for *red light*) and short-wavelength (flash and bL for *blue*) light are absorbed by a rhodopsin histidine kinase (Rhod-HK, oval), respectively, LOV histidine kinase (LOV-HK, *oval*) and affect via histidine phosphotransfer (HPT) the TOC1. There is furthermore a feedback (arrows) from the TTFL to the photoreceptors. After Pfeuty et al. (2012), McClung (2011a) and Troein et al. (2011)

The circadian clock is a simplified *Arabidopsis* clock possessing only two of its clock gene types (see Sect. 8.4 and Thommen et al. 2010; Djouani-Tahri et al. 2010; Corellou et al. 2009) and has been modeled (Dixon et al. 2014; Pfeuty et al. 2012; Troein et al. 2011) using only one feedback loop (see Fig. 8.1).

The model is based on a negative transcriptional/translational feedback loop (TTFL) between TOC1 and CCA1. The time delay is brought about by the timing of the expression of *TOC1* and *CCA1*. CCA1 represses *TOC1* expression during most of the day except during a couple of hours before dusk by binding to an evening element-sequence (EE) in the *TOC1* promoter (Morant et al. 2010). It afterward induces *CCA1* transcription, so that CCA1 is expressed in the night and early morning.

Non-transcriptional, post-translational feedback loops (PTFL) such as rhythmic protein modifications are also involved in circadian timing of animals and plants (van Ooijen and Millar 2012; O'Neill et al. 2011). They are able to sustain circadian timing in *Ostreococcus*, although normally they function together with transcriptional components. An output is the circadian rhythm of the redox state of peroxi-redoxin. Targeted protein degradation in the circadian mechanism seems to play a central role. It was proposed that the oldest oscillator components are non-transcriptional, as in Cyanobacteria, and that they are conserved across the plant and animal kingdoms (Bouget et al. 2014).

Light receptors in *Ostreococcus tauri* are a histidine kinase LOV-HK, which absorbs blue light, and a histidine kinase, Rhodopsin-HK (Rhod-HK), which senses longer wavelengths (Pfeuty et al. 2012). Using long- and short-wavelength photoreceptors (see Fig. 8.1) allows the cells to discriminate light variations due to depth changes (long wavelengths are absorbed within the first meters of the water!) from those due to the day/night cycling (Troein et al. 2011). Cryptochromes are further blue light receptors and one of it, CPF1 (protein of the cryptochrome/photolyase family), is involved in the maintenance of the *Ostreococcus* circadian clock (Heijde et al. 2010).

8.3.3 *Lingulodinium polyedra*

The unicellular dinoflagellate *Lingulodinium polyedra* is found at the surface of the oceans during the warmer part of the year. It displays several daily rhythms such as bioluminescence, photosynthesis, motility, cell division, and the synthesis of many proteins (Dagenais-Bellefeuille et al. 2008; Hastings 2007; Akimoto et al. 2005), and changes in protein phosphorylation may underlie them (Liu et al. 2012). The best studied rhythm is bioluminescence. *Lingulodinium* emits light under shear stress of water flow or shaking a culture in a flask (Maldonado and Latz 2007). This bioluminescence is due to luciferase and luciferin (Schultz et al. 2005) and involves mechanotransduction by G-proteins (Chen et al. 2007). The amount of emitted light depends on the time of shaking with a maximum during midnight. The flash rhythm continues under DD. Besides the flash rhythm, there is also a glow rhythm which can be observed in undisturbed cultures. The two rhythms are driven by two separate oscillators. They have been studied intensively. The cells communicate with each other via substance(s) in the medium (Hastings 2007).

In the fall, the *Lingulodinium* cells sink to the bottom of the sea, get rid of their shells and flagella, and form a cyst. This step is photoperiodically controlled (Balzer and Hardeland 1991) and melatonin might be involved (Hardeland and Poeggeler 2003). However, temporary cysts are also formed when *Lingulodinium polyedra* algae experience low temperature (e.g., 8 °C) or other environmental stresses (Hastings 2007). Temperatures above 16 °C prevent cyst formation.

Circadian rhythms in *Lingulodinium* are synchronized by blue and red light via two photoreceptors (Roenneberg and Hastings 1988).

8.3.4 *Chlamydomonas reinhardtii*

Another example and model organism for circadian rhythms and photoperiodic responses is *Chlamydomonas reinhardtii* (Schulze et al. 2010; Werner 2002; Mittag

2001). This unicellular alga belongs to the Chlorophyta and is of 14–22 μm size and found worldwide in freshwater, but also in the soil.

The genome of *Chlamydomonas reinhardtii* is entirely sequenced (Matsuo and Ishiura 2011). Much work is devoted to the clock network and to clarify how clock-related factors are interconnected. System biology approaches are used for this means, metabalance (Manichaikul et al. 2009) and functional proteomics (Wagner and Mittag 2009). Based on the elementary flux mode analysis, Schäuble et al. (2011) combined sequence information with metabolic pathway analysis and included circadian regulation. They were able to predict the changes in the metabolic state and hypothesize on the physiological role of circadian control in nitrogen metabolism.

Phototactic movement (swimming toward light) is driven by the flagella and controlled by a circadian clock (Gaskill et al. 2010; Bruce 1972). This clock furthermore controls UV sensitivity (Nikaido and Johnson 2000), chemotaxis (Schulze et al. 2010; Byrne et al. 1992), adherence to glass, cell division (Goto and Johnson 1995; Bruce 1972), starch (Ral et al. 2006), and nitrogen metabolism (Schäuble et al. 2011). The period length is temperature compensated, as in all circadian rhythms (Franklin et al. 2014). For modeling the *Chlamydomonas* clock, see Matsuo and Ishiura (2011) and Manichaikul et al. (2009).

The circadian rhythm is synchronized and phase shifted by blue, yellow, and red light, but not far-red light. Subproteome and phosphoproteome analyses (proteome is the entire set of proteins expressed by a genome, cell, tissue, or organism at a certain time; subproteome is a subset of a proteome) are used for finding the photoreceptors (Boesger et al. 2009). Homologous of cyanobacterial PHY, the cyanobacterial kinase CikA, and two CRYs with significant homology to the CRYs from plants and animals were detected (Beel et al. 2013; Mittag et al. 2005). The phase-shifting effect of light was studied by Johnson et al. (1991) and Kondo et al. (1991). Whether photoreceptor proteins ChR-1 and ChR-2 in the eyespot of *Chlamydomonas reinhardtii* at the outer chloroplast envelope are involved in the phase shift is not known (Hegemann 2008).

Light pulses with a certain fluence rate and wavelength applied at the breakpoint between delay shifts and advance shifts reduce the amplitude of the rhythm to such a degree that the clock stops to oscillate and reaches a "singularity" (Johnson and Kondo 1992), a situation which could be used to test the hypothesis that a circadian oscillator measures daylength (or nightlength) in photoperiodic events. The circadian clock controls critical aspects of plant development. As shown in Sect. 8.5, flowering is in many higher plants photoperiodically induced and CONSTANS (CO), a B box protein, regulates the expression of a florigenic signal. Green microalgae encode also CONSTANS-like (COL) proteins. The *Chlamydomonas reinhardtii* CO homologue (CrCO) expression is controlled by the circadian clock and involved in the timing of several circadian output processes such as the accumulation of starch or the coordination of cell growth and division (Romero and Valverde 2009; Serrano et al. 2009).

The CrCO is furthermore influenced by day length, being more expressed in short photoperiods. Germination efficiency of zygospores of *Chlamydomonas reinhardtii* depends on the photoperiod and is higher in longdays and lower in shortdays (Mittag et al. 2005; Suzuki and Johnson 2002). CO orthologs might represent ancient regulators of photoperiodic events. They arose early in evolution and are involved in the photoperiodic control of important developmental processes, which may have been crucial for their evolutionary success finally leading to flowering plants. The *Chlamydomonas reinhardtii* CO homologue (CrCO) can even complement the *CO* mutation in *Arabidopsis* (Serrano et al. 2009). In a review by Valverde (2011), the photoperiodic systems in algae and plants are compared and the general role of CO is proposed.

8.4 Photoperiodic Reactions in Higher Plants

In many higher plants, photoperiod regulates quite a number of different processes. In the following growth, succulence (Sect. 8.4.2), tuber formation (Sect. 8.4.3), and floral induction (Sect. 8.5) are chosen as examples.

8.4.1 Growth

The output of the circadian clock regulates transcription of downstream genes. They are responsible for different daily cellular processes. Maximum effects of elementary steps take place at specific times of the day. Among these processes are also the mechanisms which activate plant growth. Phytohormones, temperature conditions, light signaling, and photoperiod are involved. How they are coordinated with rhythmic changes in the environment by the clock has been reviewed by Yamashino (2013) and Nomoto et al. (2013). Ultradian growth oscillations are treated in Chap. 1 and nutations in Chap. 2 of this book.

A circadian promotion of elongation in *Arabidopsis* hypocotyls and petioles occurs via *PIF4* genes at the end of night in shortdays and can be described by an external coincidence model (see Fig. 8.2, Sect. 8.6 and Lagercrantz 2009).

Under short-day conditions, the PIF4 protein is stably accumulated, whereas under longdays, it is degraded by light-activated phyB, and the residual proteins are inactivated by proteins of the DELLA family (gene repressors, respectively, activators, inactivated by GA signaling and acting downstream of the GA receptor to modulate GA-induced growth and development).

Crucial evidence for an external coincidencein the photoperiodic control of plant growth has been provided by Yamashino et al. (2013).

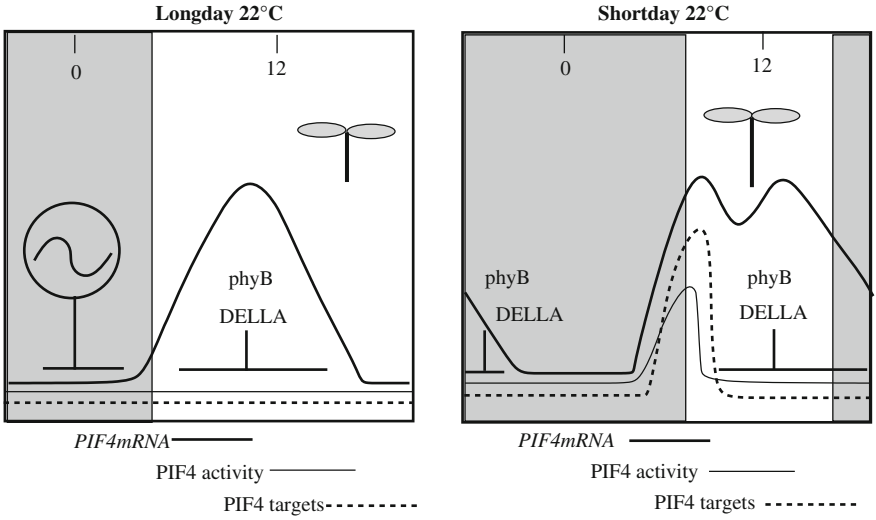

Fig. 8.2 Control of elongation by external coincidence of circadian clock (*wave in circle*) with the LD cycle (L *white*, D *gray*) under long-day (*left*) and under short-day conditions (*right*). X-axis covers a day with midnight (0) and noon (12) indicated. *PIF4mRNA* (*solid thick curve*) is produced under long-day conditions, but no PIF4 protein (*thin curve*) made because it is degraded by light-activated phyB and the residual proteins are inactivated by proteins of the DELLA family. Thus, there are no PIF4 targets (*dashed curve*) activated. The plant is short (*inset*). Under short-day conditions, the PIF4 protein is stably accumulated (*dashed curve*) during the long dark period, and the target genes are activated (*thin curve*). The plant elongates (*inset right diagram*). After Yamashino et al. (2013)

8.4.2 Succulence

Succulence (i.e., more than usually fleshy, development of water-storing tissue) of stems and leaves is also often under photoperiodic control. Under short-days, *Kalanchoe blossfeldiana* forms small, rigid, succulent leaves. Under longdays, the leaves are thinner, flexible, and large (von Denffer 1941). Water uptake and subsequent enlargement of the cells perpendicular to the leaf blade lead to the succulent appearance (Harder 1948). *Metaplasin* was proposed to induce succulence, since grafting of succulent leaves to plants under long-day conditions induces succulence in these plants (Harder and von Dassow 1940). The flower hormone florigen is, however, said to be different from metaplasin, since chloroform inhibits flower induction in shortday, but has no effect on the thickening of the leaves (Harder and Gall 1945). This could be similar to the tuberization and floral transition in potato which is controlled by StSP3D and StSP6A, two different FT-like paralogues, see next subsection. Furthermore, *Crassulaceae* are known which are succulent in shortdays, but flower in longdays. Wallrabe (1942) found red and blue lights to be effective for the photoperiodic control of succulence of *Kalanchoe* leaves, whereas green light is not; but the photoreceptors are unknown.

8.4.3 Tuber Formation in Potato

Numerous storage organs of plants are induced photoperiodically (Ewing and Struik 1998). Among them are the varieties of potatoes from South America (e.g., *andigena*). *Solanum tuberosum* is usually cultivated. After wheat and rice, potato is the third most important crop (http://faostat.fao.org/). Potato was grown in the Andes of southern Peru already 10,000 years ago. It originated from the wild species of *Solanum breviculae* (Abelenda et al. 2011), which forms tubers on their stolons (underground stems) under short-day conditions before the onset of winter (Hackbarth 1935). Diageotropical growth of the stolons is arrested and the tips swell in the subapical region to form storage organs containing large amounts of starch and other nutrients. They remain dormant in the soil during the winter and generate new plants in the spring (Sonnewald and Sonnewald 2014).

Daylength is sensed by the leaves and red is the effective light perceived by PHYB (Jackson and Thomas 1998). If the critical dark period is surpassed, tuber-inducing mobile substances are produced. They are transported via the phloem sieve elements to the underground stolons (Navarro et al. 2011). At the same time, a tuber-inhibiting substance, gibberellic acid, of the long-day loses its effect under shortdays (Tizio 1971). Growth of the stolons in the longitudinal direction is inhibited, thickening induced and starch is stored in the cells. Flowering regulators such as CO, BEL5, and miR172 play also a role (see Fig. 8.3 and Abelenda et al. 2011; Martin et al. 2009).

Tuberization under short-day conditions and flowering in potato are controlled by two members of the potato FT-like gene family, *StSP6A,* respectively, *StSP3D* (Abelenda et al. 2014; Navarro et al. 2011). They respond to different environmental cues, in case of tuberization to shortday and in case of flower induction to long-day conditions. The detailed mode of triggering flowering, respectively, tuberization in response to the mobile FT signal is under study (Abelenda et al. 2011, 2014; Navarro et al. 2011; Kloosterman et al. 2007). It is the protein and not the mRNA which is transported as a triggering signal. *StSP6A* is expressed not only in leaves but also in stolons of tuberizing plants, suggesting an autoregulatory loop for the transported protein. StCO is also involved in this loop (González-Schain et al. 2012). Two other paralogues for StSP6A were found; they have an inhibitory effect on tuberization.

A further question is how flowering (see Sect. 8.5) and tuberization are differentially triggered in response to the mobile FT signal. Perhaps StSP6A interacts in the subapical cells of stolons with an unknown transcriptional regulator or activates genes specific to these cells.

FT seems to be a key signal controlling besides flowering also a number of other meristem-associated transitions. It is for instance involved in terminating meristem growth in tomato (Shalit et al. 2009) and in the seasonal control of growth cessation in poplar trees (Böhlenius et al. 2006).

Other photoperiodic effects are known in plants such as root formation, leaf abscission, bud formation and bud dormancy of trees and shrubs, stem elongation,

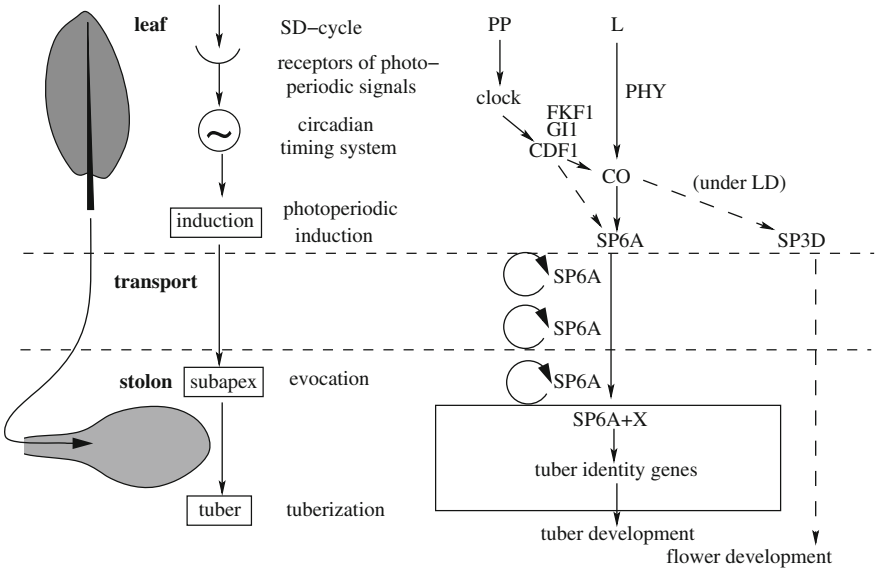

Fig. 8.3 Scheme of tuber formation in a potato plant. Photoperiod (L/D) is perceived by photoreceptors in the leaves. Under short-day SP6A, a FT homologue of *Solanum tuberosum* is induced by CO under the participation of CDF1 (which in turn is affected by FKF1 and GI) and transported to the stolons. On its way, a relay mechanism sustains SP6A synthesis. At the subapex of the stolons, it combines with a factor X and activates tuber identity genes thus initiating tuber development. The photoperiodic reaction is due to rhythmic control of *co* expression by the circadian clock, FKF1 protein functioning as a photoreceptor. PHYB plays a role in tuberization control by inhibiting it in longdays. After Abelenda et al. (2014), Kloosterman et al. (2013) and Navarro et al. (2011)

vegetative growth, cambium activity, tissue differentiation, cold resistance, fertility, sex determination, and expression, but for lack of space we refer to Nelson et al. (2010) and Thomas and Vince-Prue (1997). The most frequently studied and best known photoperiodic reaction is the induction of flowering, which will be treated next.

8.5 Seasonal Timing of Flower Induction

8.5.1 The Various Pathways

In order to flower and produce seeds at the right time of the year, plants use several control systems to evoke the transition from the vegetative to the reproductive stage of the apex (see Fig. 8.4). 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

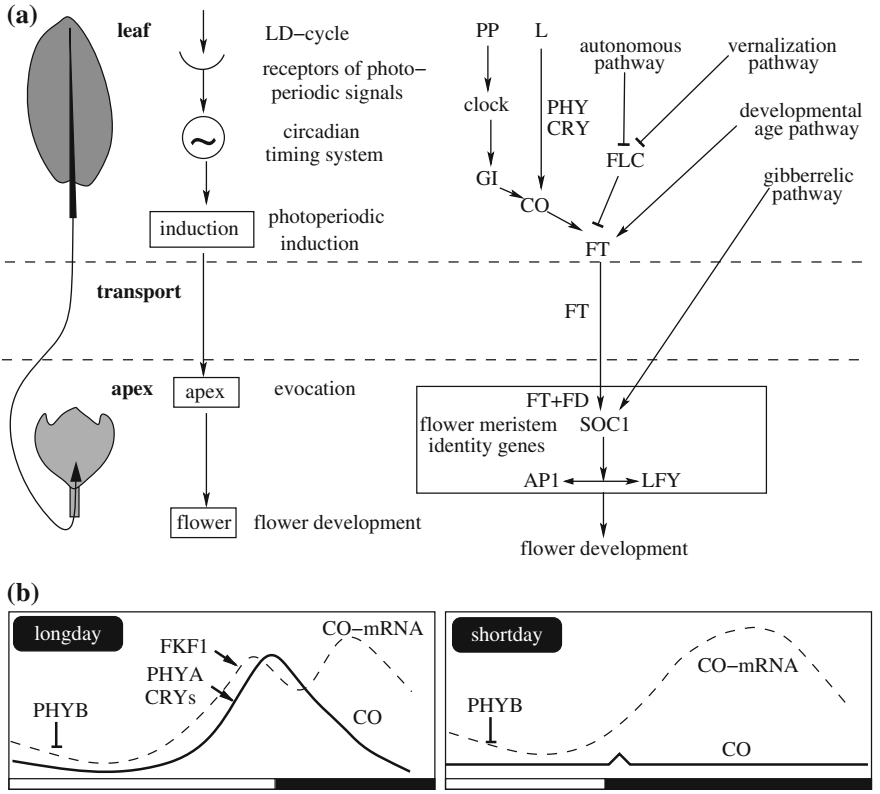

Fig. 8.4 Scheme of the flower induction of a long-day plant. Photoperiod (L/D) is perceived by photoreceptors in the leaves (PHYs and CRYs). Under longday, FT is produced and transported to the apex. There it combines with FD and activates flower meristem identity genes *ap1*, *ap2*, *cal*, and *lfy*, thus initiating flower development. There are other pathways besides the photoperiodic one which control flowering such as an autonomous pathway, which leads finally to flowering even under non-inductive photoperiodic conditions, a light quality pathway, which accelerates flowering under shading conditions, a vernalization pathway, and a gibberellic acid (GA) pathway, where GA will induce flowering. The autonomous and vernalization pathways inhibit *flc*, thus removing the blockade of FT production by FLC or activate *ft* via PFT1 in the quality pathway (which is not shown); or the GA pathway activates *soc1* and the flower meristem identity genes directly. There exists also an activation of FLC. 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 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 shortdays, there is not enough time under light to produce the CO protein (*right bottom curves*). After Más (2005)

before flowering is induced (Matsoukas et al. 2013). The age of the plant can also shorten the critical dark period, as shown for *Pharbitis nil* (Hasegawa et al. 2010). This is the **autonomous pathway** of flowering.

Another part of the control system perceives environmental signals such as daylength, ambient temperature (vernalization, a treatment where low temperature is necessary for flowering to occur (Turck and Coupland 2014; Wellmer et al. 2014; Ream et al. 2012)), humidity of the soil as well as biotic (e.g., pathogen infections) and abiotic stresses (cold, dehydration, high salinity and osmotic stress, see Yamada and Takeno 2014; Riboni et al. 2014). If this **environment-reactive pathway** reports the right conditions, flowering is induced. Whether the autonomous or the environment-reactive pathway prevails in flower induction, depends on the plants and varies a lot (Aukerman and Amasino 1996). There is furthermore a **gibberellin pathway** (Khan et al. 2014; Coelho et al. 2013).

In the following, the photoperiodic induction in the environment-reactive pathway will be treated. The transition from the vegetative to the reproductive stage of the apex can be induced by switches which become effective under longdays in so called longday plants or under shortdays in shortday plants, depending on the species or variety. The length of the light period or dark period is not important, but rather whether a critical length has passed or has fallen short. Short/longday plants such as *Bromus inernis* (Heide 1984), *Trifolium repens* (Thomas 1961), and *Echeveria harmsii* (Zeevaart 1978) need to be exposed first to shortdays and afterward to longdays in order to flower, whereas long/shortday plants such as *Bryophyllum daigremontianum* (Zeevaart 2008) need first longdays and afterward shortdays for flowering. For details, see Nelson et al. (2010), Thomas and Vince-Prue (1997) and Vince-Prue (1975).

The most frequently used plant for studying flower induction is *Arabidopsis thaliana*, a facultative long-day plant. However, other plants are also studied, such as rice *Oryza sativa*, mays *Zea mays*, morning glory *Ipomoea nil* (see page 208), and soybean *Glycine max* (Wong et al. 2013). The results of these studies illuminate the similarities and differences between and among long- and short-day plants (Shrestha et al. 2014; Zheng et al. 2009; Hayama and Coupland 2004). Photoperiodic induction of flowering in annual and perennial plants is discussed by Khan et al. (2014).

Usually, a number of inducing photoperiods is necessary in order to induce flower formation. However, in some plants, just one day is sufficient, for instance in the short-day plant *Ipomoea nil* (Chois) cv violet and *Chenopodium rubrum* (L.) selection 374, and in the long-day plant *Lolium perenne* (Evans 1969). Since the photoperiodic treatment in these plants leads rapidly to the photoperiodic reaction, they are often used for experimentation (Drabešová et al. 2014). 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).

8.5.2 *Molecular Mechanism of Flower Induction in* Arabidopsis thaliana

Figure 8.4 top shows a simplified scheme of the current molecular mechanism of the circadian clock of the long-day plant *Arabidopsis thaliana* and the photoperiodic control of flowering. First, the photoperiodically active light is perceived by photoreceptors in the leaves (and there perhaps in the mesophyll and in the epidermis as shown for instance in *Kalanchoe blossfediana* by removing the upper, respectively, lower epidermis, which reduced flowering drastically (Mayer et al. 1973), 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 special anatomical features that allow sufficient light absorption during twilight (Haberlandt 1905).

In *Arabidopsis,* it was shown that phyB perceives light stimuli in the mesophyll, whereas cry2 functions only in vascular bundles. Both photoreceptors regulate flowering by altering the expression of FT in vascular bundles. Since they are spatially separated within a leaf, the signals have to be integrated by communication between these tissues (Wang et al. 2014). There are other factors that are involved in the regulation of flowering by light such as CO, SPA, COP1, and PFT1 which link the photoreceptors and FT. They most likely function in leaves, but at which tissue level is unknown except for CO (further discussed by Endo and Nagatani (2008) and Chory (2010)).

Phytochromes are the most important photoreceptors for flower induction, e.g., in peas (Weller et al. 1997). In *Cruciferae,* additional photoreceptors for blue light are involved (Guo et al. 1998). There are opposing roles of PHYA and PHYB on flowering (by differentially regulating FT expression, see below). The signaling occurs via a network with molecular interactions. It differs at 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. 8.2 and 8.6 and Song et al. 2013; Wenden et al. 2012): If the critical dark period has been passed in short-day plants or if it falls short in long-day plants, a substance is produced by the cells in the leaf for flower induction. The 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, temperature affects the *number* of induced flowers in *Kalanchoe*: At the higher temperature, 40 times as many flowers are formed as compared to the lower temperature). This temperature compensation of the critical daylength is not only

found at flower induction, but also at other photoperiodic reactions (Franklin et al. 2014).

In photoperiodic induction of flowering in *Arabidopsis thaliana,* CO (CONSTANS) plays a central role (Putterill et al. 1995): It mediates between the environment, the clock, and the initiation of flowering (Hayama et al. 2003). CO-mRNA oscillates in a circadian manner. Under long-day conditions, the peak is at the end of day and during the night, under short-day conditions during the night (see curves, bottom of Fig. 8.4). CO in a long-day plant such as *Arabidopsis thaliana* is stabilized in the light by PHYA and CRYs at the end of day, but degraded by proteasomes in darkness. PHYB promotes CO degradation during the dark period and leads to low concentrations of CO in the morning; it will be present in longday, but not in shortday (Endo et al. 2014a; Hayama and Coupland 2004). The increase of the amount of CO in shortdays at the early portion of the dark period is very minor and is not sufficient to induce the synthesis of FT by *ft*, a target gene of CO (Valverde et al. 2004). But under longdays, sufficient CO will accumulate and lead to FT production. This is essentially what the external coincidence model requires (Suarez-Lopez et al. 2001).

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

FT and the orthologs of it is the long thought flowering signal *florigen*. It is a small globular protein transmitted from leaf companion cells through the phloem to the shoot apex (Liu et al. 2013; Turnbull 2011) where it combines with FD (a bZIP transcription factor encoded by FLOWERING LOCUS D) in the nucleus after being bridged by 14-3-3 protein (GF14) in the cytoplasm (Nakamura et al. 2014; Wigge 2011). The FT–FD complex binds to DNA and initiates the transcription of flower meristem identity genes such as *ap1*, *ap2*, *cal,* and *lfy*. It initiates reproductive development (flower evocation, Abe et al. 2005; Wigge et al. 2005) and flowers are formed according to the ABC (DE) model (Jacquot and Gadal 2014; Wellmer et al. 2014).

Key control of flowering is done by FT and TFL1. They determine, when and where flowers are made, but TFL1 is a repressor, whereas FT is an activator. Both are highly conserved and differ by one amino acid only (Mylne and Wigge 2011; Hanzawa et al. 2005).

There are further mechanisms which antagonize FT activity such as ATC (*Arabidopsis thaliana* CENTRORADIALIS homologue) which likewise moves over long distances and inhibits floral induction by competing with FT for FD (Son et al. 2014; Huang et al. 2012). Another potent floral repressor is

FLOWERING LOCUS C (FLC) (He 2012). Chromatin modifications such as histone deacetylations and methylations play a role in FT regulation preventing, e.g., precocious flowering (Shafiq et al. 2014; Gu et al. 2013). TEM1 and TEM2 (TEMPRANILLO 1 and 2), transcription factors of the RAV (=RELATED TO ABI3 AND VP1) family delay flowering by repressing the expression of FT, and gibberellins until enough reserves are formed and the plant is large enough to ensure survival of the progeny. TEMs may integrate the information from other pathways (Matías-Hernández et al. 2014).

8.5.3 Flower Induction in Short Day Plants

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

For CONSTANS-like genes and florigens in other plants, see more recent publications such as Griffiths et al. (2003); maize *Zea mays* and its florigen ZCN8 (CENTRORADIALIS, encoding both endogenous and environmental signals, but with pleiotropic functions): Meng et al. (2011) and Danilevskaya et al. (2011); soybean *Glycine max* requiring shortdays for flower induction with its florigen GmFT2a: Wong et al. (2013) and Watanabe et al. (2011); rice *Oryza sativa* as a facultative short-day plant (flowers more rapidly in shortdays, but flowers delayed also under longdays) with its florigen Hd3a and a further florigen RFT1, which induces flower induction under non-inductive long-day conditions: Itoh and Izawa (2013) and Ishikawa et al. (2011); *Chrysanthemum* as a typical short-day plant with three FT-like genes *CsFTL1*, *CsFTL2*, and *CsFTL3*: Oda et al. (2012); the short-day grass *Brachypodium* with posttranscriptional regulation mechanisms: Wu et al. (2013); tomato *Solanum lycopersicum* with its dosage sensitivity of the florigen SINGLE FLOWER TRUSS (SFT): Shalit et al. (2009) and Jiang et al. (2013).

Particularly, interesting is the absolute short-day plant *Ipomoea nil* (former *Pharbitis nil*) because of its easy rearing, rapid flower induction by just one single dark period of sufficient length in otherwise LL and its early response (Imamura 1967).

Zheng et al. (2009) investigated the role of distinct phytochromes in photoperiodic timekeeping in *Ipomoea*. The phytochrome pool consists of light labile phyA and light stable phyB and phyE. PHYC contributes to the phytochrome pool that participates in photoperiodic floral induction. Rhythmic abundance of *PHY* genes could be the molecular basis for the interaction between photoperiod and a circadian rhythm in regulating flower induction. *Ipomoea nil* expresses two orthologs of *FT*, *PnFT1,* and *PnFT2*, which promote flowering. These genes are expressed only under dark periods exceeding the critical length of 11 h. A light flash during the night reduces their expression and prevents flowering. A circadian rhythm is set by the transfer from light to dark and regulates *PnFT* expression in such a way that it rises only when the night is longer than the critical dark period. *Ipomoea* varieties with different critical dark periods express *PnFT* at different times after the transfer from LL into the dark period.

An ortholog of the *Arabidopsis CO* gene, *PnCO*, was found by Liu et al. (2001). It is expressed in a circadian pattern and the level is photoperiodically regulated. However, the *CAB* gene as a molecular marker for the circadian clock shows a different pattern and was not subject to the same photoperiodic control. *PnFT* mRNA abundance was not related to *PnCO* expression, which suggests that *PnFT* may be regulated by a different transcription factor, perhaps by *PnFL-2 (Pharbitis nil* FLOWERING-2, Kim et al. (2011)). It accumulates during the single inductive dark period in cotyledons and leaves, but is absent in other organs. A light break in the middle of the dark period eliminates the accumulation. *PnFL-2* mRNA also starts to accumulate in cotyledons and leaves 6 h after onset of the dark period and reaches a maximum between 14 and 16 h. In the subsequent light period, the concentration declines. Poor nutrition and low temperature stress induce the expression of *PnFT2* in *Ipomoea nil*, but not in another cultivar, Tendan (Yamada and Takeno 2014).

The mechanism for measuring daylength thus differs from that of *Arabidopsis* and rice (Hayama et al. 2007). It is not only *Ipomoea nil* that differs from *Arabidopsis thaliana* in respect to CO inducing FT, but also *Oryza sativa* (Hayama et al. 2007). According to Hayama et al. (2003), the evolution of long-day plants and short-day plants differed and there was a gradual adaptation of plants to environmental conditions.

The photoperiodic condition alters the methylation state of the DNA (Takeno 2010). This suggests that the expression of photoperiodic flowering-related genes could also be regulated epigenetically.

Ipomoea nil has been used for tests of the Bünning hypothesis, see section sec_Time_measurement and Kim et al. (2003), Zheng et al. (1993) and Lumsden et al. (1982). Bollig (1975) found that photoperiodic time measurement is controlled by another clock as the one timing the leaf movement rhythm. This is known also from other plants (King 1975; Wagner and Cumming 1970) and should be kept in mind in cases where the photoperiodic process displays a rhythm which differs from the one driving other circadian rhythms such as the CAB rhythm (Hayama et al. 2007). There are furthermore different circadian clocks in a plant driving various processes and/or clocks which are bound to tissues and organs of a plant (James et al. 2008; Fukuda et al. 2007; Thain et al. 2002).

8.6 Photoperiodism: Hourglass or Oscillator?

Seasonal changes in day length are used by plants and animals to synchronize annual rhythms in reproduction, physiology, and behavior to the environment.

It was the idea of Bünning (1936) that for photoperiodic regulation of a biological process, organisms have an inherited timescale at their disposal which they, using a circadian clock, compare throughout the year with the actual length of the day or night. If a critical length is reached, a photoperiodic reaction occurs (Bünning 1960). This is the *external coincidence model* as it was later termed.

Saunders (2005) shows that the idea of a circadian clock measuring daylength (or nightlength) and by comparing it with an internal critical length (Bünning hypothesis) and the idea of an hourglass-like device measuring day- or nightlength (Lees 1960) must not be mutually exclusive. The hourglass timing might well be a response of an oscillator which is, however, damped, and Saunders points to photoperiodic examples from insect studies. In further papers (Saunders 2010; Saunders and Bertossa 2011), the controversial aspects of insect photoperiodism are elucidated and it is concluded that internal and external coincidence timers are found, but in some species non-circadian hourglass-like devices are acting. The exact role of circadian rhythmicity and the molecular basis of clock genes in photoperiodism are yet to be settled in insects.

Likewise, Meuti and Denlinger (2013) point to links between circadian clocks and the clock involved in photoperiodic control of diapause in insects. Resonance experiments (Nanda and Hamner 1958) and night interruption experiments (Bünsow 1953) suggest a circadian basis for the photoperiodic control of diapause in most insects studied so far. There is furthermore a connection between circadian clock cells and centers controlling the photoperiodic diapause. On the same line, mutations and knockdown of clock genes suggest such a connection.

On the other hand, Bradshaw and his group argue on the basis of their work with *Wyeomyia smithii* that a simple interval timer (an hourglass like mechanism) is sufficient to predict the photoperiodic responses of this chironomid over a broad geographic range in North America, and that circadian clock and photoperiodism evolved independently from each other. Geographic variation in circadian genes should not be extrapolated to photoperiodism, and modern genetic approaches should be used to identify genes involved in photoperiodism (Bradshaw et al. 2012; Emerson et al. 2009; Bradshaw and Holzapfel 2008; Bradshaw et al. 2003).

Lankinen and Forsman (2006) do present further data on this line using *Drosophila litoralis* and *Drosophila montana* (Lankinen et al. 2013) from different latitudes and refer to older publications (see furthermore Koštál 2011; Takeda and Skopik 1997; Skopik and Takeda 1986). In mammals, the increasing day length during spring causes sudden changes in the reproductive system once the critical photoperiod is reached. For the molecular mechanism behind this switch, see Hut (2011). References for other vertebrates, see Nakane and Yoshimura (2014) and Mishra and Tewary (2000).

In plants, the issue of circadian clocks and photoperiodism is discussed by Edwards et al. (2010). The timing of the circadian rhythm should respond to a change in photoperiod so that a particular phase of the LD cycle can be anticipated. This has been shown to be the case by studies of Perales and Más (2007), Love et al. (2004) and Millar and Kay (1996).

One question is how the external phases such as dusk and dawn are tracked by a clock. This is not straightforward, since this particular time changes with season. Rand et al. (2004, 2006) showed by using mathematical models that multiple negative feedback loops would permit tracking and increase the flexibility and robustness of a clock (Akman et al. 2010; Kitano 2007). To the *morning loop,* an *evening loop* was added to the model (Locke et al. 2006) and further gene-regulatory loops (McWatters and Devlin 2011) and cytosolic signaling mechanisms (Dodd et al. 2014). Furthermore, tissue- and organ-specific modifications of the plant clock mechanism may increase the complexity by adding spatially distinct rhythms (Endo et al. 2014b; Thain et al. 2002).

Using a three-loop circuit and measuring expression profiles for the *Arabidopsis* clock genes across multiple photoperiods, *dusk sensitivity* was introduced as a measure for entrainment patterns (*dusk-dominant* and *dawn-dominant*) instead of dawn or dusk tracking (Edwards et al. 2010). The model consists of light inputs, the daytime effects of the morning loop upon the genes expressed in the evening, and the night effects of the evening loop upon the genes expressed in the morning and enables dawn- and dusk-dominant entrainment within a single-gene network. The observed timing of the clock components was more dawn-dominant than dusk-dominant. This speaks for a strong resetting effect of dawn, but it should be kept in mind that direct light signaling and indirect light effects (e.g., photosynthesis) are important regulators during LD cycles. The main task of the clock may be to balance direct and indirect environmental inputs and to coordinate physiological functions.

In short-day plants, dusk-dominant rhythms prevail (although they show several dawn-dominant rhythms, see Hayama et al. 2007), whereas they are less important in the gene expression profiles of the long-day plant *Arabidopsis*. Its photoperiod sensor CO shows, however, an unusual degree of dusk-dominant circadian control. There might be a circadian clock modified for dusk dominance in the companion cells expressing CO (An et al. 2004), contributing even further for complexity.

8.7 The Bünning Hypothesis

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 dark period). If a certain time span, which corresponds 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 actions (in organisms: photoperiodic reactions) could be taken.

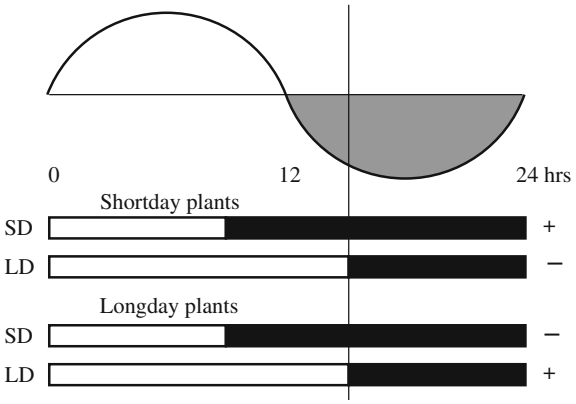

Fig. 8.5 The Bünning hypothesis explains, how a circadian clock measures the daylength. *Top curve* shows the photophilic phase (*white area*) and the skotophilic phase (*dark area*). Below: A short-day plant would be induced to flower (+) in shortdays (SD) with dark periods (*black*) exceeding a critical length, but would not flower (−) if light falls into the skotophilic phase under longdays (LD). Long-day plants would react oppositely to short-day plants (*bottom*)

However, that is often not the way organisms measure daylength. Instead, they are using oscillations as a clock, as proposed by Bünning (1936). According to the 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 (longdays or shortdays) and the photoperiodic situation of the organism (for instance, long-day plant or short-day plant; see Fig. 8.5).

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

1. A light pulse during the subjective day (portion of the circadian cycle which would correspond to the light period, if an organism is kept under DD or LL) does not shift the rhythm, since at that time the organism is normally subjected to light. A light pulse during the subjective night, however, shifts the rhythm. Light pulses of high intensity shortly before subjective midnight shift by delaying the rhythm, those after subjective 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 right strength. This is mentioned, because it has been used to test the Bünning hypothesis. If the clock measures the daylength, as claimed by Bünning, the measurement should not work any more, if the clock is stopped from running. This has been shown to be the case in *Ipomoea nil* (Bollig, 1970). A light break of 2-h white light of 2000 lx given 8 h after onset of a 58-h dark period inhibits flower induction, as shown by testing the remaining dark period with short (5 min) red light pulses ($2.3 \times 10^{-3} \mathrm{J/cm^2}$). Longer as well as shorter light breaks, likwise administered 8 h after onset of the 58 h dark period, do not inhibit flower induction.

2. Depending on the daylength, the internal oscillation with its two alternating phases (called by Bünning *photophilic*, light-loving, and *skotophilic*, dark-loving) interferes with the external light–dark cycle in different ways (see Fig. 8.5).

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

In the *internal coincidence model*, a light-on 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, respectively, by a change from light to darkness. Both rhythms interfere with each other differently depending on the length of the light period. That could be responsible for the photoperiodic reaction, as suggested by experiments in *Kalanchoe blossfeldiana* (Engelmann 1960). This model handles the varying daylengths during the year which can not be done with a model using a fixed time delay starting from just one phase such as dawn. Rand et al. (2004) point out that one oscillator with two or more loops, one of which is locked to dawn, while 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).

However, it is not necessary to consider here two rhythms (light-on and light-off rhythms, and later called M- and E-oscillator). Using a feedback model of Johnsson and Karlsson (1972) and a proper way of entering light into the feedback loop, photoperiodic reactions of flower induction in *Chenopodium rubrum* could be simulated by using just one oscillator instead of two (Bollig et al. 1976).

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 were designed and performed to test the hypothesis:

Varieties of soybeans from different geographical latitudes possess different critical daylengths for photoperiodic flower induction. At the same time, 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). The 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 already 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 1969a). Furthermore, a possible adverse effect of moonlight during the night, which could interfere with the photoperiodic reaction, is prevented (Bünning and Moser 1969b).

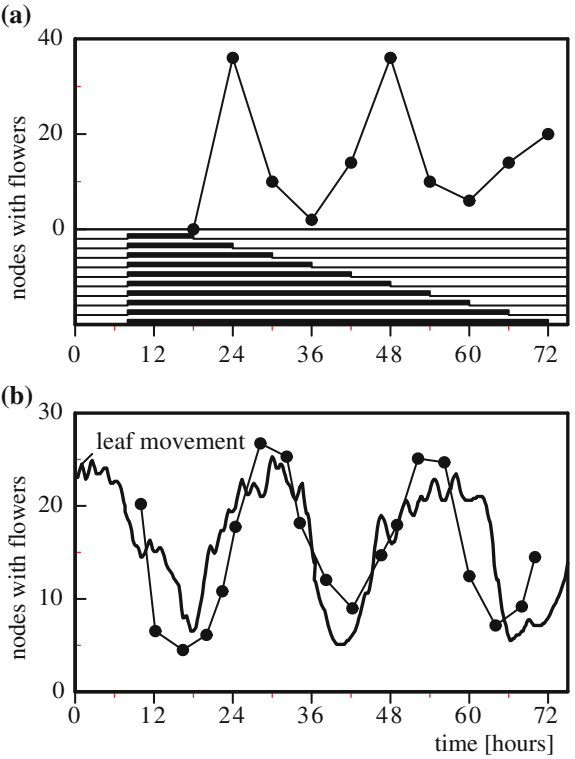

Fig. 8.6 Tests of the Bünning hypothesis. **a** Photoperiodic reaction of soybeans to dark periods of varying lengths (see *black bars*) is rhythmic (*top curve*, number of nodes with flowers). **b** Photoperiodic reaction of soybeans to light breaks in a long dark period (64 h) is rhythmic (*curve with black dots*) and the leaf movement rhythm (*black curve*) has the same time course under the same lighting conditions (i.e., 8:64 h LD). After Bünning (1954), kind permission Springer, Heidelberg

Variation of the dark period in short-day plants was used as another test of the hypothesis. If the hourglass hypothesis was correct, groups of plants receiving a fixed light period and varying lengths of dark periods should flower once the critical dark period was surpassed. It turned out, however, that the amount of flowering depended rhythmically on the length of the dark period; see experiments on *Kalanchoe* (Bünsow 1953) and soybeans (Blaney and Hamner 1957) and Fig. 8.6.

Light breaks in a long dark period: Likewise, a short light break given to different groups of plants in a long dark period leads to a rhythmic response (see Melchers 1956 with references, and Fig. 8.6).

Singular point: A special light pulse at a critical time which stops the circadian clock inhibits flower induction, as mentioned before (page SingularPoint).

Slowing the clock affects the critical daylength: If the daylength is measured by a circadian clock in photoperiodic flower induction, the critical daylength should change if the speed of the clock is changed. This was 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, 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. Whereas in the other cases of mutants mentioned, photomorphogenetic changes are also observed which could indicate changes in light signaling instead of circadian signaling; *toc1-1* lacks light-dependent morphological phenotypes (references in Yanovsky and Kay 2003). Under a 24 h LD cycle, this mutant flowers early independent of the daylength. However, under a 21 h LD cycle, which corresponds to the speed of its internal clock, flowering is induced under long-day conditions only. This is a strong argument in favor of the Bünning hypothesis.

For Pros and Cons of the hourglass hypothesis, see also Gyllenstrand et al. (2014) and in animals (King et al. 1997; Saiovici et al. 1987; Wade et al. 1986) for the Bünning hypothesis, see Menaker and Eskin (1967) and Bünning (1979). The external coincidence hypothesis and the mechanisms involved have been reviewed in Corbesier and Coupland (2006), Imaizumi and Kay (2006), Jarillo and Pineiro (2006), Bäurle and Dean (2006), Searle and Coupland (2004) and Yanovsky and Kay (2003), the internal coincidence hypothesis by Yeang (2013), Niwa et al. (2009), Sawa et al. (2008) and Nozue et al. (2007). Models can certainly help to better understand the connection between circadian rhythms and photoperiodism.

Quite a number of questions remain, such as: What are the molecular mechanisms for photoperiodic timing? What are the main differences between short-day and long-day plants on the molecular level?

8.8 Practical Aspects

In this section, horticultural and agricultural aspects of photoperiodic flower induction are mentioned. Of the large number of crop plants and plants important for the horticultural industry some are briefly mentioned here. During domestication, the tendency was to rear day neutral varieties or to change the critical day (night) length if the plants are to be bred in other geographical latitudes.

Chrysanthemum serves here as an example of a plant widely produced in the horticultural industry, but studied also intensively for the molecular basis of its flower induction (Fu et al. 2014). This short-day plant has been manipulated in such a way that their year-round commercial production by artificial lighting is enabled.

Tobacco: The genus *Nicotiana* contains day-neutral (*Nicotiana tabacum* cv Hicks), short-day (*Nicotiana tabacum* Maryland Mammoth), and long-day plants (*Nicotiana sylvestris*, Rutitzky et al. 2009). The responsible genes *NtSOC1* and *NtFUL* are similarly expressed under short- and long-day conditions in *day-neutral* and *short-day* tobaccos, but show a different expression pattern in the *long-day* plant *Nicotiana sylvestris* (Smykal et al. 2007).

Generation time can be reduced by backcrossing. Lewis and Kernodle (2009) used transgenic *FT* overexpression in tobacco which allowed flowering in an average of 39 days, in comparison with 87–138 days for non-FT plants.

Earlier flowering in the Maryland Mammoth mutant can be induced by expressing FT mRNA (Li et al. 2011). Late-flowering phenotypes of *Nicotiana attenuata* were obtained under long-day conditions by silencing *TOC1* (Yon et al. 2012).

Tomato: *Solanum lycopersicum esculentum* is an autonomously flowering day-neutral, perennial plant with early and late (e.g., mutant uniflora, Dielen et al. 2001) varieties. Plants carrying mutations in *SFT* flower delayed, grow extremely large, and produce few flowers and fruits. In heterozygous plants, yields are drastically increased due to a defect in the flowering repressor SP (Jiang et al. 2013). In modern tomato hybrids, yield is increased up to 20 % (Velez-Ramirez et al. 2014).

Potato: Allelic variation of *Solanum tuberosum* can be used for the domestication of potato in latitudes with large summer/winter day-length variation (Kloosterman et al. 2013). The mutant *ST* forms tubers even under LL (Fischer et al. 2008).

Temperate cereal crops such as **wheat** *Triticum aestivum*, **barley** *Hordeum vulgare* and **rye** *Secale cereale*: Flowering time genes such as *Vrn* and *Ppd* are involved in domestication and adaptation to local environments (Cockram et al. 2007).

The grass **sorghum** *Sorghum bicolor* is native to Africa but an important food crop also in Central America and South Asia. It is the fifth most important cereal crop in the world. Many cultivated forms exist, most of them drought and heat tolerant. Originally, a short-day plant with delayed flowering in longdays, most varieties used for grain production are day neutral and flower early to avoid adverse conditions. Energy sorghum hybrids, however, are photoperiodically sensitive, growing in longdays, thus leading to high biomass accumulation (Yang et al. 2014).

Rice: *Oryza sativa* is a facultative short-day plant that flowers more rapidly under short-days but delayed under long-days. Originally from tropical regions, it has been adapted to temperate latitudes and long-day conditions by modulating its sensitivity to day length. During domestication, *DTH2* was used in selecting rice to adapt it to higher latitudes. Furthermore, *Ehd4* plays an essential role in photoperiodic control of flowering time in rice (Gao et al. 2013).

The timing of flowering is important for successful reproduction, but also for determining the ideal balance between the duration of vegetative growth and reproductive growth. Recently photoperiod insensitive varieties have been created that grow in a wide range of latitudes and are better adapted to changing environments (Xu et al. 2014). Complete loss of photoperiodic response in a mutant *X61* of the variety Gimbozu leads to 35 days earlier flowering and to extremely early heading in the *japonica* cultivars *Hayamasari* (Nonoue et al. 2008). Some *indica* cultivars of rice such as *Nona Bokra* flower extremely late under long-day

conditions (Bin Rahman and Zhang 2013). It is tolerant to prolonged flood, submergence, and cold and is strongly photoperiodic and lacks dormancy.

Maize: Teosinte, the progenitor of *Zea mays*, is restricted to tropical environments in Mexico and Central America. It has spread to higher latitudes of the Americas by reducing the sensitivity to photoperiod. *ZmCCT*, a homologue of the rice photoperiod response regulator *Ghd7*, is the main gene responsible for it (Hung et al. 2012). There are seven *Zea mays ZCN* genes which encode FT homologous, but *ZCN8* is the only one with florigenic activity. Ectopic expression of it in shoot apices induced early flowering in transgenic plants, silencing by microRNA resulted in late flowering (Meng et al. 2011).

Soybean: *Glycine max* is an important oilseed crop used for human and animal feed. It is a short-day crop and the cultivars of it as well as its wild relative *Glycine soja* show different photoperiodic sensitivity in flowering varying with latitude. Flowering genes of soybean are discussed by, e.g., Jung et al. (2012).

Cotton: *Gossypium hirsutum* was converted from a photoperiodic perennial to an annual, day-neutral plant by ectopic expression of *FT*.

Sunflower: *Helianthus annuus* has been adapted photoperiodically during domestication (Blackman 2013). Clines varied with changes in the five *FT* paralogues, and in the expression of tissue-specific *SOC1* homologue.

8.9 Evolution of Circadian Rhythms and Photoperiodism

A few words to the evolution of circadian rhythms and photoperiodism: Several investigations have shown that circadian clocks enhance the fitness of organisms by improving their adaptation to and anticipation of environmental states such as the light–dark cycle, but perhaps also by timing and coordinating internal processes. This has been tested by Woelfle et al. (2004) using cyanobacterial strains with disrupted clocks. The authors found an adaptive value under cyclic environments, but none under constant environments. For higher plants, Yerushalmi et al. (2011) showed in *Arabidopsis* that mutants with a circadian clock possessing a 20 h period produce more viable offspring if matching a short T-cycle consisting of 13 h L and 7 h D. The same was found for mutants possessing a 28 h period if kept in long T-cycles consisting of 19 h L and 9 h D.

The role of the circadian system in photoperiodic time measurement may optimize seasonal timing and exert selection pressures at different latitudes (Vaze and Sharma 2013; Hut and Beersma 2011).

The CO signaling of the photoperiod has evolved quite early during evolution: Already, unicellular algae show photoperiodic responses in which the CO family of transcriptional activators plays a general role (Farré and Liu 2013; Valverde 2011).

For land plants, photoperiodic control of flower induction is critical. Its evolution is discussed by Simpson (2003). This process is controlled by GI and FKF1 in angiosperms. Kubota et al. (2014) report that orthologues of GI and FKF1 are present and functional already in the liverwort *Marchantia polymorpha* in the long-day-dependent growth-phase transition. Thus, plants had already acquired the GI-FKF1 system when they colonized land. This system was co-opted from the gametophyte to the sporophyte generation during evolution.

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For lack of space, the literature has been restricted by citing mainly the recent publications, in which earlier references can be found.

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Chapter 9
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 the movements of the stomatal guard cells. The guard cells close the stomatal pore to conserve water during stress. In 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 might regulate stomatal movements and the benefits that circadian regulation of stomatal behaviour could confer to the plant.

Abbreviations

ABA	Abscisic acid
ABAR	ABA receptor
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
CBF	C-REPEAT BINDING FACTOR
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
CCR2	*COLD, CIRCADIAN, RHYTHM 2*
Ci	Intercellular CO_2 concentration

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CK	Cytokinin
CK2	CAESIN KINASE 2
CO	CONSTANS
CPK	Ca^{2+}-dependent protein kinase
DD	Continuous darkness
EE	Evening Element
ELF3	*EARLY FLOWERING 3*
ELF4	*EARLY FLOWERING 4*
FT	FLOWERING LOCUS T
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
IRGA	Infrared gas analysis
LHY	LATE ELONGATED HYPOCOTYL
LKP2	LIGHT, OXYGEN, VOLTAGE/KELCH PROTEIN 2
LL	Continuous light
LUC	*LUCIFERASE*
LUX	*LUX ARRHYTHMIO*
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
OST1	OPEN STOMATA 1
PEPC	Phospho*enol*pyruvate carboxylase
PHYB	*PHYTOCHROME B*
PRR	*PSEUDO RESPONSE REGULATOR*
PP2C	Protein phosphatase 2C
PtdIns(3)P	Phosphatidylinositol 3-phosphate
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PYL	PYRABACTIN RESISTANCE-LIKE
ROS	Reactive oxygen species
SLAC1	SLOW ANION CHANNEL 1
SnRK	SNF-1-RELATED KINASE
SV	Slow vacuolar channel
TOC1	*TIMING OF CAB EXPRESSION 1*
TPC1	TWO PORE CHANNEL 1
TPK1	*TWO PORE K^+ CHANNEL 1*
VK	Vacuolar K^+ channel
ZTL	*ZEITLUPE*

9.1 Introduction

Stomata are small pores on the leaf surface that are the major point of gas exchange between the leaf and the atmosphere in higher plants. CO_2 enters through the pore before being fixed in photosynthesis, whilst water from the transpiration stream leaves the plant through the stomata. The pore is delineated by a pair of guard cells, whose movements regulate stomatal aperture. Guard cells respond to a wide range of environmental signals such as blue light, temperature, humidity and intercellular CO_2 concentration (C_i) by a change in turgor pressure that results in stomatal movements (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 cytokinin (CK) (Tanaka et al. 2006). 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 width to minimise water loss and maximise CO_2 uptake during favourable conditions.

In C3 and C4 plants, stomata open during the day to allow CO_2 uptake and are closed at night. In plants with crassulacean acid metabolism (CAM), the phasing of stomatal movements is opposite to that of C3 plants, with stomata open for part or all of the night, to allow CO_2 fixation by phosphoenolpyruvate carboxylase (PEPC). Nocturnal stomatal opening in CAM plants conserves water due to reduced transpiration in the cool of the night because the gradient for water loss is reduced by lower temperatures and increased relative humidity of the air (Webb 1998). 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, whilst 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, 2005). 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 optimisation of the plant.

9.2 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 swelling of cells along the longitudinal axis, whilst the overall length of the stomatal complex remains relatively unchanged (Sharpe et al. 1987; Shope et al. 2003). This change in cell shape

causes the opening of the pore between the guard cells and thus allows gas exchange. The accumulation and efflux of ions bring about the changes in turgor pressure through altering the water potential of the cell and driving osmosis. Early hypotheses included a role for diel changes in starch production in driving stomatal movements, but these have now been rejected in favour of models involving ion fluxes. Nevertheless, starch metabolism may 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 (Kollist et al. 2014). 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, allowing K^+ to flow down the electrochemical gradient across the plasma membrane. Cl^- and NO_3^- are taken up to counterbalance the K^+ charge, a role that is also fulfilled 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 the 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 two pore K^+ (VK) channel encoded by *TPK1* (Gobert et al. 2007) 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 $[Ca^{2+}]_{cyt}$ is proposed 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 (preventing further hyperpolarisation) (Kinoshita et al. 1995) and inhibit the inward-rectifying K^+ channel preventing further K^+ influx (Grabov and Blatt 1997). Critically, through Ca^{2+}-dependent and Ca^{2+}-independent routes, ABA also activates SLAC1 (SLOW ANION CHANNEL1) which carries the efflux of Cl^- and malate^{2-} across the

plasma membrane, making the major contribution to plasma membrane depolarisation (Negi et al. 2008; Vahisalu et al. 2008). Ca^{2+}-dependent activation of SLAC1 occurs through a Ca^{2+}-dependent protein kinase, CPK6 (Brandt et al. 2012), whereas Ca^{2+}-independent activation of SLAC1 is due to phosphorylation by an SNF-1 RELATED KINASE (SnRK2.6) also known as OPEN STOMATA1 (OST1) (Geiger et al. 2009). SLAC1 is voltage insensitive across a wide range of membrane potentials, allowing sustained activation of the anion channel resulting in prolonged depolarisation. At potentials positive of about −120 mV, the outward-rectifying K^+ channel is activated allowing 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).

The coordination of the many ion channel activities on the tonoplast and plasma membrane is brought about by a complex signalling network that includes changes in cytosolic pH, changes in $[Ca^{2+}]_{cyt}$ and the activity of protein phosphatases 2C (PP2C). 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 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) and H_2O_2 (Bright et al. 2006) also contribute to increases in $[Ca^{2+}]_{cyt}$ through multiple effects.

Another effect of ABA is to increase the cytosolic pH through a mechanism which 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), demonstrating that whilst it is not known how pH exerts its effects on ion channels, pH is likely to be a central regulator of ABA signalling.

ABA perception occurs through binding of the PYRABACTIN RESISTANCE-LIKE (PYL) family of receptors (Ma et al. 2009; Park et al. 2009). The ligand–receptor complex recruits and inactivates members of the 2C class of protein phosphatases, including ABSCISIC ACID INSENSITIVE 1 (ABI1) and ABI2 (Ma et al. 2009; Park et al. 2009). In the absence of ABA, the protein phosphatase activities of ABI1 and ABI2 are negative regulators of ABA signalling (Gosti et al. 1999; Merlot et al. 2001). ABA-dependent binding of PP2Cs to PYL ABA receptors inhibits the phosphatase activity and allows ABA signalling to proceed (Ma et al. 2009). One target for the PP2C activity is OST1/SnRK2.6 (Yoshida et al. 2006), and PP2C activity dephosphorylates the OST1 protein and inhibits its kinase

activity (Fujii et al. 2009). In the presence of ABA, the inhibition of the protein phosphatase activity permits autophosphorylation and activation of OST1 kinase activity, which results in the phosphorylation and activation of AtrbohF (the NADPH oxidase responsible for H_2O_2 production; Sirichandra et al. 2009) and SLAC1 (Geiger et al. 2009). OST1 also phosphorylates the inward-rectifying K^+ channel KAT1, resulting in the inhibition (Sato et al. 2009). KAT1 is also phosphorylated and inhibited in a Ca^{2+}-dependent manner by CPK10 (Zou et al. 2010), and SLAC1 is also phosphorylated and activated by CPK6 (Brandt et al. 2012) representing points of convergence between Ca^{2+}-dependent and Ca^{2+}-independent signalling pathways.

A number of other signalling components have been identified that can regulate guard cell closure. These include 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). The location of these components within the guard cell signalling network is currently unclear. There have also been alternative receptors of ABA postulated, such as ABA receptor (ABAR/ChlH/GUN5). However, the identification of the PYR/PYL/RCAR receptors and the saturation of ABAR with ABA at physiological concentrations suggest that ABAR might not act as a receptor (Risk et al. 2009).

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 a guard cell signalling network rather than as a component of completely distinct signalling pathways (Hetherington and Woodward 2003), and this might explain how guard cells integrate many signals to optimise the aperture of the stomatal pore. 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). Changes in the pattern of $[Ca^{2+}]_{cyt}$ oscillations might encode information that can integrate information about many stimuli to regulate stomatal aperture. The shape, period and amplitude of the $[Ca^{2+}]_{cyt}$ oscillation are related to the concentration and the type of stimulus (McAinsh et al. 1995) with the period of stimulus-induced oscillations of $[Ca^{2+}]_{cyt}$ increasing in correlation with the concentration of the external stimulus (McAinsh et al. 1995; Staxén et al. 1999; Young et al. 2006). Simultaneous multiple stimulation results in novel oscillatory patterns of $[Ca^{2+}]_{cyt}$ suggesting a mechanism for integrating information from multiple signals (Hetherington et al. 1998). The complexity of $[Ca^{2+}]_{cyt}$ signals is most likely associated with the regulation of medium-term stomatal responses because an initial increase in $[Ca^{2+}]_{cyt}$ causes ion efflux leading to rapid closure, but the regulation of the final steady-state aperture appears more complex because this is strongly dependent on the frequency and amplitude of oscillations of $[Ca^{2+}]_{cyt}$ (Allen et al. 2001). If the optimal $[Ca^{2+}]_{cyt}$ oscillation frequency is not detected, or $[Ca^{2+}]_{cyt}$ is at steady state, stomata will reopen following the closure caused by an initial rise in $[Ca^{2+}]_{cyt}$ (Allen et al. 2001). CPK3

and CPK6 act as sensors of the initial increase in $[Ca^{2+}]_{cyt}$ that brings about rapid stomatal closure (Mori et al. 2006), but the sensors that detect oscillatory $[Ca^{2+}]_{cyt}$ signals in stomata are unknown.

9.3 The Circadian Clock

Stomatal movements are regulated also by endogenous cues arising from the circadian clock, which is an internal timekeeper that enables synchronisation of molecular, physiological and metabolic events throughout the day (see Hsu and Harmer 2014 for review). Circadian rhythms have a period of approximately 24 h, are entrained by light–dark, temperature and physiological cycles to synchronise cellular events with rhythmic changes in the environment and, once entrained, can persist in constant conditions (Millar 2004; Haydon et al. 2013). In contrast to most other biological processes, the oscillator is relatively insensitive to temperature meaning that the period of the oscillator has limited variation under a 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). 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. 2005).

The Arabidopsis core circadian clock is proposed to comprise interlocking loops of transcriptional repressors such as the partially redundant MYB-like transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) (Wang and Tobin 1998), LATE ELONGATED HYPOCOTYL (LHY) (Schaffer et al. 1998; Mizoguchi et al. 2005) and the PESUDORESPONSE REGULATORS (PRRs) 1 (also known as TIMING OF CAB EXPRESSION 1 (TOC1); Strayer et al. 2000; Gendron et al. 2012), 3, 5, 7 and 9 (Eriksson et al. 2003; Matsushika et al. 2000). These transcriptional repressors are expressed in sequence from dawn starting with *LHY* and *CCA1*. The transcriptional repressors reciprocally regulate each other, for example the LHY and CCA1 proteins bind the *TOC1* promoter at the evening element (EE; AAAATATCT) and inhibit *TOC1* expression (Alabadí et al. 2001). Rhythmic abundance of the proteins is ensured not only by rhythmic transcription, but also by rhythmic turnover due to the activity of E3 ubiquitin ligases coupled to regulatory F-box proteins such as ZEITLUPE (Somers et al. 2000). The regulation of nuclear–cytosolic partitioning of the transcriptional repressors is achieved through phosphorylation, such as in the case of TOC1, and protein–protein interactions such as the function of the evening complex EARLY FLOWERING 3 and 4 proteins that regulate the repression of the night-time active LUX ARRHYTHMIO, a MYB-like transcriptional repressor of *PRR9* (Herrero et al. 2012; Helfer et al. 2011). Transcriptional activation in the network is provided by the REVEILLIE family of transcriptional activators (Hsu et al. 2013).

The complexity of the circadian oscillator and the high degree of feedback between components and autoregulation inhibits intuitive analysis of the network.

For these reasons, the data have been incorporated into mathematical descriptions of the systems that have increased in complexity as new information has come available [e.g. Locke et al. 2005a, b; 2006; Pokhilko et al. (2010; 2012); Herrero et al. (2012); Fogelmark and Troein (2014)]. Whilst the models vary in complexity and detail, in essence they describe a consensus view of interlocking feedback loops in which the cycle is considered to start in the morning with the light-mediated activation of *LHY* and *CCA1* expression, through perception by the phytochromes and cryptochromes. The transcriptional repressors are expressed in temporal waves with the morning genes *LHY* and *CCA1* first, followed by the day *PRR* genes and lastly the evening complex of *ELF3, ELF4* and *LUX*. Build-up of the repressors prevents expression of downstream targets, until time-of-day-regulated protein degradation relieves the repression allowing expression of the next in the sequence. The phase of the circadian clock is also adjusted by sugars produced from photosynthesis through the regulation of *PRR7*, but this aspect has not yet been incorporated into mathematical models (Haydon et al. 2013).

The transcriptional regulators of the core circadian clock also regulate the expression of output genes. Thus, the clock acts as a rhythm generator, and the phase of the output gene expression will be dependent on the phase of expression of the regulatory clock component. The waveform and precise phase of expression of output genes are further modified through modulation by light-dependent signalling pathways (Dalchau et al. 2010).

9.4 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 (V. *faba*) to 26 h (*Arachis*) (Ståfeld 1963; Pallas et al. 1974). Rhythms have been studied both at a whole plant level through 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 damp considerably after the first two cycles (Holmes and Klein 1986). This indicates that in C3 plants, a light-dependant 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, whilst in CAM plants, where carbon fixation by PEPC takes place at night, the stomata are open during part or all of the night (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 that investigated the advantage

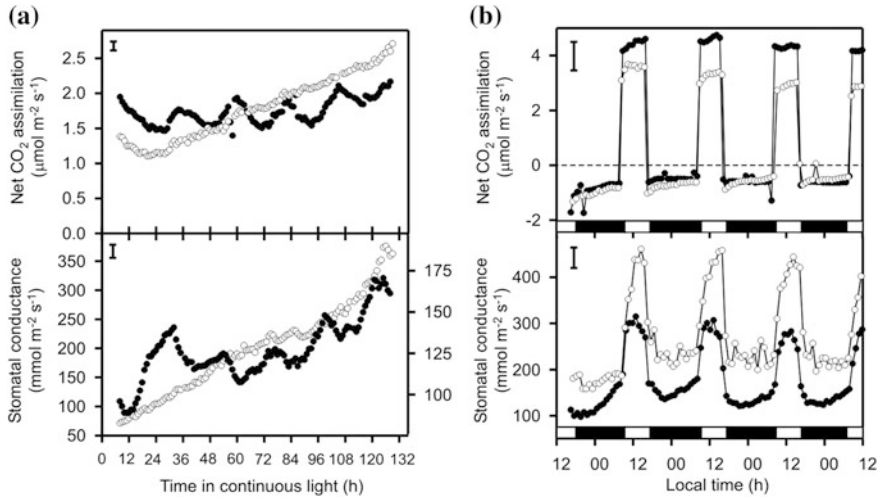
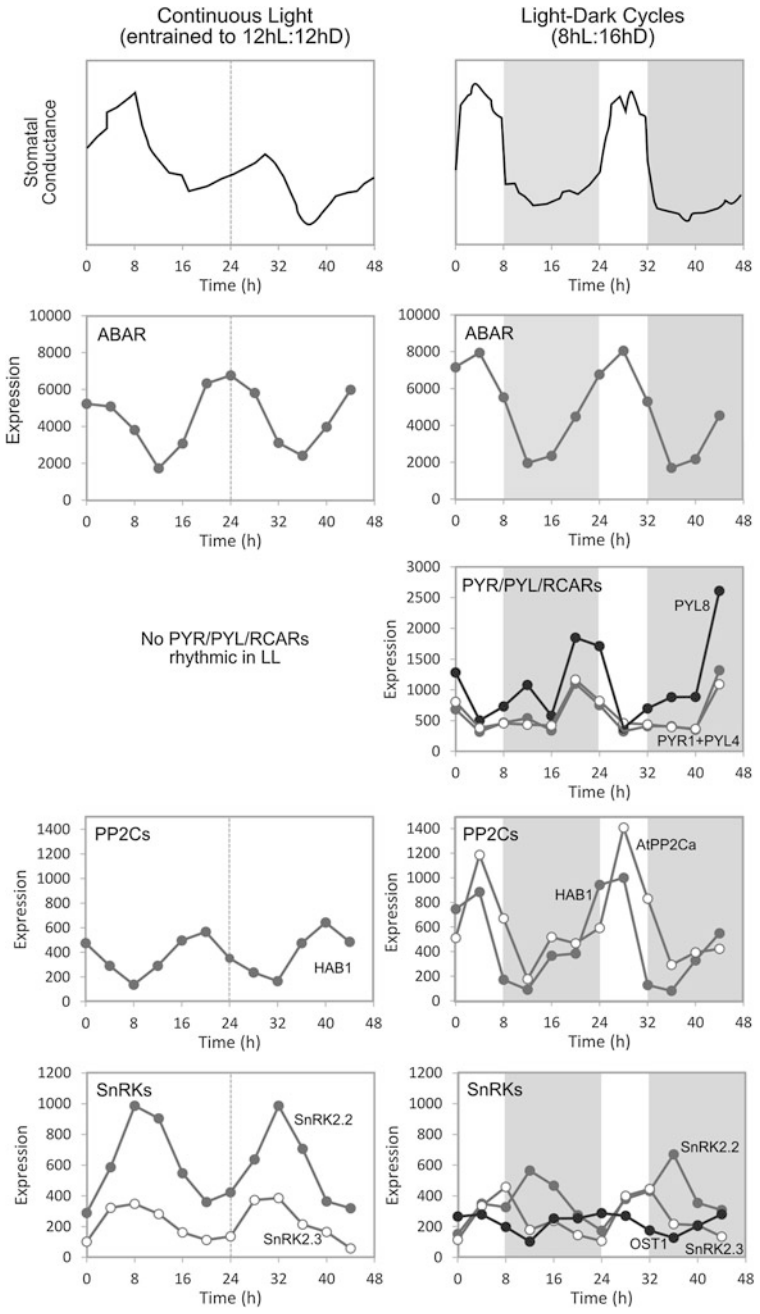


Fig. 9.1 Circadian and diurnal rhythms of photosynthesis and stomatal conductance in wild type and *CCA1*-ox plants. Infrared gas analysis of mature *Arabidopsis* rosettes. *Filled circles* represent wild-type plants (Col-0) and *open circles* are the circadian arrhythmic line *CCA1*-ox. **a** Rhythms in photosynthesis and stomatal conductance under continuous light. **b** Rhythms in photosynthesis and stomatal conductance under light–dark cycles which are represented by the *open and filled bars*, respectively. Data are means of six replicates. Largest standard errors of the mean are represented by the *vertical bars*. Reproduced from Dodd et al. (2005), reprinted with permission from AAAS

conferred by matching the endogenous clock period (τ) with the period of the exogenous light–dark cycle (T) (so-called circadian resonance; Pittendrigh and Bruce 1959; Ouyang et al. 1998; Dodd et al. 2005). 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 is resonant with $T = 24$, whereas *toc1-1* ($\tau = 20.7$ h; Somers et al. 1998) and *ztl-1* ($\tau = 27.1$–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. 2005). 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. 2005). An analysis of a population of *Brassica rapa* recombinant inbred lines suggested that there was a significant relationship between circadian period and both assimilation and stomatal conductance, but interestingly no relationship between the circadian oscillator and water-use efficiency (Edwards et al. 2011, 2012).

In C3 and C4 plants, stomatal opening anticipates dawn in both LD and LL (Dodd et al. 2005). In *CCA1*-ox plants, there are no circadian rhythms in stomatal conductance and there is also no predawn increase in conductance when *CCA1*-ox plants are grown under light–dark cycles (See Fig. 9.2, Dodd et al. 2005). 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 allowing stomata to anticipate changes in the fluctuating environment. It is particularly striking that the stomata of *CCA1*-ox

◀ **Fig. 9.2** Circadian and diurnal rhythms of stomatal conductance and transcript abundance for components of the ABA signalling pathway. Stomatal conductance redrawn from Fig. 9.1, Dodd et al. (2005). Expression values taken from the short-day and LL_LDHH data sets incuded in the DIURNAL database (http://diurnal.mocklerlab.org/). Transcripts with a correlation value greater than 0.8 and and an amplitude of 300 or above are shown

continue to open until the light signal is removed. This demonstrates that in unstressed plants, the circadian clock, not the physiological or water status of the leaf, arrests stomatal opening at midday and promotes the onset of closure in the afternoon in wild-type plants (Fig. 9.2). Control by the circadian clock rather than physiological limitation has also been proposed in studies of net ecosystem exchange of CO_2 across a range of contrasting biomes (Dios et al. 2012).

Misregulation of stomatal movements in *CCA1*-ox and plants in which the period of the clock is mismatched with that of the environment might contribute to reduced CO_2 assimilation (Dodd et al. 2005). 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 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), at least in part due to circadian regulation of the nuclear-encoded chloroplastic sigma factors (Noordally et al. 2013). Whilst 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. 2005). Despite the importance of the circadian regulation of photosynthesis and the role of stomata in regulating gas exchange, the circadian clock appears to particularly benefit events that occur at night (Dodd et al. 2014), when starch is being remobilised to support growth, and it seems that it is the timing of starch metabolism that might be the cause of the major growth benefits conferred by the circadian system of Arabidopsis (Graf et al. 2010).

9.5 Structure of the Guard Cell Clock

Models of the circadian oscillator have been generated primarily through the analysis of entire plants or on whole leaves. Whilst this has been very informative, it is possible that all of the components of the 'consensus' clock are not found in any one cell type, and there might be cell-type-specific circadian oscillator architectures. It has been recently shown that there are differences between the circadian

oscillator contained in the vasculature and the mesophyll, with the mesophyll being enriched for components of the 'morning' loop and the vasculature containing the evening loop (Endo et al. 2014). In addition, it was found that the vascular clock can modulate oscillators in other tissues, suggesting that cell-type-specific clocks might communicate with each other to modulate the circadian behaviour of the entire organism.

Whilst there is the potential for cell-type-specific variation in circadian clock architecture, the genes that form the 'consensus' circadian oscillator contribute to stomatal regulation. 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. 2005). There are also circadian oscillations in the abundance of transcripts encoding the circadian clock components *CCA1*, *LHY*, *TOC1* and *GI* in the guard cell (Yakir et al. 2011). It is therefore likely that the circadian clock in the guard cell shares the same genetic structure with the clock found in other cell types, but this oscillator might be adapted to the specialised role of guard cells because it is phased slightly later than circadian rhythms measured from whole leaf samples (Yakir et al. 2011).

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, 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; Yakir et al. 2011).

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. It 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. Whilst the oscillators that regulate 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 substomatal cavity by photosynthesis during the day in C3 and C4 plants will promote stomatal opening. Whilst there is considerable

evidence that in CAM plants the nocturnal depletion of C_i by PEPC promotes stomatal opening, 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 reinforce a number of rhythmic phenomena (Bläsing et al 2005; Haydon et al. 2013).

9.6 Mechanisms of Circadian Control of Guard Cell Physiology

There is evidence that the circadian clock both directly and indirectly regulates stomatal movements. In the direct pathways, circadian clock components or outputs generated by the circadian oscillator interact to regulate the activity of the transporters and signalling networks that regulate stomatal movements. Indirect circadian regulation of stomatal aperture occurs due to circadian gating of signal transduction pathways and as consequence of circadian alterations in the physiology of the plant.

The direct regulation of the signalling machinery is demonstrated by the circadian regulation of the transcription of components of the ABA signalling pathways (see Hubbard et al. 2010 for review). We analysed the data sets available in the DIURNAL microarray tool (http://diurnal.mocklerlab.org/) to discover that *ABAR*, *SnRK2.2, SnRK2.3* and the PP2C *HAB1* have rhythmic expression patterns under continuous light, indicating direct transcriptional control by the oscillator (Fig. 9.2). In short days (8 h light: 16 h dark), these loci also have rhythmic expression patterns, as do three members of the *PYR/PYL/RCAR* family, *AtPP2Ca* and *SnRK2.6/OST1*. This suggests rhythmic patterns of stomatal closure could be generated through circadian regulation of gene expression of key stomatal regulators. However, confirmation of this circadian regulation of ABA signalling transcript abundance in stomatal guard cells requires analysis of guard cell-enriched RNA fractions for confirmation.

One mechanism by which the circadian oscillator has been prosed to directly modulate stomatal movements is through TOC1 regulation of ABA signalling. *TOC1* overexpressing plants have higher rates of stomatal conductance than those of wild types and also have reduced levels of stomatal closure in response to ABA (Legnaioli et al. 2009). *TOC1*-ox plants have reduced expression levels of the *ABAR*, and *TOC1* expression is increased in the presence of ABA. Based on these findings, Legnaioli et al. (2009) proposed a model whereby TOC1 and ABAR reciprocally regulate one another to control stomatal closure (Legnaioli et al. 2009). The induction of *TOC1* by ABA is gated by the circadian clock, with maximal responsiveness to ABA occurring between 5 and 10 h after subjective dawn (Legnaioli et al. 2009). This ABA/TOC1 module has been incorporated into mathematical models of the circadian oscillatory network, which simulates an increased stomatal aperture in TOC1-*ox* plants, consistent with the experimental data (Pokhilko et al. 2013). How the ABAR regulates

TOC1 is unclear and the assumption that it participates in ABA signalling has been questioned (Risk et al. 2009).

FLOWERING LOCUS T (FT), a circadian-regulated output of the circadian oscillator, which has no role in circadian time generation, has recently been proposed to act as intermediary by which the circadian clock regulates the movements of stomata (Kinoshita et al. 2011). FT was originally characterised as a circadian-regulated mobile signal controlling photoperiodic-dependent flowering. In the leaf during long days, the coincidence detector protein CONSTANS (CO) accumulates in the late afternoon and activates the expression of FT, which travels to the shoot apical meristem to initiate the developmental switch to reproductive growth. FT is also expressed in guard cells and appears to regulate stomatal aperture (Kinoshita et al. 2011). Null mutants of *ft* are less able to open their stomata in response to light and have reduced phosphorylation-dependent binding of 14-3-3 proteins to the H^+-ATPase responsible for hyperpolarisation of the plasmamembrane (Kinoshita et al. 2011). Reduced stomatal opening in response to light was also observed in mutants of *co* and *gi* (Ando et al. 2013), suggesting that many of the key photoperiodic-dependent flowering regulators also regulate stomatal opening, thereby providing a mechanistic link between outputs of the circadian clock and guard cell physiology. A model has been proposed whereby circadian rhythms in *ELF3* expression drive rhythmic changes in stomatal aperture through FT, based in part on the observation that *FT* expression was significantly higher in an *elf3* mutant (Ando et al. 2013; see Fig. 9.3). Further analyses might be required to understand the relationship between ELF3, FT and the regulation of stomatal movements; although a model in which ELF3 represses *FT* is supported by the genetic data, it is not consistent with the

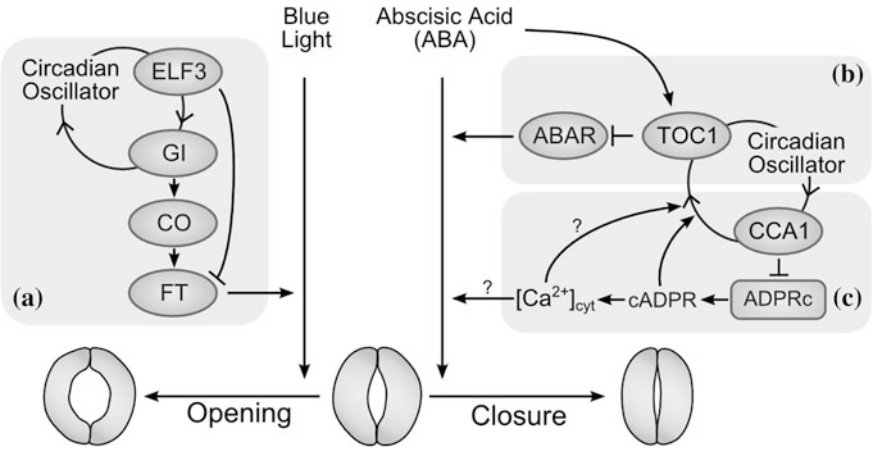


Fig. 9.3 Models for interactions between the circadian oscillator and guard cell movements. **a** The circadian clock regulates blue-light-induced stomatal opening through the action of FT, as proposed by Kinoshita et al. (2011) and Ando et al. (2013). **b** TOC1 regulates stomatal closure through the ABAR signalling network, as proposed by Legnaioli et al. (2009). **c** The circadian clock regulates guard cell dynamics through the secondary messengers cADPR and $[Ca^{2+}]_{cyt}$

coincidence peak of *ELF3* and *FT* transcript abundance in the late afternoon in arial tissues in LD cycles (Michael et al. 2008). It is possible that the relative timing of expression of *ELF3* and *FT* is different in the guard cell or that genetic analysis of regulatory relationships in oscillatory networks has proven to be misleading, as turned out to be the case with the incorrect assumption that TOC1 acts a transcriptional activator (Gendron et al. 2012).

An example of a hypothesis of indirect circadian regulation of stomatal movements is the proposal of Tallman (2004) 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 Ci, 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), therefore reducing the guard cell cytosolic ABA pool. The ABA precursor violaxanthin (Xiong and Zhu 2003) is converted to zeaxanthin for photoprotection (Eskling 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 allow sucrose accumulation in the cytosol to maintain turgor; 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, sucrose transporters are inhibited so 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. Suppression of ABA catabolism will increase levels of both violaxanthin and ABA, and the stomatal aperture will decrease through ABA signalling pathways. This model may not explain self-sustained free-running rhythms of stomatal opening that occur under constant conditions, since its perpetuation is at least partially dependent upon light–dark cycles.

9.7 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).

The circadian $[Ca^{2+}]_{cyt}$ oscillation is driven by the secondary messenger molecule cyclic ADP ribose (cADPR) (Dodd et al. 2007). There is a circadian oscillation in the concentration of cADPR which peaks at the same time of day as that of $[Ca^{2+}]_{cyt}$ and is dependent on a functional circadian oscillator. The activity of ADP ribosyl cyclase is abolished by the treatment with 50 mM nicotinamide, and in the presence of this metabolite, the circadian $[Ca^{2+}]_{cyt}$ oscillation is abolished. Treatment with nicotinamide also increases the period of the circadian oscillator, indicating that cADPR/$[Ca^{2+}]_{cyt}$ are not just outputs of the circadian clock, but also feedback to the transcriptional oscillator to modulate its behaviour (Dodd et al. 2007). There has also been a suggestion that diurnal $[Ca^{2+}]_{cyt}$ oscillations are controlled by the chloroplastic Ca^{2+}-sensing receptor CAS, which controls the concentration of IP3 in the cell (Tang et al. 2007). However, this pathway does not appear to feed back onto the oscillator itself and is more responsible for the amplitude of diurnal $[Ca^{2+}]_{cyt}$ oscillations than their phase or period (Tang et al. 2007).

There are therefore two models through which $[Ca^{2+}]_{cyt}$ may regulate circadian rhythms of stomatal movements; $[Ca^{2+}]_{cyt}$ may be an output of the guard cell circadian clock which also regulates known Ca^{2+}-dependent components of the guard cell signalling network, or alternatively cADPR/$[Ca^{2+}]_{cyt}$ may regulate the circadian oscillator which interacts with other guard cell control proteins through alternative mechanisms. These two models are not mutually exclusive, as $[Ca^{2+}]_{cyt}$ may regulate both the oscillator and stomatal response elements.

Supporting evidence for a role for Ca^{2+}-based signalling pathways in the circadian regulation of stomata comes from the studies of inhibition of phosphatidylinositol kinase activity and 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). Phosphoinositide metabolism provides a potential link between the circadian clock and ABA signalling because PtdIns kinase activity inhibited ABA-induced increases in $[Ca^{2+}]_i$ in the guard cell (Jung et al. 2002).

To fully understand the role of circadian oscillations in stomatal control, it is important to ascertain whether there are circadian $[Ca^{2+}]_{cyt}$ oscillations in the guard cells. Most analyses of circadian $[Ca^{2+}]_{cyt}$ oscillations have been performed using the $[Ca^{2+}]_{cyt}$ reporter APOAEQUORIN expressed under the control of the constitutive CaMV 35S promoter that does not permit specific detection of guard cell $[Ca^{2+}]_{cyt}$. To increase the tissue specificity of the aequorin reporter, the GAL4-enhancer trap system has been used to target AEQUORIN expression to particular cell types (Kiegle et al. 2000; Gardner et al. 2009; Dodd et al. 2006). Analysis of a number of different enhancer trap lines revealed that circadian $[Ca^{2+}]_{cyt}$ oscillations are likely to originate from the spongy mesophyll, as this was the only tissue type where $[Ca^{2+}]_{cyt}$ oscillations with the same dynamics as those detected from whole plants could be detected (Martí et al. 2013). Circadian oscillations of $[Ca^{2+}]_{cyt}$ were not detected when APOAEQUORIN was expressed

specifically in the guard cells, although diel oscillations of $[Ca^{2+}]_{cyt}$ were detected in the guard cells under light–dark cycles (Martí et al. 2013). This analysis does not exclude the possibility that circadian $[Ca^{2+}]_{cyt}$ oscillations occur in guard cells, but that these could not be detected due to the low signal-to-noise ratio when AEQUORIN is targeted to small populations of cells.

An alternative to technically demanding long-term measurements of cellular processes, such as changes in $[Ca^{2+}]_{cyt,}$ might be a prediction based on simulations derived from mathematical models. Two classes of mathematical model to explain guard cell signalling have been developed, a Boolean network based on logic gates of connectivity (Li et al. 2006) and OnGuard, a quantitative kinetic model (Hills et al. 2012). OnGuard predicts that predawn stomatal opening might be an emergent property of the homeostatic balancing of ions through the night (Chen et al. 2012). However this homeostatic regulation of solute flux alone is insufficient to explain full circadian regulation of stomatal behaviour because OnGuard does not simulate correctly rapid stomatal opening in the morning, nor the predusk onset of closure (Chen et al. 2012).

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

Rather than there being a direct mechanistic connection between the circadian oscillator and the processes responsible for guard cell movements, it may be that the oscillator regulates the sensitivity of the guard cells to acute environmental signals, thereby modulating changes in aperture indirectly. Guard cells are able to integrate a wide variety of signals that promote both opening and closure 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. As a consequence 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, so the underlying circadian clock enables maximal light-induced stomatal opening at a time when 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. 2005).

A number of other gated responses in stomatal guard cells have been reported. These include rhythmic sensitivity to ABA (Correia et al. 1995), external K^+ (Snaith and Mansfield 1986), auxins and fusicoccin (Snaith and Mansfield 1985) and pathogens (Zhang et al. 2013). Gating of these diverse stimuli ensures phase-appropriate response of the guard cells to external stimuli. For example, guard cells are least responsive to ABA in the early morning (Correia et al. 1995), allowing high levels of CO_2 uptake in the morning before water becomes limiting in the late afternoon. Similarly, gating of cold-induced increases in $[Ca^{2+}]_{cyt}$ has been detected 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 therefore require induction of photoprotective mechanisms to prevent ROS overproduction resulting from an excess of energy within the light-harvesting apparatus. This hypothesis is supported by the observation that the low temperature induction of expression of the C-REPEAT BINDING FACTOR (CBF) 1-3 transcription factors is also gated by the clock (Fowler et al. 2005). This is due to a requirement for CCA1 in the pathway by which cold upregulates CBF expression (Dong et al. 2011).

The mechanistic basis of circadian gating is still relatively poorly understood. In addition, gating may not just represent the clock influencing the sensitivity of output pathways, but might also include differential sensitivities of feedback pathways back into the oscillator. For example, ABA acutely induces the expression of the central oscillator gene *TOC1* which is a gated response, with greatest induction occurring 5–10 h after dawn (Legnaioli et al. 2009). Uncovering the molecular interactions responsibly for rhythmic sensitivity to external signals will give us a much deeper understanding of the relationship between the oscillator and guard cell movements.

9.9 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. Whilst the physiological processes that underlie guard cell movements are well understood, how the clock regulates physiology at a molecular level is still being uncovered. 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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Chapter 10
Time to Network: The Molecular Blueprint of the Circadian Timing System in Plants

Selahattin Danisman, Julieta Mateos and Dorothee Staiger

Abstract Plants are exposed to periodic changes of light and darkness in their habitats through the Earth's rotation around its axis. Accordingly, plants have evolved an endogenous system to schedule their day in synchrony with their surroundings. Here, we review the blueprint of the core clockwork in plants and how the core clockwork orchestrates time-of-day-specific gene expression programmes.

10.1 Introduction

Like most organisms, plants are exposed to periodic changes of light and darkness in their habitats through the Earth's rotation around its axis. Accordingly, plants have also evolved an endogenous timing system to schedule their day consonant with their surroundings.

Chronobiology has a long tradition in plants. The first report on a time-of-day-dependent process dates back to the era of Alexander the Great. The Greek writer Androsthenes in his records on the marches to the Indus noted that *Tamarindus indicus* on the island of Bahrein folds its leaflets at the end of the day and that the entire leaf takes a "sleep position" during the night (Bretzl 1903). Already at that time, the term "sleep movement" was coined.

Two millennia later, in 1729, it was shown by a French astronomer, De Mairan, that this periodic leaf movement is not a mere reaction of the plant to the light–dark transition (De Mairan 1729). He placed *Mimosa pudica* plants in a cabinet for

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several days and observed persistence of the leaf movement rhythms in constant darkness, as if the plants were still seeing day and night, and sunrise and sunset. This pointed to an endogenous origin of the rhythms. A century later, in 1832, De Candolle found a slight change in period length under constant external conditions, resulting in a so-called free-running rhythm with a period close to, but not exactly equal to, 24 h—an important insight that the rhythms in fact are under endogenous control (de Candolle 1832). Another century later, in 1935, Bünning noted that the period of leaf movement rhythms in *Phaseolus vulgaris* is heritable and thus has a genetic basis (Bünning 1935). Such endogenous rhythms that persist in the absence of the day–night cycle with a period of about 24 h were termed "circadian" rhythms, from Latin circa diem, about a day.

Research on circadian rhythms entered the molecular era with the first description of rhythms in gene expression in pea: In 1985, Klaus Kloppstech discovered that the light-induced transcripts encoding LIGHT HARVESTING CHLOROPHYLL BINDING PROTEINS (LHCPs) undergo oscillations with a peak in the morning that persist in constant light (Kloppstech 1985). Subsequently, differential cDNA screening for time-of-day-dependent changes in gene expression uncovered genes with peaks at other times of the day (Heintzen et al. 1994; Carpenter et al. 1994; Heintzen et al. 1994; Zhong and McClung 1996). Later, microarray analyses showed that a large part of the transcriptome is under control of the endogenous clock (Harmer et al. 2000; Covington et al. 2008).

Overall, the circadian timing system that allows an organism to promote daily rhythms in many physiological and metabolic processes can be conceptualized by three components: the input pathways that integrate the environmental signals and transmit them to the clock, the central oscillator that generates the rhythms (the clockwork) and the output pathways that control the responses. Here, we present the current view how the circadian clock globally regulates gene expression programmes in plants to govern physiological rhythms and how the molecular core clockwork functions.

10.2 The Core Clockwork

In all eukaryotes, the principles that make the clock run properly rely on similar molecular mechanisms based on transcriptional–translational feedback loops (TTFL). This simple concept turns much more complicated when examined closer at a molecular level. Indeed, among eukaryotes, the central oscillator architecture of plants is found to be the most complex. Morning-, afternoon- and evening-phased genes form a series of interconnected feedback loops, though recent work suggests that the plant clock should be seen more as a highly interconnected network rather than discrete loops (Hsu et al. 2013).

The first clock proteins identified in Arabidopsis form a transcriptional negative feedback loop where two closely related morning-phased Myb transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED

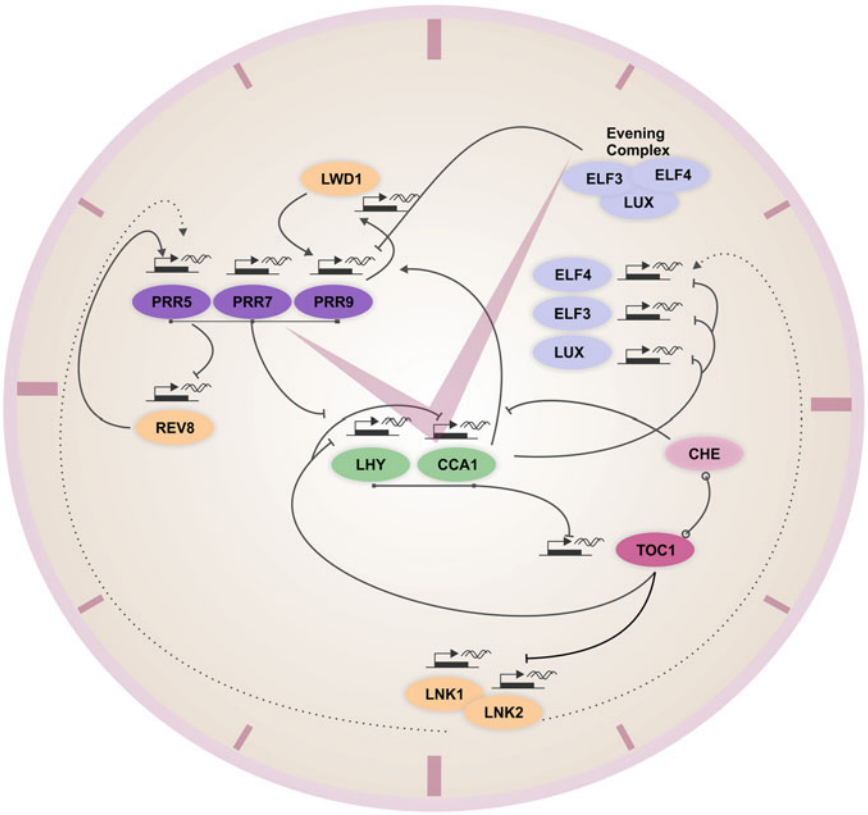

Fig. 10.1 Blueprint of the core clockwork in the model plant *Arabidopsis thaliana.* The core clock loop is based on reciprocal regulation of CCA1 and LHY on the one hand and TOC1 on the other. Interlocked with this loop is the morning loop with PRR9, PRR7 and PRR5 sequentially repressing *CCA1* and *LHY* in the course of the day. PRR5 forms a feedback loop with RVE8. PRR9 forms a feedback loop with LWD1. *PRR9* is also repressed by the evening complex formed by LUX, ELF3 and ELF4. At dawn, *LUX*, *ELF3* and *ELF4* are kept low by CCA1 and LHY. CCA1 and LHY form a feedback loop with CHE. CCA1 and LHY repress *CHE,* and CHE directly represses *CCA1*. TOC1 interacts with CHE antagonizing CHE repression of *CCA1*. TOC1 also represses *LNK1* and *LNK2* by direct binding. LKN1 and LNK2 act as activators of *ELF4* and *PRR5* transcription. Direct regulation is shown by *solid lines* and indirect regulation by *dashed lines*. PRRs: purple; evening genes: light blue. Core loop genes: green and magenta; activators: light orange

HYPOCOTYL (LHY) (Wang and Tobin 1998; Schaffer et al. 1998), repress the expression of the evening-phased *TIMING OF CAB EXPRESSION 1* (*TOC1*) gene (Alabadi et al. 2001). TOC1 was identified in a screen for mutants that show aberrant rhythms of a *LHCP* promoter-driven LUCIFERASE transgene and is also known as *PSEUDO-RESPONSE REGULATOR 1* (*PRR1*) (Millar et al. 1995). In the current model of the clock, TOC1 feeds back to directly repress *LHY* and *CCA1* expressions (Gendron et al. 2012; Huang et al. 2012) (Fig. 10.1). TOC1 additionally indirectly

activates *CCA1* expression by interacting with CCA1 HIKING EXPEDITION (CHE), a TCP (TEOSINTE BRANCHED 1, CYCLOIDEA, PCF1) transcription factor known to repress *CCA1* (Pruneda-Paz et al. 2009). Additionally, *CHE* is repressed by LHY and CCA1, forming another loop. Together with TOC1, other members of the PRR family play an important role in the core clock and form another segment of the circuit: *PRR* genes are expressed during the day peaking sequentially after dawn (first *PRR9*, followed by *PRR7*, *PRR5* and finally *TOC1* in the evening) to directly repress expression of *LHY* and *CCA1* by binding to their promoters and restricting their accumulation to the morning (Nakamichi et al. 2010; Nakamichi et al. 2012). Through another interconnected loop, LHY and CCA1 may play a role as activators by inducing the expression of *PRR9* and *PRR7* (Farré et al. 2005). LHY and CCA1 also restrict the expression of several other evening-expressed genes such as *GIGANTEA* (*GI*), *LUX ARRHYTHMO* (*LUX*), *EARLY FLOWERING 3* (*ELF3*) and *EARLY FLOWERING 4* (*ELF4*). A complex of LUX, ELF3 and ELF4 known as the "evening complex" in fact represses expression of the morning gene *PRR9* (Dixon et al. 2011; Helfer et al. 2011; Herrero et al. 2012; Chow et al. 2012).

Until recently, the connections between the clock genes described in the previous paragraph involved almost exclusively direct repressive interactions. Recent work now sheds light onto transcriptional activators revealing novel loops in the Arabidopsis clock. The CCA1/LHY1 homologue REVEILLE 8 (RVE8) binds to evening elements (EE) of the *PPR5* promoter to activate its transcription (Hsu et al. 2013; Rawat et al. 2011). Other related Myb transcription factor genes such as RVE4 and RVE6 have a minor role than RVE8 in the core clock, yet they can also bind EE elements, and the triple mutant *rve4 rve6 rev8* enhances the *rev8* long-period phenotype (Hsu et al. 2013). PRR5 binds to the *RVE8* promoter (Nakamichi et al. 2012), and genetic evidence suggests that *PRR5* feeds back to repress *RVE8* (Rawat et al. 2011), thus forming another interlocked transcriptional loop. The identification of two other transcriptional activators revealed a new limb in the core clock. LIGHT-REGULATED WD 1 (LWD1) and LWD2 have dual functions both in the input pathway and as part of the central oscillator (Wu et al. 2008; Wang et al. 2011). In the core clockwork, LWD1 and PRR9 form a positive feedback loop. Direct binding of LWD1 to the *PRR9* promoter causes its activation, while the triggering of *LWD1* expression by PPR9 appears to be indirect, probably by repressing an unknown *LDW1* repressor (Wang et al. 2011). Finally, two members of a family of transcription factors that are clock-regulated and induced by light have been recently connected to the clock function. *Night light-inducible and clock-regulated 1* (*lnk1*) and *lnk2* mutants show a long-period phenotype and downregulation of many evening-phased genes. *PRR5* and *ELF4* are significantly reduced in *lnk1 lnk2*, implying that LNK1 and LNK2 act as activators (Rugnone and Faigan 2013). LNK proteins lack a putative DNA-binding domain, and recent data suggest that they act as transcriptional coactivators by interacting with RVE4 and RVE8 (Xie et al. 2014). Furthermore, PRRs including TOC1 directly bind to the *LNK1* and *LNK2* promoters, and *LNK* transcript levels are high in *prr7*, *prr9* and *toc1* mutants. Hence, the *PRRs* and *LNKs* form the last but not least of the transcriptional loops in the Arabidopsis clock.

The accurate synchronization and defined action of each wheel of this molecular gear guarantees the plant clock ticking at the right pace.

10.3 Post-translational Regulation of Clock Proteins

Apart from transcriptional regulation exerted by the clock proteins, processes at the post-translational level are important to generate a period of 24 h (Schöning and Staiger 2005; Gallego and Virshup 2007; Seo and Mas 2014). These include post-translational modification of clock proteins, regulated protein stability or subcellular localization. CCA1 and LHY undergo phosphorylation by casein kinase 2, a serine/threonine protein kinase consisting of two α subunits with enzymatic activity and two β subunits with regulatory function. The CK2 β subunits interact with CCA1 and LHY facilitating their phosphorylation (Sugano et al. 1998; Sugano et al. 1999; Daniel et al. 2004). Overexpression of the CK2 β subunits leads to enhanced CK2 activity and a short-period clock as in the *cca1* mutant, suggesting that CK2 may impair clock function partly through CCA1. Indeed, CCA1 phosphorylation reduces CCA1 binding to target promoters (Portoles and Mas 2010).

Another important mechanism within the core clockwork was unveiled with the notion that *toc7*, a long-period mutant recovered in the same genetic screen as *toc1*, has a mutation in an F-box protein (Millar et al. 1995; Somers et al. 2000). F-box proteins are subunits of SCF type E3 (ubiquitin ligase) complexes that target cellular proteins for ubiquitination and subsequent proteasomal degradation. TOC7/ZEITLUPE (ZTL) contributes to the 24-h period of the clock by causing rhythmic degradation of the TOC1 protein (Mas et al. 2003). ZTL interacts with TOC1 through its N-terminal LOV domain with the flavin mononucleotide chromophore involved in blue light perception to promote TOC1 degradation in the dark. The physical interaction of TOC1 with ZTL is abolished in the *ztl-1* mutant, resulting in constitutive TOC1 levels. ZTL interacts with PRR5 to mediate its rhythmic degradation (Kiba et al. 2007).

TOC1 and PRR5 also undergo phosphorylation which favours their interaction with ZTL and thus ultimately their degradation (Fujiwara et al. 2008). Another outcome of TOC1 phosphorylation is its uptake into the nucleus so that TOC1 escapes from ZTL-mediated degradation in the cytoplasm (Wang et al. 2010). Clearly, more detailed biochemical studies are needed for a complete understanding of the opposing effects of TOC1 phosphorylation.

Rhythmic expression of ZTL is necessary for the rhythms in TOC1 and PRR5, and ZTL protein oscillation despite a constant *ZTL* mRNA level is caused through phase-specific degradation of ZTL (Kim et al. 2003). Another clock protein, GIGANTEA (GI), is necessary to sustain ZTL protein oscillations. In the presence of blue light, GI interacts with the ZTL LOV domain to stabilize ZTL (Kim et al. 2007). In the dark phase, the interaction of ZTL with GI is abolished. Thus, the GI oscillations promote the time-of-day-dependent degradation of ZTL and thus ultimately the oscillations of PRR5 and TOC1. In turn, GI rhythms in turn appear to be

determined through interaction between ELF3 and the ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 that targets GI to proteasomal degradation in the dark (Yu et al. 2008).

10.4 Post-transcriptional Regulation

In addition to transcriptional and post-translational control, clock genes were found to undergo regulation at the RNA level. RNA-binding proteins involved in splicing or RNA stability that themselves show circadian oscillations were assumed to contribute to post-transcriptional regulation of circadian genes (Staiger 2001; Perez-Santángelo et al. 2014).

Today, a widespread role for alternative splicing in the circadian clock has been firmly established (Staiger and Green 2011). Transcripts of several core clock genes undergo extensive alternative splicing in particular upon changes in light or temperature, suggesting that alternative splicing may mediate clock adjustment to varying light and temperature conditions (James et al. 2012; Filichkin et al. 2010). Moreover, mutants defective in components associated with the splicing process show clock phenotypes. PROTEIN ARGININE METHYL TRANSFERASE 5 (PRMT5), a protein that methylates arginine residues in histone and non-histone targets, has been shown to contribute to circadian period determination in Arabidopsis at least in part by regulating alternative splicing of *PRR9* (Sanchez et al. 2010; Hong et al. 2010). PRMT5 non-histone targets include splicing-related factors such as heterogeneous nuclear ribonucleoproteins, snRNP, SmD1, SmD3, and LSm4, and global splicing defects observed in *prmt5* mutant are proposed to be caused by the reduced methylation status of these factors (Deng et al. 2010). Moreover, mutants of the core spliceosomal components LSM4 and LSM5 display long-period phenotypes (Perez-Santángelo et al. 2014). Indeed, *LSM5* transcription is at the same time clock-regulated (Perez-Santángelo et al. 2014). This study suggests that clock-regulated transcription of core splicing factors might be a mechanism by which transcriptional and post-transcriptional regulation is linked to control clock function. A still open question in plants is to which extent post-translational modifications of spliceosomal components are needed to regulate splicing and whether this affects clock function.

Aberrant splicing of core clock genes and a long period of the clock were also observed in two mutants that are defective in proteins with homology to mammalian splicing-associated factors, Ski-interacting protein (SKIP) and SPLICEOSOMAL TIMEKEEPER LOCUS 1 (STIPL1) (Wang et al. 2012; Jones et al. 2012).

An RNA-binding protein in clock output, *At*GRP7 (*Arabidopsis thaliana* glycine-rich RNA-binding protein 7), is part of a feedback loop that negatively autoregulates by binding to its own pre-mRNA and causing its alternative splicing. The alternative splice form retains a premature termination codon and is rapidly degraded via the nonsense-mediated decay pathway (Staiger et al. 2003; Schöning

et al. 2007; Schöning et al. 2008). This is the first example of a clock-regulated feedback loop based on post-transcriptional regulation. *At*GRP7 in turn regulates a suite of circadian-regulated transcripts (Streitner et al. 2010; Streitner et al. 2012).

10.5 Epigenetic Regulation of Clock Gene Expression

Besides the transcriptional and post-transcriptional mechanisms described earlier, epigenetic regulation is emerging as a novel additional level of circadian control, certainly not only in plants (reviewed in (Henriques and Mas 2013)). In fact, plants, as sessile organisms that have to adjust to profound fluctuations in the environment where they established, particularly depend on epigenetic mechanisms to adapt and survive. Epigenetics relies on changes in chromatin status at specific loci that is known to dictate the degree of transcription. Responsible in part for changes between silent and active chromatin are post-translational modifications decorating histones, the central components of the nucleosomes. The surface of the nucleosomes is studded with a variety of modifications among which acetylation and methylation at lysine or arginine histone residues are the most prominent. Histone 3 acetylation (H3Ac) and histone 3 trimethylation at lysine 4 (H3K4me3) are well-known markers for active transcription, and increased H3K27 methylation is associated with silent chromatin (Kouzarides 2007). The role of several histone marks is not described yet, and others, such as H3K36me, are still ambiguous. Most modifications are highly dynamic, and different enzymes coordinately determine the extent of methylation and acetylation of histones to achieve a functional outcome (Kouzarides 2007). Indeed, histone acetyltransferases (HAT), methyltransferases and histone deacetylases (HDAC) are involved in many developmental programmes in plants (Stratmann and Mas 2008).

In the circadian timing system, time-of-day-dependent activity of the core oscillator genes involves chromatin-based regulation (Perales and Mas 2007; Farinas and Mas 2011). *TOC1* mRNA peaking at dusk is preceded by high levels of H3Ac at the *TOC1* promoter, a hallmark of active transcription (Perales and Mas 2007) (Fig. 10.2). Furthermore, STRUCTURE SPECIFIC RECOGNITION PROTEIN 1 (SSRP1), a component of the facilitates chromatin transcription (FACT) complex that assists transcription by modulating chromatin structure (Duroux et al. 2004), associates with the *TOC1* promoter following a circadian pattern similar to the *TOC1* mRNA oscillation (Perales and Mas 2007). At the same time, CCA1 binding to the same DNA region leads to a decrease in H3Ac levels, reducing *TOC1* expression (Perales and Mas 2007). RVE8 similarly binds to the *TOC1* promoter albeit with opposite consequences (Hsu et al. 2013; Farinas and Mas 2011), i.e. promoting acetylation at the locus and boosting its transcription (Farinas and Mas 2011). Although it is intriguing how the two morning-phased genes with similar MYB domains, CCA1 and RVE8, cause antagonistic effects on the same core clock gene *TOC1*, this demonstrates interplay between transcriptional and epigenetic controls to ensure proper circadian oscillations (Fig. 10.3). Besides

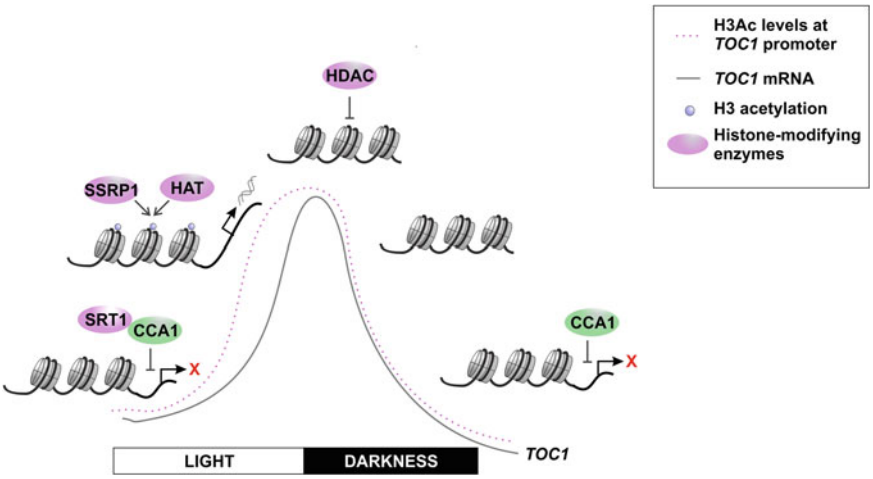

Fig. 10.2 Epigenetic modification of the *TOC1* promoter in the course of the day. Rhythmic expression of *TOC1* is achieved by transcriptional and epigenetic mechanisms at its locus. *TOC1* levels (*grey line*) are maintained low at dawn by the circadian binding of CCA1 to *TOC1* promoter. SIRTUIN 1 (SRT1), an HDAC enzyme, interacts with CCA1 in vitro and is suggested to contribute to rhythmicity (Malapeira et al. 2012). *TOC1* transcriptional activation is accompanied by cyclic levels of histone acetylation that precede the *TOC1* burst (*purple dotted line*). STRUCTURE SPECIFIC RECOGNITION PROTEIN1 (SSRP1) associates with *TOC1* promoter with a circadian pattern similar to *TOC1* expression (Stratmann and Mas 2008). Following *TOC1* peak expression at dusk, transcriptionally silenced chromatin status mediated by histone deacetylase leads to the declining phase of *TOC1*. Nucleosomes are shown as *grey cylinders* and histone acetylation marks as *blue circles*. Histone-modifying enzymes are represented by purple ovals and transcription factors by *green ovals*. *Red cross* indicates repressed transcription. HAT, histone acetyltransferases; HDAC, histone deacetylases

TOC1, several other clock genes were recently surveyed for epigenetic regulation. Rhythmic oscillations of *LHY*, *CCA1*, *PRR9* and *LUX* mRNAs are closely associated with rhythmic histone acetylation and methylation on their promoters (Song and Noh 2012; Malapeira et al. 2012). Transcript levels positively correlate with H3Ac and H3K4me3 levels and negatively correlate with high H3K36me2 marks, all features for active chromatin (Song and Noh 2012; Malapeira et al. 2012). These patterns were maintained under free-running conditions, suggesting that the Arabidopsis clock is regulated by rhythmic histone modifications. Nevertheless, besides SSRP1 and the observation that HDAC activity is needed to cause *TOC1* levels to drop (Perales and Mas 2007), the chromatin remodelling factors responsible for the dynamic oscillation of histone marks within core clock genes are largely unknown. In a recent work, Lee et al. scanned among hundred chromatin remodelling factors and found 17 genes that displayed circadian oscillation, regardless of the environmental input (Lee et al. 2014). One of these, SIRTUIN 1 (SRT1), a member of the HDAC enzymes, physically interacts with CCA1 in vitro, suggesting that this interaction can be critical for circadian physiology (Lee et al. 2014).

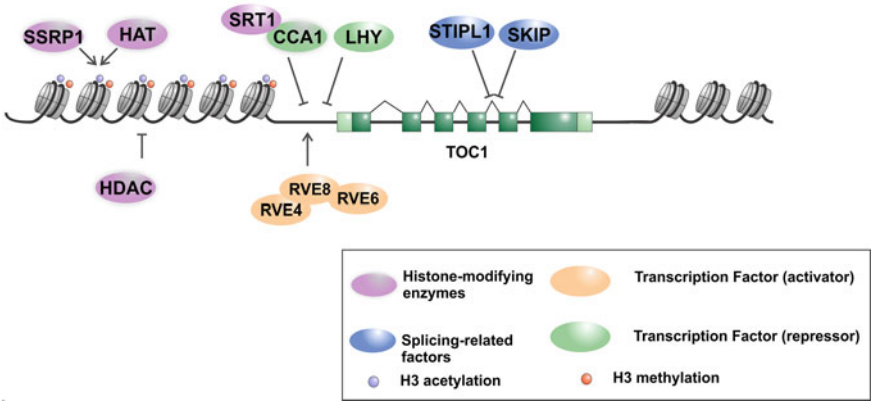

Fig. 10.3 Regulatory events at the *TOC1* promoter. CCA1 and LHY repress *TOC1* transcription at dawn by direct binding to the *TOC1* promoter. SRT1 interacts with CCA1, but the biological relevance of the interaction is not determined yet. Conversely, RVE8, RVE4 and RVE6 act as activators of *TOC1* expression, although direct binding to the promoter has been only demonstrated for RVE8. Releasing CCA1 from the promoter allows epigenetic changes to occur, and access of HAT mediates deposition of permissive histone modifications in *TOC1* chromatin, leading to *TOC1* activation. Active *TOC1* chromatin shows high levels of H3 acetylation and H3K4me marks. The whole picture of histone-modifying enzymes involved in TOC1 regulation is not complete yet, but SSRP1 associates with the *TOC1* promoter in a circadian pattern. HDAC mediates deacetylation of chromatin reverting it to a silent state. SKIP and STIPL1 affect *TOC1* transcript accumulation of by regulating alternative splicing events at exon 4. *Boxes* represent exons, and *lines* represent introns. *Diagonal lines* represent splicing events

10.6 Entrainment of the Core Clockwork to the Environment

Synchronization of endogenous physiological processes with the periodic environment is of utmost importance for plant performance. Ambient light is the prominent environmental cue that synchronizes the circadian clock with the day outside (Nagy and Schaefer 2002; Fankhauser and Staiger 2002). Light is perceived by a suite of photoreceptors including the red/far-red light-sensing phytochromes (Somers et al. 1998; Yanovsky et al. 2000), the blue light-sensing cryptochromes CRY1 and CRY2 (Yanovsky et al. 2001), as well as ZTL and its family members FLAVIN-BINDING, KELCH REPEAT, F-BOX 1/LOV KELCH PROTEIN 2 (Imaizumi et al. 2003; Baudry et al. 2010). To reset the endogenous time, light signalling ultimately affects steady-state abundance of core clock components.

Clock progression has been analysed in a mutant lacking all five phytochromes. The *phyA phyB phyC phyD phyE* quintuple mutant has a long period at high fluence rates of continuous red light but a short period at low fluence rates. This differential behaviour was proposed to rely on opposite functions of the Pfr (far-red light absorbing) and the Pr (red light absorbing) forms of phytochromes with Pfr accelerating the speed of the clock and Pr slowing down the clock (Hu et al. 2013).

A recent study used far-red light to study the effect of phyA signalling on the clock in the absence of signalling through the other phy family members (Wenden et al. 2011). Under these conditions, evening-phased genes showed a higher expression and morning-phased genes showed a lower expression and ELF4, a component of the evening complex, was identified as a target of phyA signalling. EFL4 has been shown to be activated by the phyA signalling components FAR-RED ELONGATED HYPOCOTYL 3 (FHY3), FAR-RED IMPAIRED RESPONSE 1 (FAR1) and LONG HYPOCOTYL 5 (HY5), elucidating a possible phyA signalling cascade to the core clock (Li et al. 2011). Another input into the clock comes from ZTL that affects the TOC1 and PRR5 protein levels through time-of-day-specific degradation (Mas et al. 2003; Baudry et al. 2010).

In addition to light, ambient temperature affects clock function. On the one hand, circadian rhythms are well buffered against changes in environmental temperature, a feature designated as temperature compensation (Ruoff et al. 2000). Several clock components have been implicated in this temperature compensation of the Arabidopsis clock although the exact mechanisms are not well understood at present (Edwards et al. 2006; Gould et al. 2006; Salome et al. 2010). On the other hand, regular temperature cues entrain the clock. When seedlings grown from seed in constant light are exposed to alternating phases of high and low temperatures with as little as 4 °C, transcript oscillations are observed with a phase relationship similar as in light–dark cycles (Heintzen et al. 1994; Heintzen et al. 1994; Michael and McClung 2002; Somers et al. 1998). Moreover, these oscillations persist in constant temperature and thus show the features of circadian rhythms. How temperature is perceived and signalled to the clock is largely unknown (McClung and Davis 2010). However, we begin to understand the molecular targets in the clock. In the absence of functional PRR7 and PRR9, plants do not entrain to temperature cycles (Salome et al. 2010). HEAT SHOCK FACTOR B2b (HsfB2b) binds the *PRR7* promoter and is involved in heat input into the core clockwork (Kolmos et al. 2014).

10.7 Clock Output

The circadian clock has a pervasive impact on the expression of a large number of genes, which in turn mediate the time-of-day-dependent output processes in the plant. The first genome-wide analysis of the transcriptome around the clock using the Affymetrix AG1 microarray that represents about 8000 Arabidopsis genes thus provided insights how the circadian clock controls physiological rhythms (Harmer et al. 2000). It was found that transcripts for metabolic pathways are rhythmic so that components with similar functions are expressed in synchrony at a certain phase of the light–dark cycle, e.g., components of photosynthetic light absorption peak early in the day, enzymes involved in sugar metabolism peak later and starch-degrading enzymes in the middle of the night. Functions for cell wall synthesis are expressed in the middle of the night. Before dawn, the flavonoid

biosynthetic pathway is activated to produce UV-absorbing secondary metabolites that accumulate in the epidermis and serve as sunscreen.

Subsequent studies using the Affymetrix ATH1 microarray representing most of the Arabidopsis genes unveiled circadian oscillations of about a third of the transcripts (Covington et al. 2008). Interestingly, hormone-responsive genes were highly enriched among the oscillating genes, including genes induced by auxin, cytokinin, abscisic acid (ABA), salicylic acid (SA) and methyl jasmonate (MJ) or genes downregulated by ABA, 1-aminocyclopropane-1-carboxylic acid (ACC), brassinolide, auxin, cytokinin, gibberellic acid (GA), SA and MJ (Covington et al. 2008). This suggests that the circadian clock restricts the response to hormones to distinct times of the day, a phenomenon known as "gating" (Hotta et al. 2007). Indeed, auxin signalling and auxin responses are gated by the circadian clock (Covington and Harmer 2007; Nozue et al. 2007). Such a temporal organization of growth processes has been proposed to optimize allocation of resources and thus contribute to plant performance.

Furthermore, responses to external threats have been found to be controlled by the circadian clock. Plants are constantly exposed to a plethora of abiotic and biotic stress factors that bear the risk of lowering plant performance, i.e. reduce resources available for photosynthetic production and growth. To integrate the reactions to external disturbances with the endogenous metabolism, these reactions are restricted according to the likelihood of the stimulus to occur. For example, the response to cold in Arabidopsis is higher in the subjective evening than during the day (Fowler et al. 2005).

Recently, an interconnection has been described between circadian timekeeping and the innate immune system that contributes to plant performance by combatting invading pathogens (Wang et al. 2011). The circadian clock gates the defence against *Pseudomonas syringae* to distinct times of the day (Bhardwaj et al. 2011; Zhang et al. 2013; Korneli et al. 2014). Moreover, resistance against the herbivore *Trichoplusia ni* correlates with rhythms in JA accumulation (Goodspeed et al. 2012).

10.8 Rhythm Generation in Plants Follows the Same Principle as in Other Eukaryotic Model Organisms

The clocks in other model organisms such as mammals, Drosophila and Neurospora follow the same fundamental principle as in plants: i.e. circadian oscillation is based on transcriptional–translational feedback loops of the clock genes. However, the players are different. In mammals, the brain governs daily rhythms for the entire body through the master circadian clock present in the suprachiasmatic nucleus (SCN) of the hypothalamus (Ralph et al. 1990) and reviewed in (Coomans et al. 2014). Still, recent studies have shown that most tissues and cell types display circadian patterns of gene expression when isolated from the SCN (Yamazaki et al. 2000; Brown and Azzi 2013; Abe et al. 2002).

The mammalian transcriptional–translational central feedback loop involves at least ten genes. Clock (circadian locomotor output cycles kaput) and Bmal1 (brain and muscle Arnt-like 1) form the positive branch of the loop, while Period (Per1, Per2 and Per3) and Cryptochrome (Cry1 and Cry2) cover the negative limb. CLOCK and BMAL1 dimerize to trigger transcription of *Per* and *Cry* (Dibner et al. 2010; Ukai and Ueda 2010) via E-box enhancer elements in their promoters. PER/CRY complexes translocate to the nucleus and in turn inhibit the transcriptional activity of the CLOCK/BMAL1 dimer, preventing their own transcription. Later, degradation of PER/CRY releases the repression on CLOCK/BMAL1 which allows a new cycle to start. The stability of PER and CRY proteins is key to setting the period of the clock. Degradation pathways driven by phosphorylation of PER and CRY target these proteins for ubiquitination and subsequent proteolysis (Camacho et al. 2001; Eide et al. 2005; Shirogane et al. 2005). Mutations in several of the kinases and phosphatases (CKα, CKδ, CKε, PP1, PP5 and AMPK) involved in these pathways result in period lengthening, shortening or arrhythmia (reviewed in (Reischl and Kramer 2011)). CLOCK/BMAL1 also initiate a supplemental feedback loop by activating transcription of orphan nuclear receptor genes Rev-Erbα/β and RORα/β. REV and ROR proteins activate and repress Bmal1 transcription, respectively, ensuring the rhythmic transcription of Bmal1 (Guillaumond et al. 2005).

In the circadian clock of *Drosophila melanogaster*, transcription of the clock genes *Period* (*Per*) and *Timeless* (*Tim*) is activated by the transcription factors CLOCK and CYCLE. After a time delay, PER and TIM proteins enter the nucleus where the association of PER with CLOCK and CYCLE inhibits their transcriptional activity. Gradually decreasing levels of TIM and PER relieve the inhibition again through a series of post-translational modifications and initiate another round of transcription (Allada and Chung 2010). In addition to the principal PER/TIM feedback loop, a second loop regulates *Clk* transcription. CLOCK/CYC activates CLOCKWORK ORANGE (CWO), PAR DOMAIN PROTEIN 1 (PDP1) and VRILLE (VRI) by binding to their promoters (Cyran et al. 2003; Kadener et al. 2007; Matsumoto et al. 2007; Lim et al. 1082). CWO feeds back to repress CLOCK/CYC activation. PDP1 and VRI have opposite effects on *Clk;* whereas PDP1 activates *Clk* transcription, VRI competes with PDP1 binding to repress it.

The molecular components of the feedback loop that command the internal clock of the fungus *Neurospora crassa* consist of four core proteins, two proteins in the negative arm of the loop, FREQUENCY (FRQ) and FREQUENCY-INTERACTING RNA HELICASE (FRH), and two proteins in the positive arm, WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2). Similar to what is known for Drosophila and mammals, WC-1 interacts with WC-2 forming the white collar complex (WCC) to promote *frq* transcription. A new protein complex formed by FRQ/FRH attracts WCC and in turn inhibits the activity of WCC by recruiting several kinases. The time delay needed to achieve a circadian period (or rhythmic output) is facilitated by phosphorylation of FRQ that influences the stability of FRQ. After FRQ turns over, *frq* can be again activated by newly synthetized WCC (reviewed in (Baker et al. 2012)).

TTFLs are thought to be crucial molecular processes for daily timekeeping in higher eukaryotes. However, this view was recently confronted with several findings that proved the presence of rhythms in the absence of TTFL mechanisms, renewing to some extent the view of the clock. The first circadian rhythms shown to persist without transcription were described in cyanobacteria. In *Synechococcus elongatus* where the *KaiABC* operon is responsible for generation of circadian rhythms (Ishiura et al. 1998), the clock can run independent of de novo transcription and translation processes (Tomita et al. 2005). Twenty-four-hour oscillations are seen through periodic phosphorylation of a key clock component KaiC that persists even in darkness where transcription is interrupted (Tomita et al. 2005). Indeed, these rhythms can be reproduced in a test tube by mixing the KaiA, KaiB and KaiC clock proteins with ATP (Nakajima et al. 2005). Likewise, in the unicellular algae *Ostreococcus tauri* and in human red blood cells, circadian rhythms in the form of redox reactions can run without any transcriptional–translational mechanisms (O'Neill and Reddy 2011; O'Neill et al. 2011), demonstrating that what was previously observed in cyanobacteria was not restricted to prokaryotes. Interestingly, these redox rhythms are coupled with TTFLs, as long-period mutants show alteration in redox oscillations (O'Neill et al. 2011).

10.9 Organization of Circadian System Is Different in Plants Compared to Mammals

In mammals, the master clock resides in the SCN. The SCN receives light input from melanopsin photoreceptors located in retinal ganglion cells via the retinohypothalamic tract. In turn, the SCN governs rhythms in peripheral cells via hormones or neuronal signals (Dibner et al. 2010).

In contrast, most plant cells harbour a clock thought to be entrained by light input (McWatters et al. 2001). However, it was not clear whether clocks in different cells would be identical. Using transgenic Arabidopsis plants expressing CCA1 tagged with a fluorescent reporter protein to measure rhythmicity in individual leaf cells, stomatal guard cells were shown to have a different period from surrounding epidermal and mesophyll leaf cells. Oscillators of individual cells in the leaf are robust, but become partially desynchronized in constant conditions. These results suggest that at the level of individual cells, there are differences in the canonical oscillator mechanism (Yakir et al. 2011).

The core clockwork in roots of mature Arabidopsis plants is organized differently from that in shoots (James et al. 2008). In roots, the core clockwork runs only on the morning loops because CCA1 and LHY are able to inhibit gene expression in shoots but not in roots. The root clock is synchronized by a photosynthesis-related signal transmitted from shoots. Thus, the plant clock is organ specific.

Recently, differences between clocks in leaf mesophyll cells and cells of the vasculature have been unveiled (Endo et al. 2015). In the mesophyll cells, the

morning loop of the clock is prevalent and rhythmically expressed genes are mostly morning-phased, whereas in the vasculature, the evening loop is prevalent and rhythmically expressed genes are mostly evening-phased. Notably, the clock in the vasculature is dominant over the clock in mesophyll cells. Thus, the plant circadian system may also show a hierarchical organization reminiscent of the clock system in mammals.

10.10 Conclusion/Perspectives

Following the description of the first core clock loop, more and more interconnected feedback loops were discovered, arriving at a view that the circadian clock system represents a highly interconnected network structure. As new experimental evidence on regulatory mechanisms and molecular components of plant clock continues to emerge, the ultimate description of the molecular blueprint of circadian systems benefits from state-of-the-art mathematical modelling that may provide fresh insights into the understanding of the intricate relationships among all experimental findings (Pokhilko et al. 2012; Bujdoso and Davis 2013; Schmal et al. 2013; Locke et al. 2006). Among exquisite levels of regulation not yet explored in plants is the organization of genes that are expressed with circadian rhythms in functional territories within the nucleus, as recently demonstrated for mammals (Aguilar-Arnal and Sassone-Corsi 2014). Furthermore, comparative analyses provide insights into the make-up of the circadian timing system in crop plants (Campoli et al. 2012; Calixto et al. 2015; McClung 2013; Staiger et al. 2013).

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Part III
Theoretical Aspects and Modelling

Chapter 11
Noise-Induced Phenomena and Complex Rhythms: A Test Scenario for Plant Systems Biology

Marc-Thorsten Hütt, Ulrich Lüttge and Michel Thellier

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. Empirical case studies of rhythmicity are derived from three areas of study, (i) Crassulacean acid metabolism, (ii) stomatal pore regulation by guard cells and (iii) plant memory. Biorhythmicity is underlying the former two, whose spatiotemporal dynamics can be documented by, among other techniques, chlorophyll fluorescence imaging. The third one, plant memory, is intimately related to rhythmicity and the biological clock with its set points and phase regulation. All three case studies reveal nonlinear performance with synchronization/desynchronization leading to modelling and theoretical concepts.
2. In modelling, maximal 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

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and coherence resonance, where intermediate noise intensity generates quasi-rhythmic behaviour of systems from arrhythmicity.

This merges into a new path towards systems biology, where extensive data currently provided by analytical progress are integrated 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 more advanced models.

11.1 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 initially been derived from theoretical constructs and then 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 concept 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 Gierer (1998) writes, to understand the system properties of the living world, one needs experimental observations and data, an appropriate abstract perception of the experienced reality and—last but not 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 non-trivial mathematical tools involved.

Systems composed of modules can develop characteristics which the components themselves do not have. Integration of modules in networks with nonlinearity leads to the emergence of completely novel properties (Lüttge 2012). To grasp these emergent 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). Systems biology attempts to incorporate this systemic view into its modelling and data analysis endeavours. In its simplest form, systems biology is the systemic contextualization of large numbers of individual observations (see, e.g. Smith and Hütt 2010; Hütt 2014). At the core of systems-thinking in biology is the concept of networks (Barabási and Oltvai 2004; Gallos et al. 2007).

How does network architecture help analyse and understand rhythmic phenomena? It is well established now that the synchronizability of a network depends strongly on its architecture (see, e.g. Arenas et al. 2008). Furthermore, dynamical processes on networks can also have a pattern-like organization (Müller-Linow et al. 2008; Hütt et al. 2014). Such network equivalents of classical spatiotemporal patterns include waves organized around highly connected components ("hubs") and synchronized patches coinciding with the network's topological modules. Lastly, regulatory components in networks (like feedback loops) can specifically encode certain dynamical functions (Alon 2007).

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 decade 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 the 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 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. We shall organize this in seven sections of this chapter. We will begin with explaining aims and approaches of modelling (Sect. 11.2), followed by the two case studies of systems-theory-modelling of Crassulacean acid metabolism (Sect. 11.3) and stomatal patterns (Sect. 11.4). Then, we shall consider the role of rhythmicity for fitness (Sect. 11.5), the functions of biological clocks in plant memory (Sect. 11.6) and a third case study of systems-theory-modelling with a conceptual model of plant memory (Sect. 11.7). Finally, we shall advance at integrating the empirical and theoretical considerations in an attempt of finding a new path towards systems biology (Sect. 11.8).

11.2 Aims and Approaches of Modelling

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 *maximal 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.

Interesting accounts of the aims of maximal models are given, for example, 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

$$\frac{\mathrm{d}x}{\mathrm{d}t} = f(x) \tag{11.1}$$

[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 analysed. 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 accommodated in 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 (Sect. 11.4).

Given an initial value $x(0)$, the function yields the change dx/dt from this point on, leading to a new value of x a little later and, thus, to a new function value $f(x)$ which, in turn, determines a new change dx/dt. In this way, the differential equation, Eq. (11.1), *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.

The theory of self-organization is an important theoretical building block of our understanding of complex systems. Elements interact locally and thus, on the system-wide level, produce coherent collective behaviour. Such collective system states are "emergent", i.e. they occur spontaneously when critical parameter values (e.g. in the interaction strength) are reached.

The internal parameters of the system affecting collective behaviour are called control parameters. Quantities capturing the collective behaviour are called order parameters. Mathematically speaking, it is a remarkable feature of self-organized processes that systems consisting of a multitude of components (large number of degrees of freedom) "synchronize" to comparatively few collective states (few possible values of an order parameter). Self-organization is an important property of diverse natural processes. The most striking examples are forms of synchronization (Strogatz 2004), human mobility patterns (Helbing et al. 1997, 2000) and aspects of neuronal activity (Deco et al. 2011).

In the case of coupled phase oscillators, the control parameter is the coupling strength and the order parameter is the clustering of phases on the circle (see below Fig. 11.7 and the discussion around Figs. 11.7, 11.8 and 11.9).

11.3 Case Study I of Systems-Theory-Modelling—Crassulacean Acid Metabolism (CAM)

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 lipoprotein 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 (Lüttge 2000), clearly points to the nonlinear dynamics of CAM.

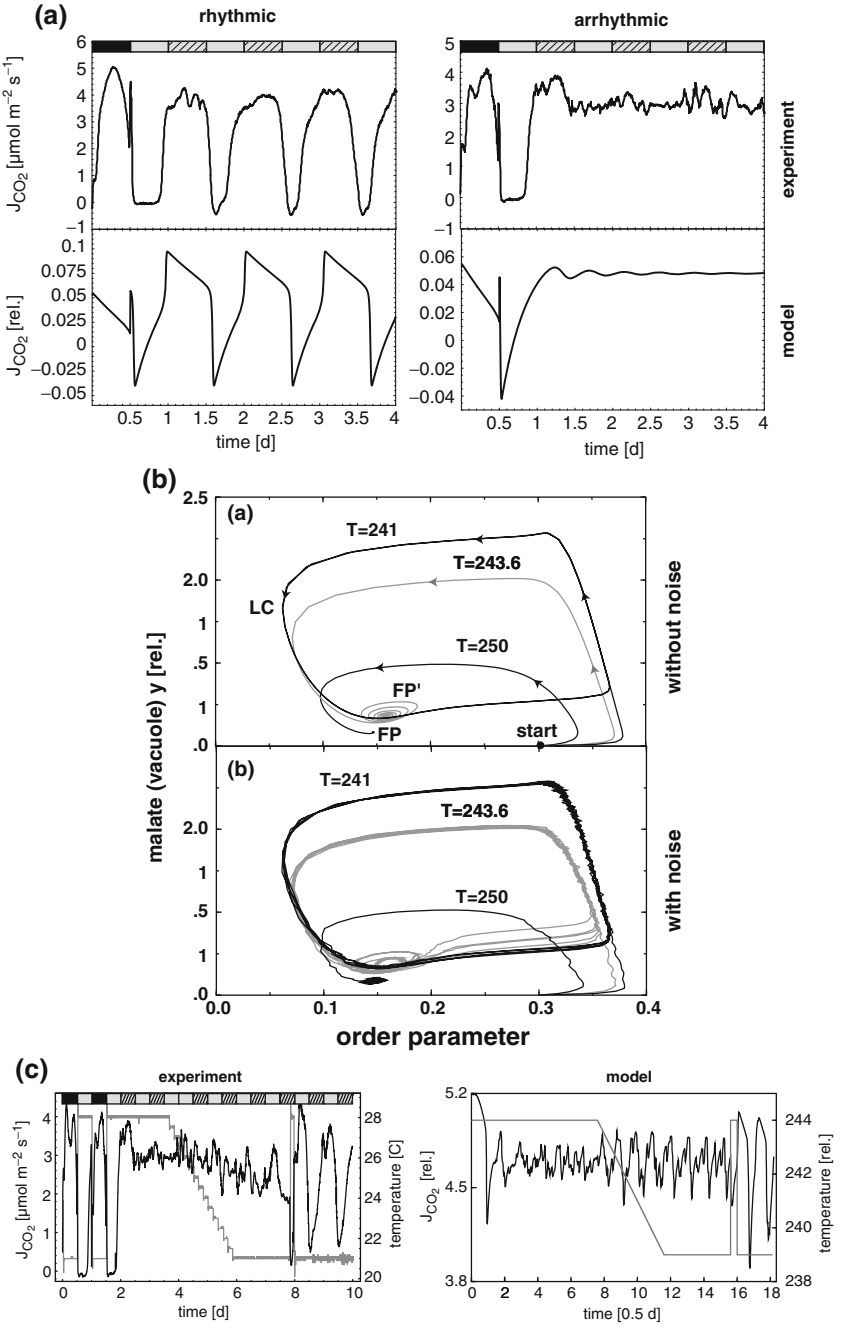
Biorhythm research is dominated by the quest for the biological clock or circadian oscillator on a genetic basis (for a review of the plant biological literature, see Lüttge 2003a). Some central oscillator genes or master genes have been identified. Although we must keep in mind that, in *Drosophila*, both the circadian rhythm of eclosion and locomotor 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). 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 one 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), i.e. subject to downstream control. However, the transcription of this apparent key gene is under metabolic control of malate, the product of PEPC activity (Borland et al. 1999), i.e. subject to upstream control. This is an example of the general principle of nonlinear interplay between the clock and metabolism, where in feedback loops the circadian clock controls metabolism and is itself controlled by metabolites (Müller 2014). Thus, although there is evidence of a *TOC1/CCA1/LHY*-oscillator to control oscillations in the CAM pathway (Hartwell 2005), it is seen that the gene hierarchies simultaneously work top–down (downstream regulation) and bottom-up (upstream regulation) in integrated network-type relations. Moreover, CAM is also an example where functioning of a biophysical circadian oscillator downstream of the direct involvement of genetic control can be demonstrated. Its dependence on malate levels makes its regulation subject to biophysical compartmentation between cytoplasm and vacuole, i.e. dependent on

vacuolar loading/unloading via the tonoplast membrane (Lüttge 2000; Nimmo 2000). At the bottom level, a direct involvement of CCGs appears to be excluded and the primary oscillator of the CAM cycle is based on post-translational functions.

Therefore, application of nonlinear dynamics tools was an important choice for advancing our understanding of the CAM oscillator. Over one and a half decade, 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, 1998, 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. 11.1a).

Due to the combination of a highly controllable experimental set-up 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 earlier experimental results for the CAM plant have been reproduced by this model and refinements thereof. Examples are shown in Fig. 11.1. 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 analysed 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


(a)
rhythmic
arrhythmic
experiment
model
J_{CO_2} [µmol m^{-2} s^{-1}]
J_{CO_2} [rel.]
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
J_{CO_2} [µmol m^{-2} s^{-1}]
temperature [C]
J_{CO_2} [rel.]
temperature [rel.]
time [d]
time [0.5 d]

◀ **Fig. 11.1** 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 (*a*) and with noise (*b*), illustrating the dynamical properties of the model more clearly. A phase diagram shows 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)

order undergoing a phase transition when this parameter is varied. The lipid order parameter thus 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. 11.1b).

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 taken from a purely temporal to a spatiotemporal dynamics. It concerns the inclusion of stochastic processes and dynamics at a small timescale, which formally enter the description as *noise*.

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 would decrease. A schematic example of such a *stochastic resonance* is given in Fig. 11.2a. This phenomenon is possible only in a nonlinear system (in our schematic example of Fig. 11.2a, 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). For circadian oscillations in plants, it was suggested that the desynchronization between noisy oscillations in single cells contributes to the observed damped oscillations at the level of the cell population (Guerriero et al. 2012).

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 scenarios, such as a slow (rather than abrupt) temperature variation, are simulated by the model, yielding high agreement with experimental data (Fig. 11.1c).

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 for 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. 11.1b). 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. 11.2b).

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 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 spatio-temporal analysis tools (see Hütt and Neff 2001; Hütt and Lüttge 2002) have been formulated and extended to a method for estimating connectivity from the time

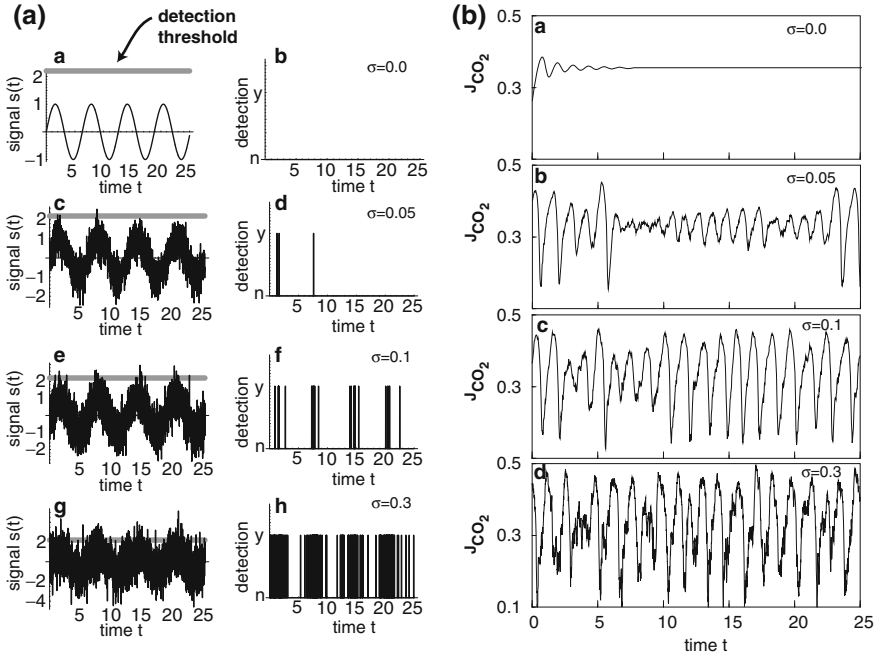


Fig. 11.2 **a** Schematic example of a system displaying stochastic resonance. A signal $s(t)$, consisting of a sine wave and additive noise, enters some detection mechanism with a threshold (*a*, *c*, *e* and *g* for different noise intensities σ). At each point in time, the signal is translated into 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. 11.1, shows a similar behaviour with respect to recovering the circadian oscillation in the noisy fixed point. Data in *a* were schematic representation of the system without noise ($\sigma = 0$) reaching the fixed point. Data in *b*, *c* and *d* were obtained by numerical simulation with increasing noise intensities (σ of 0.05–0.3). 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)

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).

In CAM, the major interrelated subsystems are (1) malate compartmentation, (2) stomatal movements regulating CO_2 uptake and (3) CO_2 assimilation via Rubisco (Bohn et al. 2003). The overwhelming complexity here is illustrated by observations that the vacuolar malate accumulation/remobilization oscillator discussed 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.

11.4 Case Study II of Systems-Theory-Modelling—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. 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 or receptor has not been definitely identified as yet, it is clear that increased and decreased internal CO_2 concentrations affect 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; Haefner et al. 1997). Under particular conditions, and rather than anatomical features, it is physiological constraints which may lead to physiological homobary (Duarte et al. 2005; Lüttge and Hütt 2006).

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). Giersch (1994) considered the various possible metabolic control points and discussed 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 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 11.3 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 in 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. 11.3a). 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 state space. Figure 11.3b 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. 11.3b 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 analyse the statistical features of a rather famous cellular automaton designed to perform a specific computational task, and compare these

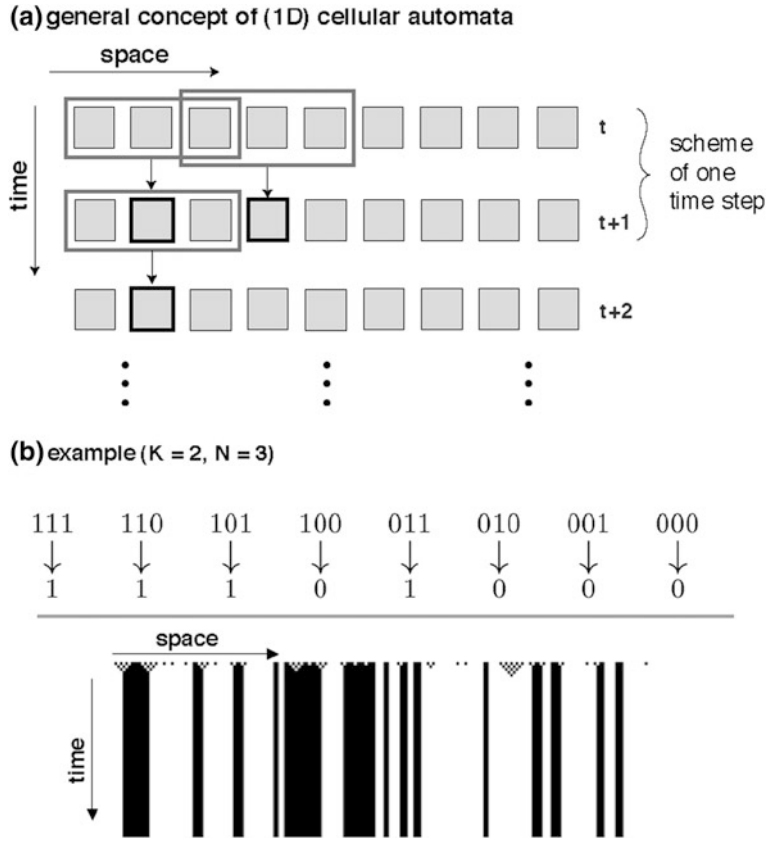


Fig. 11.3 Concept of cellular automata (CA) illustrated for one spatial dimension (**a**) and an example of a CA simulation for a binary state space and a three-element neighbourhood (**b**). For this selection of update rules, one obtains fixed-point dynamics after a short transient (adapted from Lüttge and Hütt 2006)

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

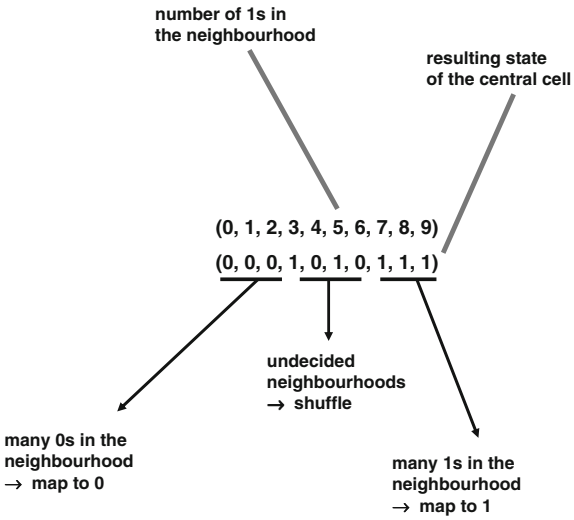


Fig. 11.4 Summary of the update rule for a totalistic CA suitable for density classification. The *upper line* of digits in parentheses gives the number of 1*s* in a spatial (nine-element) neighbourhood, whereas the *lower line* lists the corresponding state of the central cell in the next time step (adapted from Lüttge and Hütt 2006)

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 11.4 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 11.5 shows spatial snapshots at 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. 11.5. The capacity of this automaton to classify initial densities by converging to a specific state is summarized in Fig. 11.6. 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 analysed with methods of nonlinear dynamics. Here, the mathematical 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,

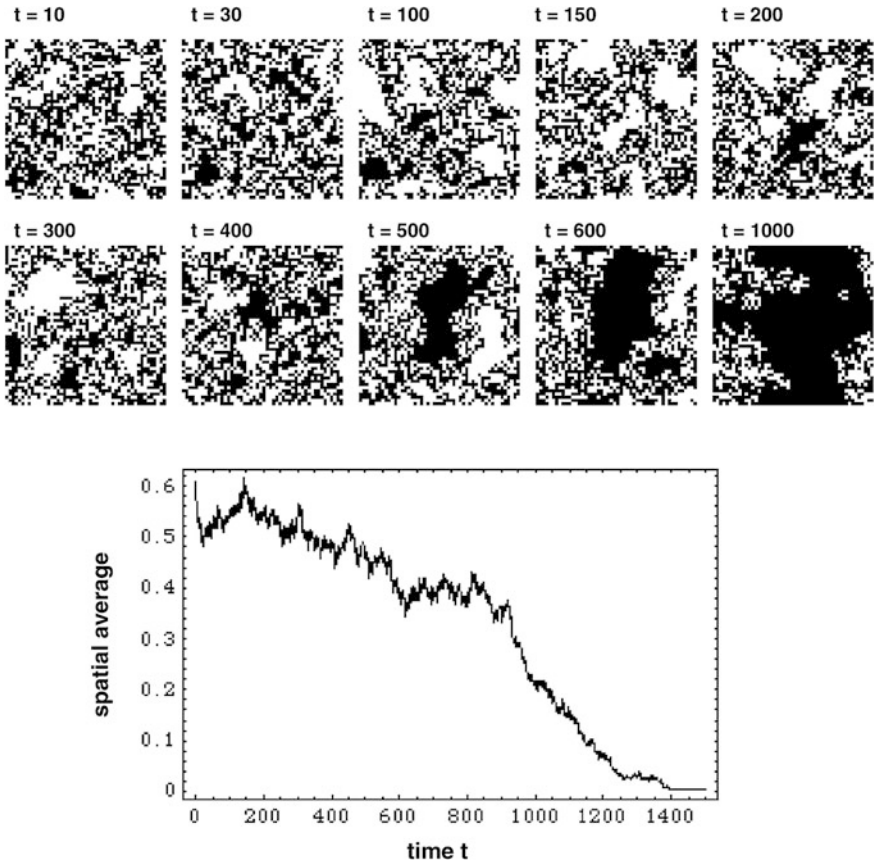


Fig. 11.5 Spatial snapshots of the CA in Fig. 11.4 at different time points (*top*) and time course of the density $r(t)$ of $1s$ (i.e. of the spatial average over the lattice at each time point; adapted from Lüttge and Hütt 2006)

Peak et al. (2004) analyse event sizes, the scaling of transients and the distribution of interpeak intervals in the average opening state (see also West et al. 2005). Such "complex systems that perform tasks" (Mott and Peak 2006) have a multitude of additional applications across many scientific disciplines (see Moreira et al. (2004) for a more stylized example).

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).

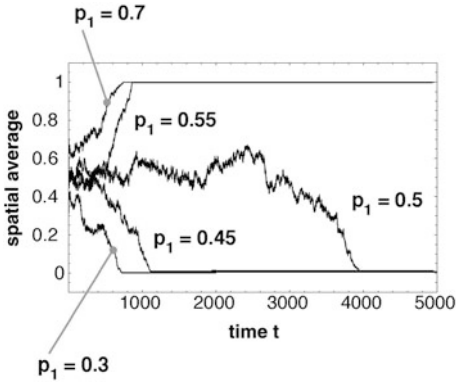


Fig. 11.6 Density $r(t)$ as a function of time t for different values of the initial density of 1s, p_1. The cellular automaton is the same as in Figs. 11.4 and 11.5 (adapted from Lüttge and Hütt 2006)

11.5 Rhythmicity and Fitness

The biological clock communicates alertness to organisms ensuring their punctual performance under rhythmically changing external conditions. This particularly relates to the fluctuations of day/night conditions. That this is a major function for providing fitness somewhat is common-place wisdom also repeated time and again in the scientific literature. However, we must check, what is the solid evidence for this (Sect. 11.5.2)? Moreover, we must ask, what is fitness? Is it success of reproduction for selection in the vein of the evolutionary theory of Charles Darwin and Alfred Russel Wallace? Is it sustaining of ecophysiological performance with adaptive responses to variable spatiotemporal environmental stress? Is it competitiveness given by productivity of biomass and hence growth? If we consider fitness in a broad sense comprising a variety of these aspects, we shall see that indeed rhythmicity does support it. Thus, correlations between rhythmicity and fitness are given not only under the direct action of the circadian biological clock (Sect. 11.5.2) but also with ultradian (Sect. 11.5.1) and annual or phenological oscillations (Sect. 11.5.3).

11.5.1 Ultradian Rhythms

Ultradian rhythms lie below a lot of biophysical, biochemical and physiological functions as listed and reviewed by Lüttge and Hütt (2004), namely

- biophysical functions of membranes,
- biochemical functions of isolated enzymes and metabolic networks,
- cell growth and development (e.g. pollen tubes),
- movements of stomatal guard cells and photosynthesis,

- circumnutation of shoots and tendrils,
- leaf movements and
- cell elongation and extension growth.

We can see that ultradian rhythmicity supports robustness of a plethora of essential functions and therefore sustains performance as a basis of fitness.

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. Oscillations of membrane functions and biochemical reactions in the cells never occur in isolation. The discovery and understanding of ultradian oscillations of glycolysis goes back to the work of Britton Chance (Ghosh and Chance 1964; Chance et al. 1964; Betz and Chance 1965) and has received interest over decades (e.g. Goldbeter and Lefever 1972; Berridge and Rapp 1979; Olsen and Degn 1985; Rapp 1986; Goldbeter 1996). These oscillations involve ultradian rhythms of the redox state of the pyridine nucleotide system (Betz and Chance 1965). More recent work with yeast cells by the group of David Lloyd shows that the complete redox poise of the cells is affected where H_2S has signalling functions (Lloyd and Murray 2006; Lloyd 2008). Hence, this group speaks of an "ultradian clock" (Lloyd and Kippert 1993). The redox poise is considered as an overarching aspect. Koukkari et al. (1997) have searched among ultradian rhythms for a "biological hour". Among ultradian oscillations in bacteria, amoebae, yeast, higher plants and mammals they found periods ranging from 30 to 240 min averaging 95 min. However, for the redox poise the Lloyd group notes a period length of 40 min which they think is the basic period of the ultradian clock. With the central importance of the redox poise in cell biology, we may ask if one can construct circadian rhythms from ultradian rhythms (Lüttge and Hütt 2004). Indeed, the Lloyd group argues that interference of many fast ultradian oscillations can produce robust circadian oscillations: the period length of 40 min is contained exactly 36 times in the natural diurnal 24-h rhythm. The large number of known ultradian rhythms provides a huge experimental data basis for system analysis to unravel governing principles of nonlinear dynamics. This is generic, and it may be one of the reasons for the great success of studies of model systems such as glycolysis and also the oscillations of cyclic adenosine monophosphate (cAMP) secretion regulating the attraction and pattern formation of amoebae of the slime mould *Dictyostelium* (e.g. Berridge and Rapp 1979; Lüttge and Hütt 2009).

A concrete hint for the interaction of rhythmicity and fitness is the general role of oscillations in encoding information and signalling in transduction chains or networks (McAinsh et al. 1995; Berridge et al. 1998; Shabala et al. 2001). In an ecophysiological context of fitness, it is noteworthy that natural light fluctuations in Tasmania due to cloud dynamics showed frequency peaks corresponding to periods of 3, 6 and 14 min in the same range as those of ultradian oscillations of photosynthesis (Shabala et al. 1997).

11.5.2 Circadian Rhythms

With the first case study of systems-theory-modelling above, CAM was introduced as an example of free running circadian rhythms in plants (Sect. 11.3). The molecular structure of the clock of higher plants is studied in the greatest detail in *Arabidopsis thaliana* (Nakamichi 2011). The master genes CCA1/LHY and TOC1 (see Sect. 11.3) are expressed in the morning and evening, respectively. However, master genes per se do not make up an all governing central oscillator. In a non-linear network fashion, a large number of rhythmic functions of genes and biochemical/biophysical processes are involved. There are further morning and evening elements which function as transcription factors (Harmer and Kay 2005; Kikis et al. 2005; McClung 2006; Nakamichi 2011). These genes determine the phase of the clock. Downstream a vast number of other genes are controlled by the clock, the so-called clock-controlled genes (CCGs). In the cyanobacterium *Synechococcus elongatus*, almost all genes and, in the angiosperm *Arabidopsis thaliana* (L.) Heynh., very many genes are CCGs (Liu et al. 1995; Michael and McClung 2003). Almost all functions of life appear to be subject to circadian regulation. The plant circadian clock regulates virtually every plant biological process and most prominently hormonal and stress response pathways (McClung and Gutiérrez 2010).

That this provides fitness primarily comes from a generally accepted logical contention of common sense. In support of the argument, mostly the paper of Dodd et al. (2005) is cited in the scientific literature. The most solid evidence has been obtained in experiments with period-length mutants. The rationale was that mutants with different period lengths should perform best and have competitive advantage over other mutants when the period length of an external light/dark rhythm is close to or identical to their endogenous period length. S.S. Golden and collaborators used period-length mutants of the cyanobacterium *Synechococcus elongatus* PCC7942. They grew mutants having different circadian periods of their endogenous clocks in co-culture and subjected these cultures to different external light–dark rhythms. The mutants having the correct endogenous period, i.e. closest to the imposed external light–dark rhythm, out-competed the others during growth. Under constant environmental conditions, this competitive advantage disappeared. Evidently, the endogenous circadian rhythm of the cyanobacteria provided them competitive fitness under external fluctuations of dark and light periods (Ouyang et al. 1998; Johnson and Golden 1999; Woelfle et al. 2004). Yerushalmi et al. (2011) crossed *Arabidopsis thaliana* mutants with different circadian period lengths and subjected the F2 and F3 generations to altered light/dark cycle periods. They found that under such environmental pressure selection occurred favouring the plants which had an endogenous circadian rhythm that resonated with the environmental one. Thus, this suggests that endogenous rhythmicity matching environmental rhythmicity can confer fitness to the plants and provide a selective advantage (Green et al. 2002; Hotta et al. 2007; Yerushalmi and Green 2009; Yerushalmi et al. 2011). Nevertheless, matching of internal circadian periods with

periods of external rhythmicity is not sufficient for guaranteeing positive clock effects on growth and competitiveness. This is due to the complex nonlinear network dynamics involved, where metabolomics controlled by gene expression exert feedback by controlling transcription (Sect. 11.3; Müller et al. 2014). As a result of a detailed systems-level analysis of circadian rhythms, it was shown that 12-h rhythms can be linked to physical binding properties of transcription factors in the presence of a circadian clock (Westermark and Herzel 2013).

Summarizing, circadian rhythmicity can therefore be viewed as a phenomenon organized across several system scales. On the cellular level, rhythms can be associated with feedback loops in the underlying regulatory networks. Due to this deep relationship between the architectural ("hardware") properties of the regulatory networks and the dynamics (i.e. the diverse types of rhythmic behaviour), cellular rhythms have, over the last few years, become an important topic in systems biology (see, e.g. Goldbeter et al. 2012). In the last few years, the relevance of transcriptional regulation for circadian oscillations has been re-evaluated. In particular, it was shown that non-transcriptional mechanisms are sufficient to sustain circadian timekeeping in eukaryotes (O'Neill et al. 2011). Circadian rhythms can thus be regarded as a multilevel phenomenon that cannot be related to a single layer of cellular organization.

Additionally, it is well established by now that circadian rhythms in plants can have a complex underlying spatiotemporal organization, leading to propagating waves along leaves, coordinating photosynthetic activity (Rascher et al. 2001). The stomatal cells of plants respond with complex patterns (termed "stomatal patchiness") to changes in the environment, which effectively implement a density classification algorithm (Peak et al. 2004).

11.5.3 Annual Rhythmicity of Phenological Patterns

The clearest evidence for the absolute necessity of the biological clock for plant fitness comes from phenological patterns which are subject to annual rhythmicity. Among the most important examples of phenological phenomena are plant growth and development, flowering and seed production, initiation of frost hardiness and preparation for leaf shedding of deciduous woody plants (Lüttge and Hertel 2009). These processes are subject to regulation by day-length or photoperiod. Their appropriate annual periodicity is directly related to the various aspects of fitness. It requires measurement of day-length, and this is mediated via the biological clock, whose phase adjustment is an essential mechanism in photoperiod perception (Frankhauser and Staiger 2002; Roden et al. 2002; Love et al. 2004; Ogudi et al. 2004; Fujiwara et al. 2008; Niwa et al. 2009; Ibáñez et al. 2010). The genetic basis of phase information is revealed by the identification of phase information genes (Michael and McClung 2002; Salomé et al. 2002), phase mutants (Onai et al. 2004) and genetically encoded phase variations in populations (Darrah et al. 2006).

11.6 Biological Clocks and Plant Memory

Plants are sensitive to many different stimuli (such as wind, rain, touch, drought, cold shock, heat shock, wounding and attack by fungi, bacteria or viruses). They are even sensitive to low-intensity 0.9 and 105 GHz electromagnetic radiation (Tafforeau et al. 2002, 2004; Roux et al. 2006; Vian et al. 2006). When they have perceived a stimulus, plants generate a final response consisting sometimes of a macroscopic movement and most usually of growth and/or metabolic modifications. Upon perception of a stimulus, in most cases the plant reacts almost immediately by Ca^{2+} invasion of the cytosol in the cells in the stimulated area (Knight et al. 1991). The intermediate steps between Ca^{2+} invasion of the cytosol and the final response are still not completely unravelled, but they implicate the modification (especially the phosphorylation) of some existing proteins, the activation/inactivation of ionic channels and changes in genome expression.

Quite often, the final response is stereotyped and follows the perception of the stimulus almost immediately (with only the delay for the intermediate steps to take place). This may be termed a "direct" response. However, it has been recognised since the beginning of the 1980s (Thellier et al. 1982), and often confirmed afterwards, that cases exist in which a "memory" is also involved. More precisely, it has appeared (Trewavas 2003) that in plants we may distinguish memory function with capacities of priming and store/recall [STO/RCL] memories (Thellier 2011; Thellier and Lüttge 2013; Thellier et al. 2013). Stimulating the priming memory changes the way the plant transduces one or several subsequent stimuli, with the effect of "familiarization" ignoring subsequent harmless stimuli or, conversely, with "sensitization" producing increasingly violent responses to harmful stimuli. The memory with STO/RCL functions is more sophisticated: it involves (i) storage of information as a consequence of the perception of a first stimulus and (ii) recall of that information at a later time, allowing the plant to make use of that information in the control of its growth and metabolism. To some extent, the STO/RCL function of plant memory resembles the store/evocation function of animal and human memory.

The characteristics of the STO/RCL memory were studied mainly in three different systems (I–III):

- In system I, seedlings of *Bidens pilosa* L. were grown under conditions of low light and deficient mineral nutrition (Thellier et al. 1982; Desbiez et al. 1984, 1991a, b). When the terminal bud of the seedlings was removed by decapitation at the onset of daylight, there was outgrowth of both lateral buds in the axils of the cotyledons. Preferentially, one of the two buds was taking the lead, but symmetry was given in that it was impossible to predict which one it was. If one of the cotyledons was stimulated mechanically or chemically, this broke the symmetry and there was preferential outgrowth of the bud in the axil of the opposite cotyledon. To obtain this effect, decapitation could be done up to 2 weeks after cotyledon stimulation (Fig. 11.7). This proves that there is memory, which in fact has separate functions of signal storage (cotyledon

stimulation) and recall (asymmetric bud outgrowth), respectively. Whether STO (pricking stimulus) was stimulated before or after RCL (decapitation) made no difference, which shows that the STO and RCL boxes work independently of each other. When a dissymmetrical stimulus (pricks administered to a single cotyledon of each plant) was combined with one or several symmetrical stimuli (same number of pricks administered simultaneously to both cotyledons), it was possible to repeatedly recall the stored information.

- In system II, the inhibition of hypocotyl elongation of *B. pilosa* was studied (Desbiez et al. 1987) shortly after seed germination (Fig. 11.8). The stimulus was pricking (STO-function). However, the reaction of hypocotyl elongation only occurred when the seedlings were in water and not in nutrient solution. When the seedlings were in water, there was an immediate effect of the stimulus. When the stimulus (STO) occurred on day 6 and without effect with the seedlings in nutrient solution, a transfer two days later on day 8 elicited an immediate effect (RCL on).
- In system III, the production of epidermal meristems in hypocotyls of flax seedlings was studied (Verdus et al. 1997, 2007, 2012) shortly after seed germination (Fig. 11.9). Here, the stimulus (STO) was manipulation by transfer to a new nutrient medium and the recall function was activated by transient removal of calcium (RCL). The 1st control was an immediate reaction (RCL on) upon a stimulus (STO) and after 2 days in the absence of Ca^{2+}. The 2nd control was giving only the stimulus (STO) when there was no reaction. The 3rd control was transient removal of Ca^{2+} only with no stimulus and naturally again no reaction. The 1st interesting response was after giving the stimulus (STO) followed 4 or 8 days later by transient removal of Ca^{2+} (RCL on) then eliciting the reaction. This shows the existence of memory. Again (see system I), repeated recall is possible as shown by sequences of transient Ca^{2+} removal (RCL on) followed

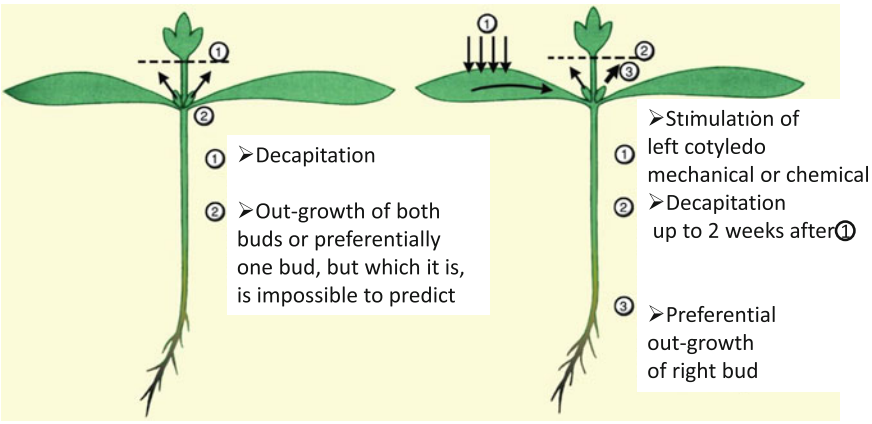


Fig. 11.7 Storage of information breaking the symmetry of lateral bud outgrowth in the axils of cotyledons of *Bidens pilosa* (L.) seedlings and its recall after decapitation

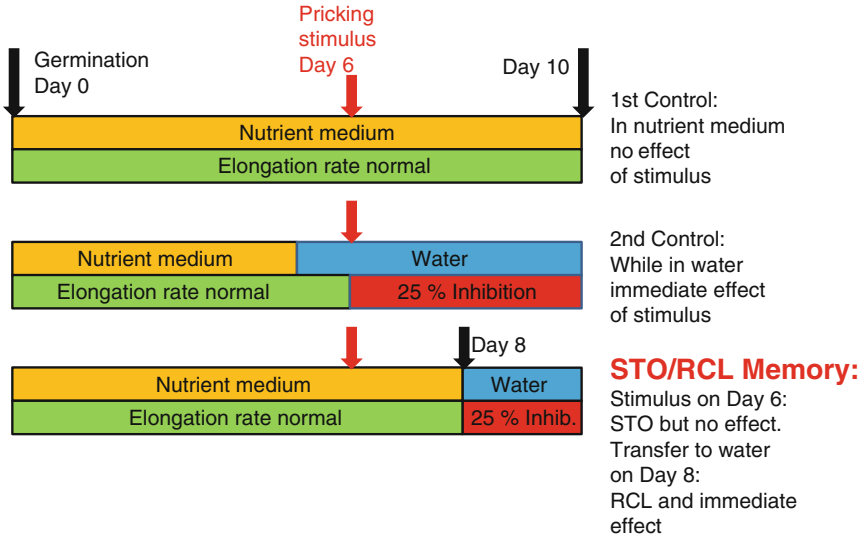


Fig. 11.8 Store/recall (STO/RCL) functions revealed by inhibition of hypocotyl elongation in *Bidens pilosa* (L.)

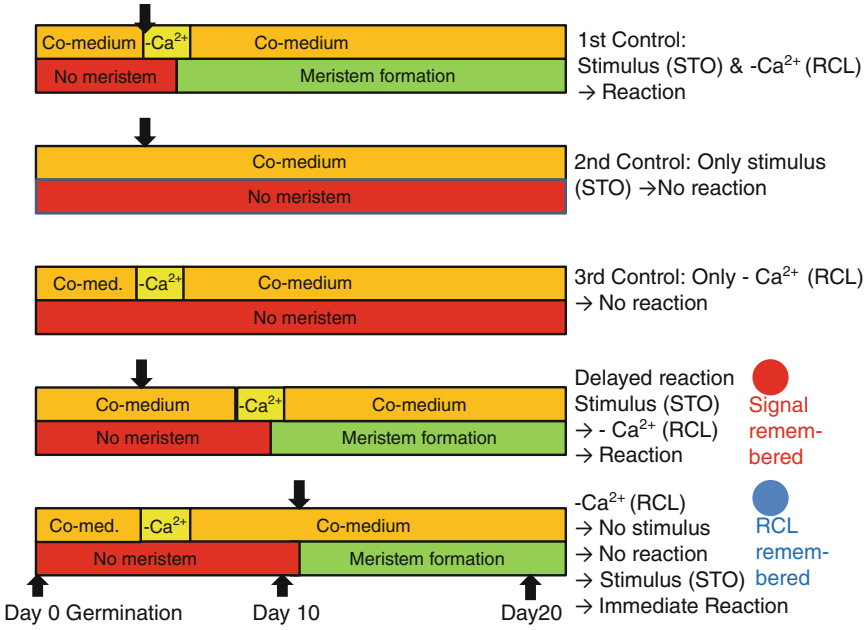


Fig. 11.9 Independent operation of store (STO) and recall (RCL) boxes shown by epidermal meristem formation in hypocotyls of flax seedlings. *Black arrows pointing downwards* stimulus by transfer to new culture medium of the seedlings. Co = control medium

by transient Ca^{2+} excess (RCL off) followed by transient Ca^{2+} removal (again RCL on). The 2nd interesting response was transiently removing Ca^{2+} (RCL on) without stimulus, and hence without response followed later by a stimulus (STO) eliciting an immediate reaction. This shows that the recall function was remembered. Again (see system I and Fig. 11.7), these experiments show that the STO and RCL boxes work independently of each other.

From the compilation of data as indicated above (Thellier and Lüttge 2013) or found in the literature, one may select a few interesting characteristics of the STO/RCL memories.

- The shape, amplitude and duration of the Ca^{2+} wave invading the cytosol immediately after stimulus perception have been found to be specific of the perceived stimulus [e.g. touch, cold shock or drought] (Dolmetsch et al. 1997; Knight et al. 1998; McAinsh and Hetherington 1998). Some of the early protein modifications (e.g. phosphorylation) and changes in genome expression are also specific of the perceived stimulus (Tafforeau et al. 2006). However, at a longer-term, it becomes impossible to find a memorized trace of the perceived stimulus. The only information that can eventually be recalled (even when recalled repetitively) is a sort of instruction controlling the final response (breaking of the symmetry of bud growth in system I, inhibition of hypocotyl growth in system II and production of epidermal meristems in system III) to be done in reaction to the perceived stimulus. Hence, it seems that the early reactions of the plant would orient the plant towards a response appropriate to the perceived stimulus. Briefly, by contrast to animals and humans that memorize facts and events, plants memorize the response to be done to stimulation.
- It has been observed with system III that the combination of several different stimuli changes the final response. This means that a STO/RCL memory can elaborate an integrated, updated response to the variety of stimuli and their fluctuations that are perceived in the course of time. This has definitely a better ecological value than memorizing the stimuli themselves and their fluctuations.
- The logic of the three types of final responses that a plant can make to stimulation then begins to get clearer. Direct, stereotyped responses are appropriate to react to rare or unknown stimuli. Responses involving priming memory permit the plant to economize producing defence substances against innocuous stimuli (familiarization) or increase the rapidity and efficacy of their reaction to harmful attacks (sensitization). Responses involving STO/RCL memory are particularly useful for acclimatizing the plant to the environmental conditions existing at the place where it has rooted. Moreover, the RCL function can serve to synchronize the use of stored information with the progress of other processes or reactions within the plant.

The functioning of plant STO/RCL memory is apparently associated with plant rhythmicity.

- With system I, the combination of an asymmetrical pricking stimulus with appropriate stimuli after varied intervals of time tends to turn the RCL box “on”

when this interval of time (h) is in the range of 0.5–2, 7–10 or 1 > 14, while it tends to turn the RCL box "off" when it is in the range of 2–4 or 10–14. The functioning of the RCL box thus is associated with an ultradian rhythm reset by the asymmetrical pricking treatment (Fig. 11.10).

- Again with system I, it was stated above that using seedlings that were asymmetrically stimulated (e.g. by pricking only one of the plant cotyledons) then decapitated sometime later at the onset of daylight, there was preferential outgrowth of the bud in the axil of the opposite cotyledon. This means that information subsequent to the asymmetrical stimulus was stored (box STO) in the plant and that, under these conditions of plant decapitation, the RCL box was "on". Now, when plant decapitation was carried out in the middle of the day both buds had equal chances to be the first to start growing, meaning that the RCL box was then switched "off" (Desbiez et al. 1986, 1991b; Thellier et al. 2013). Moreover, when decapitation was carried out at the end of the day, the RCL box tended to reopen (M. O. Desbiez, personal communication of preliminary results). The RCL box thus is clearly connected to the circadian rhythm.
- With system III (Verdus et al. 1997), when using plants all subjected to transient Ca^{2+} depletion, a very significant increase of the production of meristems has been observed to take place in the period of April to June whether these plants were stimulated or non-stimulated; however, the number of meristems produced in the stimulated plants remained at least 5 to 10 times larger in the stimulated than in the non-stimulated plants. By contrast, when using plants non-subjected to transient Ca^{2+} depletion, the number of meristems produced was always close to zero, whether the plants were stimulated or not and whatever the period of the year. It may thus be inferred that the functioning of the RCL box is somehow linked with an annual rhythm of the plants.

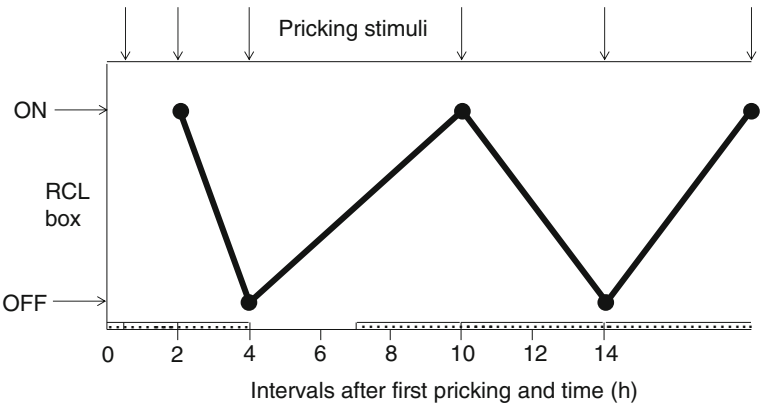


Fig. 11.10 Ultradian rhythm of switching the RCL box on and off, respectively, when pricking stimuli (*arrows* at the *upper* abscissa) are given after different time intervals (*dotted lines* at the *lower* abscissa)

In conclusion, the switching on/off (and inversely) of the RCL box thus appears to be intimately linked with all the types of rhythms of the plants. This reinforces the idea that the RCL box of the STO/RCL types of plant memory plays a part in the synchronization of the response to stimuli with the ultradian, circadian and seasonal evolutions of the metabolism and growth of the plant.

11.7 Case Study III of Systems-Theory-Modelling—Towards a Conceptual Model of Plant Memory

The data above suggest connecting memory to the biological clocks in the plants. However, basically a clock allows measuring the flow of time, but as such this is not memory. A clock becomes part of the structure and function of memory if it contains set points. An alarm clock set on a specific point in time is a reminder: it causes us to remember. An essential aspect is resetting of the clock. We experience this with our own endogenous biological clock after jetlag. We may change existing and introduce new set points of the memory and reminder functions of the clock. Resetting in general terms is the basis of new entrainment when phases of environmental rhythms have changed. Therefore, phase information genes (see Sect. 11.5.3) may constitute an important link between biological clock and memory, which are both basic building blocks in systems biology. Currently, this does not appear to be studied, but it should be a fascinating theme for future research deepening our understanding of biological systems.

We may now endeavour to draw a hypothetical, minimal model, which we may term a conceptual model, of plant memory (Thellier et al. 2013; Thellier and Lüttge 2013). Certainly, we expect that STO and RCL functions must have genetic bases, and we hypothesize the existence of STO and RCL genes. Proteome changes, i.e. up-regulation and down-regulation of the expression of genes for some proteins, and transient protein phosphorylation shortly after a stimulus are related to the STO/RCL memory (Henry-Vian et al. 1995; Tafforeau et al. 2006; Thellier 2011; Thellier et al. 2013). Studies of substrate induction of biochemical reactions in organisms led to the regulator–operator theory of gene regulation of François Jacob and Jacques Monod (Nobel Prize in 1965). Addition of the β-galactoside lactose to the prokaryotic cells of the bacterium *Escherichia coli* induced β-galactoside uptake and metabolism by inactivating a repressor. The induction was still effective, i.e. remembered, sometime after removal of the substrate until turnover produced an active repressor again and the induction was then forgotten. Glucose uptake by the eukaryotic algal cells of *Chlorella* is another example of substrate induction stable or memorized for more than 10 h after substrate removal (Tanner et al. 1970).

The genetically best studied genetic basis of memory functions is that of the epigenetic memory (Thellier and Lüttge 2013). Molecular epigenetics is a system of reading the genetic information of DNA. The molecular mechanism of epigenetic regulation is based on modifications of structure and conformation properties of

DNA and nucleosomal histones in the chromatin and changed acetylation/methylation equilibria (Zhang and Reinberg 2011; Yaish et al. 2011). Methylation leads to silencing of the genes (Chinnusamy and Zhu 2009). It controls biological functions of genes (Zhang et al. 2006). Epigenetic modifications are not associated with changes in DNA sequences. They can be triggered by the input of environmental cues, and they can be stable during cycles of mitosis and even meiosis. This means that there is epigenetic memory even through generations (Bird 2002; Hauser et al. 2011; Bilichak et al. 2012). From this, a minimal model of epigenetic memory can be derived as shown in Fig. 11.11 (Thellier and Lüttge 2013). Stimuli and environmental cues via various signals lead to modification of histones and chromatin and changed methylation/demethylation states. The methylation/demethylation state is then transmitted during mitotic or meiotic cell divisions. Alternatively, the histone/chromatin conformation is transmitted which then is followed by changed methylation/demethylation equilibria.

It is one of the basic approaches of systems biology to integrate modules into networks to study the emergent properties and to produce models for simulations (Lüttge 2012). With the modules of plant memory described above, a hypothetical minimal model can be built (Thellier and Lüttge 2013; Fig. 11.12). Separate STO and RCL genes are responsible for the separate functions of the STO/RCL memory. Stimuli and environmental cues via various signals and effectors, via the biological clock or via the epigenetic memory, unlock locked genes of the priming or the STO/RCL memory. Ca^{2+} waves (Berridge et al. 1998) derived from a

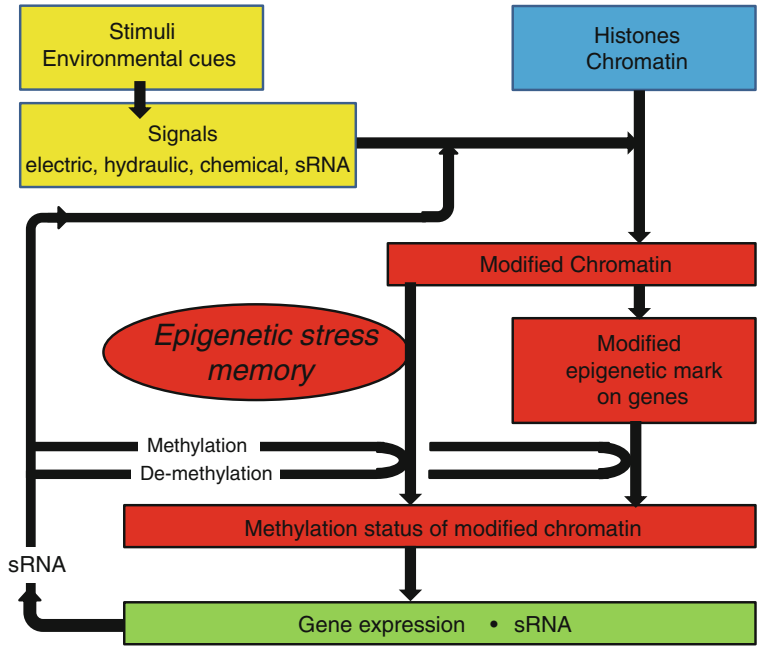


Fig. 11.11 Minimal model of the epigenetic stress memory

threshold-dependent Ca^{2+} condensation/decondensation on fixed negative charges and not obeying the mass-action law may be involved in activating the RCL-genes (Verdus et al. 2007; Ripoll et al. 2009; Thellier and Lüttge 2013; Thellier et al. 2013). The RCL-gene products affect the activation of the STO-genes. The STO-gene products are steering the processes of life such as those described above, i.e. growth (e.g. hypocotyl elongation, bud outgrowth), development (e.g. meristem formation) and differentiation.

The diverse observations on plant memory discussed in the previous section could in principle serve as a starting point for a mathematical model incorporating the notion of plant memory. Starting from the conceptual model of plant memory, it is interesting to try to elaborate a more mathematical model to become able to make quantitative predictions that can be tested experimentally. Several approaches have already been followed (Kergosien et al. 1979; Desbiez et al. 1994; Demongeot et al. 2000, 2006; Thellier et al. 2004). However, two challenges are the existence of multiple timescales and the requirement to go beyond a single type of biological processes (e.g. by including epigenetic signals, in addition to standard signal transduction and transcriptional regulation). Here, the diverse theoretical literature on modelling memory phenomena (Henke 2010), and multiscale phenomena (Twycross et al. 2010), as well as the progress in understanding epigenetic signals (Bonasio et al. 2010), might provide some guidelines in this direction.

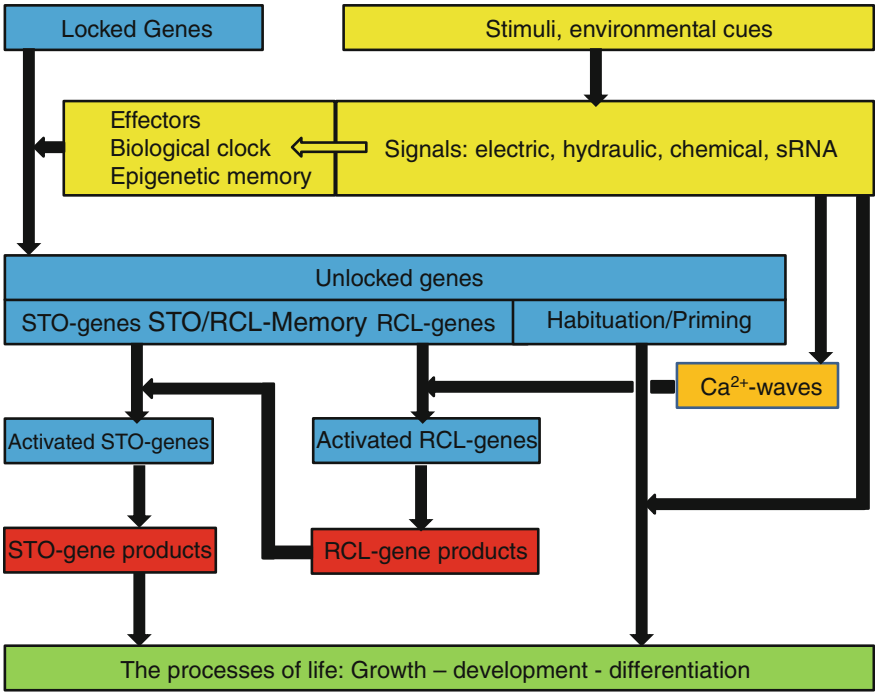


Fig. 11.12 Minimal model of the habituation/priming and store/recall (STO/RCL) memories of plants

11.8 A Path Towards Systems Biology

Systems biology has diverse subfoci, e.g. the formulation of minimal models (strongly influenced by statistical physics, theoretical biology and nonlinear dynamics), the development of detailed process ("in silico") models (inspired by engineering) and the integration and interpretation of high-throughput (multi-"omics") data (at the interface to bioinformatics). In plant biology, the systems view has by now established itself as an important research focus (see, e.g. Liu et al. 2010; Lucas et al. 2011; McClung and Gutiérrez 2010; Collakova et al. 2012; Bassel et al. 2012). Recently, this trend has been emphasized by two striking developments in the systems biology of *Arabidopsis thaliana*: the formulation of a compartmentalized metabolic model (Mintz-Oron et al. 2012) and the publication of a multiscale digital model capable of reproducing the effects of environmental regulators on *Arabidopsis* growth (Chew et al. 2014). Genome-scale metabolic models in plant science have also been reviewed in Collakova et al. (2012). In Liu et al. (2010) some recent progress in modelling of metabolism, growth, signalling and circadian rhythms in plant cells is summarized. How interaction networks can facilitate a systems-level view on many organizational scales in plant science is described in Lucas et al. (2011).

What are the origins of the high complexity of biorhythmicity when viewing it as an inherent property of systems? 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 feedback 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!

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. Initially, this 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 maximal model (Sect. 11.2). Subsequently, however, 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. The process of integrating system-wide information from several scales is the core competence of systems biology. In terms of a slightly narrower definition based on (computational) systems biology, one needs to understand the step from laboratory experiments (either for living organisms, in vivo; or for 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 high 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 within which systems 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 incorporated 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 synchronize 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 (Lüttge 2003a). Generally, 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 timescales 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 timescales, again we may return to stomata. Synchronization/desynchronization, of course, is involved in stomatal non-patchiness/patchiness (see Sect. 11.3 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 synchronous oscillations in net gas exchange (H_2O and CO_2) which changed into non-synchronous oscillations due to slight local variations in the period. Obviously, oscillations in net gas exchange eventually disappeared 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. 11.3; 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). Leaf cells of the CAM plant *Kalanchoë daigremontiana* oscillating in phase at high internal CO_2 levels desynchronize when internal CO_2 levels decrease (Rascher et al. 2001). When oscillations are desynchronized above an upper temperature threshold and leaves show overt arrhythmicity abrupt but not gradual reduction of temperature below the threshold triggers synchronization and overt rhythmicity (Rascher et al. 1998, see also above Sect. 11.3).

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 11.13 shows the "generator" of our periodic signal, which will be the focus of our discussion. A point moves with uniform (angular) velocity along a circle of radius r in one 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

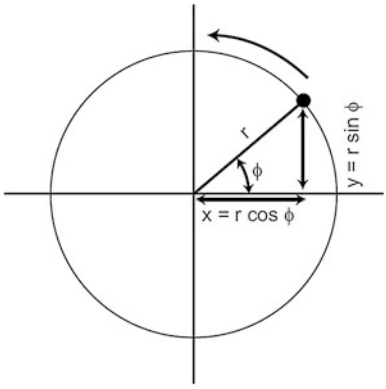


Fig. 11.13 Concept of a phase oscillator: schematic representation of a uniformly rotating point on a circle, together with the position given in Cartesian coordinates (x, y) and polar coordinates (r, ϕ; adapted from Hütt and Dehnert 2006)

$$\frac{\mathrm{d}\phi}{\mathrm{d}t} = \text{const} = \omega \tag{11.2}$$

This simple system is called a phase oscillator. Here, the parameter ω is the eigenfrequency of this oscillator. A highly non-trivial 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

$$\frac{\mathrm{d}\phi_1}{\mathrm{d}t} = \omega_1 + \varepsilon \sin(\phi_2 - \phi_1), \quad \frac{\mathrm{d}\phi_2}{\mathrm{d}t} = \omega_2 + \varepsilon \sin(\phi_1 - \phi_2) \tag{11.3}$$

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 11.14 shows the phase *difference* $\Delta\phi = \phi_1 - \phi_2$ at fixed 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 crosstalk 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. 11.14, one can visualize this process by analysing 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 11.15a 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. (11.3), 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.

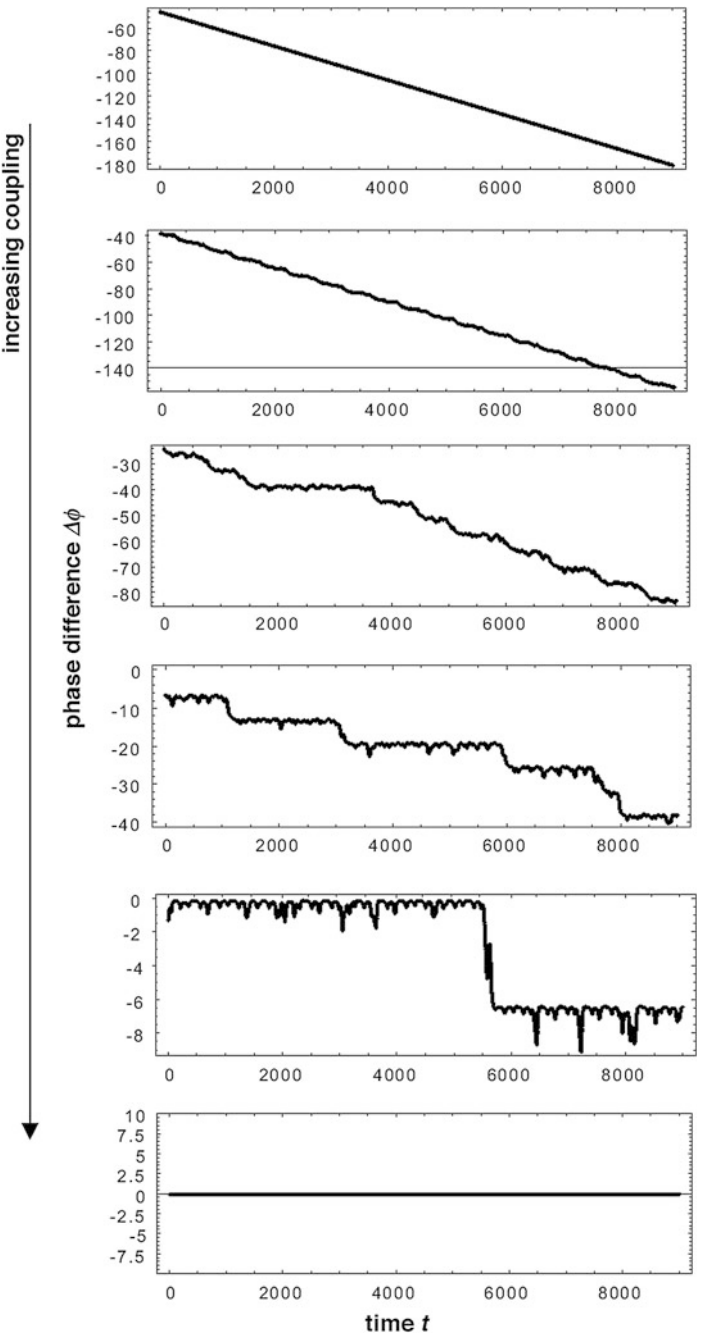


Fig. 11.14 Phase difference $\Delta\phi$ as a function of time t at different values of the coupling strength ε for a system of two coupled phase oscillators. The eigenfrequencies are $\omega_1 = -0.7$ and $\omega_2 = -0.4$. Coupling strength ε has been increased from $\varepsilon = 0$ (*top*) to $\varepsilon = 1.0$ (*bottom*) in steps of 0.2 (adapted from Hütt and Dehnert 2006)

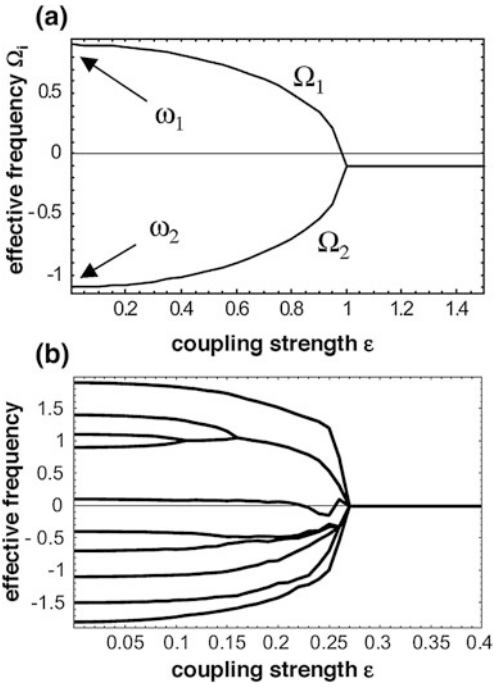


Fig. 11.15 **a** Effective frequencies Ω_1 and Ω_2 as a function of the coupling strength ε for the system of two coupled phase oscillators from Fig. 11.14. **b** Same as (**a**) but for ten coupled phase oscillators (adapted from Hütt and Dehnert 2006)

In a similar form, we can now discuss a larger set of phase oscillators. In Fig. 11.15b, the effective frequencies for a group of ten such oscillators are shown as a function of the coupling strength ε. A route towards complete synchronization paved by a stepwise 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.

11.9 Conclusions

Among the three empirical examples chosen in our test scenario for plant systems biology two are directly displaying rhythmicity, namely CAM and stomatal patterns, while the third is intimately coupled with rhythmicity, namely memory. The concept of systems biology first emerged from technical advances which allowed analysing completely or almost completely all genes (genomics), all transcripts (transcriptomics), all metabolites (metabolomics), etc. of living systems. It then soon turned out that it was insufficient to know all those building blocks and that for understanding systems in addition all functions needed to be assessed. Progressively, this is still seen to be unsatisfactory because building-blocks and functions are nested in networks which always show nonlinear behaviour. This is particularly obvious where rhythmicity is involved, such as the ultradian, circadian and annual oscillations borne out by the case studies considered in this chapter. Such oscillations are intrinsically nonlinear phenomena. This requires searching for a new path towards systems biology where the three legs of the tripod—experimentation, modelling, theory—interact intimately and where coupling and synchronization of phase oscillators within networks allow to understand why the nonlinear functions of the biological systems with their enormous complexity are sustained.

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Chapter 12
Emergent Oscillatory Properties in Modelling Ion Transport of Guard Cells

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Abstract Transport across eukaryotic cell membranes, including those of plant cells, is so complex that it defies intuitive understanding. Quantitative systems modelling is essential in these circumstances, to test our understanding of the system through the experimental scrutiny of predictions. OnGuard is the first, fully integrated and quantitative modelling package developed for the study of cellular homoeostasis in guard cells and its relevance to stomatal aperture dynamics. It offers a unique tool for exploring the properties arising from interactions between plasma membrane and tonoplast transporters with the processes involved in the control of pH, ionic and neutral osmolite concentrations, membrane potentials and ion buffering in cytoplasm and vacuole. OnGuard has already yielded detail sufficient to guide phenotypic and mutational studies, opening the way towards 'reverse-engineering' of stomatal guard cells with the aim of improving water-use efficiency. We focus here on the fundamental insights OnGuard models offer for understanding the physiological phenomena of oscillations in membrane voltage, cytosolic-free Ca^{2+} concentration, and their roles in stomatal movements. OnGuard models faithfully reproduce differences in stomatal closure with oscillation

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frequency, much as observed in vivo and including an optimal cycle period. Analysis shows that these oscillations arise from the balance of transport activities at the plasma membrane and tonoplast to generate a range of resonant frequencies. The lowest frequencies are of sufficient duration to permit substantial changes in cytosolic-free Ca^{2+} concentration. Thus, we demonstrate the oscillations as an emergent property of the system of transport at the two dominant membranes of the guard cell.

12.1 Introduction

Our intuitive understanding of cellular homoeostasis is hampered by the complexity of the interactions between all the relevant transport, metabolic and buffering components active in the different cellular compartments. Handling this level of complexity demands an integrative computational framework from which the dynamic behaviour of the cell in physiological and experimental conditions may be derived from a full and quantitative accounting of each of the contributing process. A substantial body of data now exists enabling integrated modelling of cellular homoeostasis in many eukaryotic cells. The physico-chemical principles that govern and connect this information have been developed and successfully implemented in a variety of cell systems in recent decades (Lew et al. 1979; Lew and Bookchin 1986; Mauritz et al. 2009; Hills et al. 2012).

The critical components in all these models are the primary ion and solute transporters of the cell membranes, encoded by unique sets of kinetic and regulatory descriptors. Every transport process that carries charge across a membrane will affect—and will be affected by—the voltage across that membrane. In some cases, this relationship is a consequence of mass action and the movement of the charged ions it carries; in others, it reflects direct effects of voltage and ion concentrations on regulatory sites on the channel. In either case, we are forced to consider voltage and solute concentration as both substrates and products of transport. Furthermore, the voltage—and often the ion concentration(s)—is shared between all of the charge-carrying transporters at the membrane, and the dependencies on voltage of the individual transporters are frequently nonlinear (Blatt et al. 1987; Blatt and Slayman 1987; Sanders 1990; Weiss 1996). Models of cellular homoeostasis in plants have been applied to validate a role for K^+ transport in phloem loading of sucrose as an 'energy reserve' (Gajdanowicz et al. 2011) and to describe oscillatory characteristics in H^+ and K^+ fluxes of roots (Shabala et al. 2006). However, the real test of any systems model is its capacity for predicting unexpected behaviours that are experimentally testable.

The Homoeostasis, Transport and Signalling (HoTSig) libraries (Hills et al. 2012) address this issue. HoTSig is built on an open, expandable structure that incorporates user-definable libraries for transporter kinetics, chemical buffering, macromolecular binding, metabolic reactions and macroscopic equations to couple

these processes to solute content, cell volume and turgor. Its open structure makes the platform adaptable to any single-cell system for which an accurate description of these macroscopic relations is known, and it offers the potential for applications to multicellular situations that are best scaled from the cellular to whole-tissue and organ structures. The OnGuard software (Chen et al. 2012; Hills et al. 2012) is the first implementation of the HoTSig libraries and is freely available at www.psrg.org.uk. OnGuard focuses on guard cell mechanics and on the control of stomatal aperture. It includes a graphical user interface for real-time monitoring of the individual transport and homoeostatic processes under simulation. A unique feature of OnGuard is its Reference State Wizard which serves as an aid to defining a starting point for experimental simulations with sensible outputs for all known variables.

OnGuard models of *Vicia* and Arabidopsis guard cells have reproduced many of the known properties of ion transport, solute content and stomatal aperture in response to well-defined experimental manipulations of these cells. They have also generated a number of unexpected and emergent outputs, notably demonstrating a homoeostatic 'communication' between the plasma membrane and tonoplast independent of ad hoc signal transduction networks, counterintuitive changes in cytosolic-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and pH over the diurnal cycle, and an exchange of vacuolar Mal^{2-} with Cl^- subject to the availability of the anion, all of which found direct support in independent experimental data (Raschke and Schnabl 1978; MacRobbie 1991, 1995, 2000, 2006; Thiel et al. 1992; Blatt and Armstrong 1993; Willmer and Fricker 1996; Frohnmeyer et al. 1998; Dodd et al. 2005, 2006). Furthermore, OnGuard has been shown to possess true predictive power. Two examples suffice here. In studies with the Arabidopsis *slac1* Cl^- channel mutant, Wang et al. (2012) found that eliminating the Cl^- channel, which greatly slows stomatal closure, also suppresses inward-rectifying K^+ channel activity, enhances outward-rectifying K^+ channel activity, and slows stomatal opening. OnGuard analysis predicted these effects to arise from anion accumulation in the mutant which in turn affects the H^+ and Ca^{2+} loads on the cytosol, elevating cytosolic pH and $[Ca^{2+}]_i$, both factors that are well known to regulate the K^+ channels. These predictions were confirmed experimentally, and redressing the balance of cytosolic pH and $[Ca^{2+}]_i$ was shown to be sufficient to restore both the K^+ currents and wild-type stomatal opening kinetics. Thus, OnGuard modelling uncovered a homoeostatic network that was unexpected and which connects three unrelated ion channels in the guard cell. In a second study, Wang et al. (2014a) addressed the question of whether guard cell function might be manipulated through genetic means to improve stomatal dynamics. This study represents a crucial step towards the use of OnGuard modelling for in silico design and reverse-engineering of guard cell physiology, notably to improve water-use efficiency during photosynthesis (Lawson et al. 2012; Blatt et al. 2014; Lawson and Blatt 2014). These studies confirmed independent and concurrent experimental work (Wang et al. 2014b) that reported no substantial effects on water-use efficiency of overexpressing either the AHA2 H^+-ATPase or the dominant K^+ channels. Instead, the modelling predicted that enhanced stomatal dynamics and gains in water-use efficiency were most likely

to be realized through modification of the voltage- and K^+-dependent gating of the K^+ channels.

We focus now on one intriguing, and as yet unresolved question concerning the role of Ca^{2+} oscillations in stomatal behaviour. The $[Ca^{2+}]_i$ of guard cells at rest is about 50–200 nM, as it is in virtually all living cells, but can be induced to rise above 1 μM—and locally, most likely above 10 μM—following stimulation (Blatt 2000). Such elevations are often short-lived, lasting for periods of tens of seconds to a few minutes, and may be periodic (McAinsh et al. 1995; Webb et al. 1996; Grabov and Blatt 1998, 1999; Staxen et al. 1999; Allen et al. 2001). These transients in $[Ca^{2+}]_i$ have frequently been associated with stomatal closure. They are understood to accelerate closure by promoting controlled, often cyclic release of K^+, Cl^- and malate (Mal^{2-}) from the guard cell (Chen et al. 2012). Here, we examine one aspect of $[Ca^{2+}]_i$ oscillations, namely how different frequencies in the oscillation might arise, what the consequences are for osmotic solute flux, and whether specific frequencies are critical for stomatal closure.

12.2 Sources of Ca^{2+} for $[Ca^{2+}]_i$ Elevation and Oscillations

A brief review of our experimental knowledge of Ca^{2+} and $[Ca^{2+}]_i$ signalling in guard cells is useful. The topic has been reviewed extensively (Blatt 2000; Hetherington and Brownlee 2004; Martinoia et al. 2007; Roelfsema and Hedrich 2010), and we make no attempt to be exhaustive in our coverage, but instead highlight elements most relevant to oscillatory behaviours. Three fundamental characteristics are prerequisite for the transience of $[Ca^{2+}]_i$ increases and their cyclic appearance: (1) Ca^{2+} flux into the cytosol must arise from at least two sources or pathways with different flux kinetics; (2) each of these pathways must incorporate mechanisms for self-limitation; and (3) each source must include mechanisms for Ca^{2+} recovery following a rise in $[Ca^{2+}]_i$. All three conditions must be met for any oscillatory behaviour in $[Ca^{2+}]_i$. Much controversy initially surrounded the origins of Ca^{2+} for $[Ca^{2+}]_i$ elevations, if only because Ca^{2+}-evoked signalling was frequently observed to be sensitive to Ca^{2+} depletion in the apoplast (De Silva et al. 1985). It is now generally accepted that guard cells draw on both Ca^{2+} from outside and on one or more endomembrane stores of Ca^{2+}. So the first of the three conditions is met. Similarly, the third condition is clearly satisfied by the distribution of energy-coupled Ca^{2+} transporters, both mediating Ca^{2+}/H^+ exchange—the so-called CAX antiporters—and the ACA Ca^{2+}-ATPases across all of the major membranes within the plant cell (Lopez-Marques et al. 2004; Pardo et al. 2006; Martinoia et al. 2007; Bonza and De Michelis 2011; Pittman 2011). Not only do these transporters provide the essential capacity for Ca^{2+} recovery but also the affinity to scavenge Ca^{2+} across a wide range of free concentrations [see Appendix 2 and Tables 1–6 of Hills et al. (2012)]. It is the second condition that has proven the more difficult to demonstrate based on the physiological and biophysical characteristics of known Ca^{2+} transport pathways in plants.

The major pathway for extracellular Ca^{2+}-entry is via Ca^{2+}-permeable channels at the plasma membrane—notably the highly Ca^{2+}-selective, hyperpolarization-activated Ca^{2+} channels of low conductance—and potentially via a number of other non-selective, Ca^{2+}- and cation-permeable channels (Very and Sentenac 2002), including several of the glutamate receptor-like channels (Qi et al. 2006; Cho et al. 2009). The hyperpolarization-activated Ca^{2+} channels mediate Ca^{2+} influx in a strongly voltage-dependent manner, especially at voltages negative of approximately −150 mV, and their activity (open probability) is suppressed as $[Ca^{2+}]_i$ rises to 1 μM and above (Grabov and Blatt 1997, 1998, 1999; Hamilton et al. 2000, 2001), thus satisfying the second condition for these channels to contribute to $[Ca^{2+}]_i$ transients and oscillations. Indeed, to date, the hyperpolarization-activated Ca^{2+} channels of guard cells are the only channels in plants known to exhibit such self-limitation.

Of interest, these Ca^{2+} channels are activated by ABA, which shifts the apparent threshold for activity to more positive voltages and, intriguingly, ABA acts on the channels even when isolated in membrane patches (Hamilton et al. 2000). These characteristics fit closely the observations that ABA affects the apparent voltage threshold for $[Ca^{2+}]_i$ elevations (Grabov and Blatt 1998). It may be that this shift in voltage sensitivity is connected to (de-)phosphorylation of the channels or proteins that associate with them. Certainly, Ca^{2+} influx is regulated by Ca^{2+}-dependent protein kinases, especially the Ca^{2+}-dependent protein kinases CPK3 and CPK6 (Mori et al. 2006). However, until the molecular identity of these Ca^{2+} channels is resolved, a clear knowledge of this regulatory process is hampered. This point not withstanding, it is clear that these channels are activated by reactive oxygen species (ROS), as their enhancement by ABA is impaired in the Arabidopsis *atrbohd* and *atrbohf* mutants which encode NADPH oxidases (Kwak et al. 2003). In a recent study, the ABA receptor quadruple mutant *pyr1/pyl1/pyl2/pyl4,* which shows a much reduced response to ABA (Park et al. 2009; Nishimura et al. 2010), was found to suppress $[Ca^{2+}]_i$ elevation in response to ABA. This impairment was linked to a loss in ABA-evoked ROS production, as adding the ROS hydrogen peroxide (H_2O_2) was sufficient to recover both enhanced activity of the Ca^{2+} channels and stomatal closure (Wang et al. 2013). Thus, at least one pathway for ABA to elevate $[Ca^{2+}]_i$ is probably via a ROS intermediate, although neither these studies, nor the earlier work with the *atrbohd* and *atrbohf* mutants, distinguish between the roles for ROS as a primary intermediate and as a 'permissive' factor.

Until recently, it was less obvious what the contributions are from Ca^{2+}-permeable but non-selective cation channels and their regulation. Two genes, *OSCA1* (Yuan et al. 2014) and its close homologue *AtCSC1* (Hou et al. 2014), are now known to encode such non-selective cation channels at the guard cell plasma membrane, and both show significant permeability to Ca^{2+}. The *OSCA1* mutant is impaired in Ca^{2+} signalling in response to hyperosmotic stress, suggesting that OSCA1 is likely to be important for $[Ca^{2+}]_i$ increases associated with mechanical or osmotically induced stress at the plasma membrane. However, the mutant was

found to respond normally to ROS (H_2O_2) treatments, and it showed a normal ABA response in stomatal closure. Furthermore, no evidence of self-limitation has come to light for OSCA1 as yet. These observations underscore the specificity of stimulus-specific Ca^{2+} signalling pathways in $[Ca^{2+}]_i$ elevation. They also suggest that these channels are unlikely to contribute to cyclic elevations in $[Ca^{2+}]_i$.

Calcium entry is important to trigger increases in $[Ca^{2+}]_i$, but best estimates (Chen et al. 2012) suggest that Ca^{2+} from outside accounts for some 5 % or less of the total Ca^{2+} entering the cytosol during $[Ca^{2+}]_i$ transients. The bulk of the cation flux comes from intracellular stores that are sequestered within endomembrane compartments. Like animals (Bezprozvanny et al. 1991; Hille 2001), guard cells clearly incorporate endomembrane channels for Ca^{2+} release. Furthermore, there is unequivocal evidence for Ca^{2+} release as $[Ca^{2+}]_i$ rises with Ca^{2+} influx across the plasma membrane (Grabov and Blatt 1998, 1999), a process often identified as Ca^{2+}-induced Ca^{2+} release (CICR). Channels that respond to cytosolic Ca^{2+} and ligands, including IP_3, cyclic ADP-ribose (cADPR), nitric oxide (NO) and inositol hexakisphosphate (IP_6), are known or have been implicated as Ca^{2+} flux pathways at the endoplasmic reticulum and tonoplast of plant cell types, including those of *Vicia* guard cells (Alexandre et al. 1990; Muir and Sanders 1996; Wu et al. 1997; Leckie et al. 1998; Grabov and Blatt 1999; Garcia-Mata et al. 2003; Lemtiri-Chlieh et al. 2003). Again, the prerequisite for self-limitation implies that the predominant pathways for Ca^{2+} release, like the animal counterparts, should show a Ca^{2+}-dependence that leads to flux suppression at elevated $[Ca^{2+}]_i$. It is of interest, therefore, that none of the pathways for Ca^{2+} release identified at a molecular level to date satisfy this second condition for $[Ca^{2+}]_i$ oscillations.

Consider, for example, the slow-vacuolar (SV) channels that have been identified with the *TPC1* gene product in Arabidopsis (Peiter et al. 2005). SV channels are well known as a Ca^{2+}- and K^+-permeable pathway (Peiter et al. 2005; dacz-Narloch et al. 2011) and have been repeatedly proposed as an important Ca^{2+} release pathway. Arguments for its potential regulation by dephosphorylation (Allen and Sanders 1995) not withstanding, however, SV channels in general are unsuited to the task of physiological Ca^{2+} release precisely because their Ca^{2+}-dependence is not linked to $[Ca^{2+}]_i$ nor is there evidence of suppression when $[Ca^{2+}]_i$ is elevated. Other endomembrane stores, including mitochondria and chloroplasts, may contribute as sources of Ca^{2+}, subject to their capacities both for Ca^{2+} sequestration and its release (Subbaiah et al. 1998; Coelho et al. 2002; Loro et al. 2012). However, their dominance in any process of CICR will be defined by the ability for elevated $[Ca^{2+}]_i$ to suppress the activities of the associated Ca^{2+} release pathways. Indeed, we predicted before (Chen et al. 2012) that one or more channels eventually will surface exhibiting these characteristics and consequently have used our knowledge of $[Ca^{2+}]_i$ dynamics to 'reverse-engineer' a hypothetical Ca^{2+} release channel in our models with the characteristics of the animal IP_3 and ryanodine receptor channels essential for self-limitiation.

12.3 $[Ca^{2+}]_i$ Control of Osmotic Solute Flux

Although a $[Ca^{2+}]_i$ increase has not always been observed with stomatal closure [cf. Gilroy et al. (1991); Armstrong et al. (1995); Romano et al. (2000); also discussion by Lemtiri-Chlieh and MacRobbie (1994)], the consensus is that its elevation is important to accelerate closure in the face of biotic and abiotic stress. For transport across the plasma membrane, the primary effects of $[Ca^{2+}]_i$ elevation are to suppress current through the inward-rectifying K^+ channels and the H^+-ATPase, and to enhance the activity of anion channels mediating Cl^- and Mal^{2-} efflux (Schroeder and Hagiwara 1989; Blatt et al. 1990, 2007; Kinoshita et al. 1995; Blatt 2000; Chen et al. 2010; Roelfsema and Hedrich 2010). At the tonoplast, increasing $[Ca^{2+}]_i$ suppresses H^+ transport via the K^+-activated pyrophosphatase (Darley et al. 1998) and the FV-type K^+ channels, and it activates CAX Ca^{2+}/H^+ exchange (Pittman 2011), the TPK1 K^+ channel (Gobert et al. 2007), the K^+- and Ca^{2+}-permeable TPC1 channel and vacuolar anion channels (Pei et al. 1996, 1999; Beyhl et al. 2009; Rienmuller et al. 2010). Of course, raising $[Ca^{2+}]_i$ also enhances Ca^{2+}-ATPases at both membranes to drive Ca^{2+} out of the cytosol (Geisler et al. 2000; Sze et al. 2000; Bonza and De Michelis 2011; Pittman 2011). Thus, the overall effects of raising $[Ca^{2+}]_i$ are to reduce direct, metabolically driven H^+ transport energizing both membranes and to increase their conductances for net export of K^+, Cl^- and Mal^{2-} from the vacuole, through the cytosol, and out across the plasma membrane. The exceptions of the inward-rectifying K^+ channels at the plasma membrane and the FV channels at the tonoplast also fit with this pattern, as both normally contribute to K^+ uptake rather than its release from the guard cells. In short, elevated $[Ca^{2+}]_i$ adjusts the balance of much of transport across both membranes to enhance the capacity for solute efflux from the guard cell and, at the same time, it reduces the energization of both membranes that might otherwise counter this efflux. There remain a smaller number of transporters that are demonstrably $[Ca^{2+}]_i$-insensitive or are anticipated to be so, notably the outward-rectifying K^+ channels and H^+-coupled transporters at the plasma membrane and the V-type H^+-ATPase and CLC Cl^-/H^+ antiporters at the tonoplast. These transporters are either regulated by the parallel signalling pathway of cytosolic pH or under direct substrate control of the H^+ concentration. They are also controlled directly by membrane voltage. An exhaustive list of the relevant kinetic properties and $[Ca^{2+}]_i$ sensitivities for all of these transporters will be found in Appendix 2 and Supplemental Tables 1–6 of Hills et al. (2012).

12.4 The Importance of Voltage Control

Membrane voltage, especially across the plasma membrane, is a major factor determining $[Ca^{2+}]_i$ at rest, during transient elevations and its recovery. Clearly, voltage exerts its control on Ca^{2+} entry by moderating the open probability of the hyperpolarization-activated Ca^{2+} channels and, hence, the Ca^{2+} influx they will

facilitate. Hamilton et al. (2000, 2001) reported an apparent gating charge—that is, voltage sensitivity—near unity, implying a twofold increase in Ca^{2+} permeability with each −18 mV shift in voltage. The result is to impose a substantial kinetic constraint on the rate of $[Ca^{2+}]_i$ rise in vivo that is related directly to the prevailing voltage and to the chemical driving force for Ca^{2+} entry (Grabov and Blatt 1998). Voltage will also exert control on Ca^{2+} efflux through the ACA Ca^{2+}-ATPases at this membrane, but antiparallel to its effect on the Ca^{2+} channels. These pumps almost certainly couple charge (Ca^{2+}) flux with ATP hydrolysis in a 2:1 ratio (Geisler et al. 2000; Sze et al. 2000; Bonza and De Michelis 2011), implying a reversal voltage near −200 mV at resting $[Ca^{2+}]_i$ with 1 mM Ca^{2+} outside and a significant kinetic enhancement as the membrane depolarizes from −150 mV towards voltages near 0 mV (Hills et al. 2012). Thus, voltage transitions across the physiological range from −200 to −50 mV can be expected to bias the plasma membrane alternately for Ca^{2+} influx and $[Ca^{2+}]_i$ elevation on the one hand and for Ca^{2+} efflux and $[Ca^{2+}]_i$ recovery on the other. Indeed, this is precisely what has been observed in the intact guard cell (Grabov and Blatt 1998; Hamilton et al. 2000; Garcia-Mata et al. 2003), and the effect is demonstrably coupled through endomembrane Ca^{2+} release (Grabov and Blatt 1999).

Equally, voltage exerts a primary control on osmotic solute flux across the guard cell plasma membrane, both as a driving force for transport and as a regulatory factor affecting the gating of several ion channels that contribute to these fluxes. K^+ and Cl^- (anion) channels at plasma membrane. These transporters dominate trans-membrane solute flux in many circumstances (Willmer and Fricker 1996; Blatt 2000; Blatt et al. 2007; Pandey et al. 2007; Melotto et al. 2008; Ward et al. 2009; Lawson and Blatt 2014) and are complemented by H^+-coupled transport of K^+ (Blatt 1988) and, undoubtedly, of Cl^- as well as malate (Mal^{2-}) (Barbier-Brygoo et al. 2011; Chen et al. 2012; Hills et al. 2012). They facilitate K^+ and anion uptake for stomatal opening and their efflux to the apoplast for stomatal closing. What determines the bias between these solute fluxes is largely dependent on energization of the membrane and H^+-ATPase activity. Indeed, guard cells, such as many plant cells studied to date, show two states of the membrane voltage. One state is characterized by voltages close to the K^+ equilibrium voltage (E_K), with a conductance that is dominated by currents through the GORK K^+ channel in Arabidopsis (Hosy et al. 2003; Eisenach et al. 2014) and similar outward-rectifying K^+ channels ($I_{K,out}$) (Blatt and Armstrong 1993; Blatt and Thiel 1994; Blatt and Gradmann 1997) and by the anion current (I_{Cl}) that in Arabidopsis is characterized by SLAC1 and its homologous (Negi et al. 2008; Vahisalu et al. 2008; Geiger et al. 2009; Wang et al. 2012). This state is typical of the guard cell during solute loss for stomatal closure. The second state is characterized by voltages well-negative of E_K with a conductance that derives from the activities of the AHA H^+-ATPases (Lohse and Hedrich 1992; Thiel et al. 1992; Gradmann et al. 1993), primarily AHA1 and AHA2 (Merlot et al. 2007; Blatt et al. 2014), and by inward-rectifying K^+ channel current ($I_{K,in}$) including that through the channels KAT1 and KAT2 (Nakamura et al. 1995; Pilot et al. 2001). This second state reflects a bias for K^+ and Cl^- (anion) accumulation and is typical of the guard cell during stomatal opening. So, much as it affects Ca^{2+} flux and $[Ca^{2+}]_i$

dynamics, transitions in plasma membrane voltage across the same physiological range generally define whether the guard cell is actively accumulating solute or losing it. Again, such oscillations in voltage are known and have been associated with stimuli that trigger the transition from open to closed stomata (Blatt and Armstrong 1993; Gradmann et al. 1993; Blatt and Thiel 1994).

In short, plasma membrane voltage is a common denominator that connects $[Ca^{2+}]_i$ and the osmotic solute fluxes that drive stomatal movements within an elegant counterpoint. At its negative extreme, voltage favours K^+ and anion uptake but also triggers Ca^{2+} entry to elevate $[Ca^{2+}]_i$; at its positive extreme, voltage activates the channels mediating K^+ and anion efflux as well as engaging Ca^{2+}-ATPases to restore $[Ca^{2+}]_i$ to its resting level. Blatt (2000) originally described this relationship as a 'signalling cassette' in four steps which can now be updated with new kinetic detail pertaining to the H^+-ATPase (Merlot et al. 2007) and to the anion channels (Chen et al. 2010): (1) with resting $[Ca^{2+}]_i$ low, negative membrane voltage triggers Ca^{2+} influx across the plasma membrane, stimulating intracellular Ca^{2+} release to elevate $[Ca^{2+}]_i$; (2) the rise in $[Ca^{2+}]_i$ inactivates $I_{K,in}$, the Ca^{2+} channels, and it inactivates the H^+-ATPase and activates I_{Cl} to promote membrane depolarization; (3) depolarization favours K^+ and Cl^- efflux and, with the Ca^{2+} influx suppressed, permits the Ca^{2+}-ATPases and CAX transporters to resequester Ca^{2+} and restore $[Ca^{2+}]_i$; and finally, (4) with a falling $[Ca^{2+}]_i$, I_{Cl} declines, $I_{K,in}$ and the H^+-ATPases recover sufficiently to repolarize the membrane and enable K^+ and H^+-coupled anion uptake. At the core of this process is the intrinsic feedback of membrane transporters operating across a common membrane, and so sharing the common electrical driver of membrane voltage; it also incorporates a second feedback loop of $[Ca^{2+}]_i$-dependent controls on several of the major transporters that dominate osmotic solute flux and on the H^+-ATPase, not just on the Ca^{2+} channels at the plasma membrane and those predicted to associate with endomembrane stores. The overall consequence is that voltage and $[Ca^{2+}]_i$ oscillate in a syncopated rhythm with K^+ and anion flux, solute content and stomatal aperture.

12.5 An Oscillatory Optimum for Stomatal Response

However intuitively the preceding description may appear, it hides much subtlety in the oscillatory behaviour of guard cells. In the simplest sense, we might anticipate, for example, that the time-averaged ratio of residence in the hyper- and depolarized states should determine whether the guard cell shows a net uptake or loss in solute. In other words, averaged over a period of 30 min, three cycles of $[Ca^{2+}]_i$ elevation—and membrane depolarization—totalling a period of 24 min should promote solute loss and stomatal closure; similar cycles totalling a period of 15 min of $[Ca^{2+}]_i$ elevation and membrane depolarization should have no net effect on solute content or aperture; and, with a total of 6 min $[Ca^{2+}]_i$ elevation and depolarization, the solute content and aperture should increase. To an extent, this pattern has been found to hold true. Allen et al. (2001) imposed oscillations in $[Ca^{2+}]_i$ and found that

increasing the period of its elevation promoted stomatal closure. Intriguingly, however, the most effective cycle period was of 10 min with $[Ca^{2+}]_i$ elevations of 5 min interspersed with 5-min recovery periods. Shorter recovery periods were less effective, whether they were interspersed with the same or with longer periods in $[Ca^{2+}]_i$ elevation.

Interpreting these observations is complicated by the use of external buffers that varied both the Ca^+ and K^+ concentrations. Not only would elevated K^+ outside have affected membrane voltage, but it would also have greatly increased K^+ availability, the associated K^+ conductance (Blatt 1987; Schroeder 1988; Blatt 1992) and the rates of its uptake (Willmer and Fricker 1996). Even so, that there should exist an optimal $[Ca^{2+}]_i$ oscillation frequency for stomatal closure is not intuitively obvious, and it underscores two points. First, coupling between $[Ca^{2+}]_i$, voltage and osmotic solute flux is not linear. Indeed, this nonlinearity is amply demonstrated in the voltage dependence of the component currents, as we have noted above, but its consequences are less obvious. Second, the response of solute flux to $[Ca^{2+}]_i$ and voltage depends on the recent history of both, in other words solute flux almost certainly relaxes with time following transitions in $[Ca^{2+}]_i$ and voltage. This second point almost certainly relates to emergent properties that arise from the interactions between the different transporters, and this interpretation makes sense in context of related studies of the *det3* mutant (Allen et al. 2000). The *det3* mutation, which suppresses the vacuolar H^+-ATPase, showed prolonged $[Ca^{2+}]_i$ increases without oscillation and reduced stomatal closure in the face of high Ca^{2+} outside. Thus, both observations beg the question of what we can learn from quantitative systems modelling of these oscillations.

12.6 Modelling Oscillations and Stomatal Responsiveness

Detailed information is available on the construction of the *Vicia* and *Arabidopsis* models (Chen et al. 2012; Hills et al. 2012; Wang et al. 2012), including a complete listing of the transporters and their characteristics incorporated in these models [see Appendix 2 and Supplemental Tables 1–6 in Hills et al. (2012)]. Also available is a video introduction to OnGuard (www.psrg.org.uk) and a step-by-step guide to its basic application (Blatt et al. 2014). We used the Arabidopsis model with the published settings to analyse the $[Ca^{2+}]_i$ and voltage oscillations. In the model, the kinetics of primary ATP-dependent transport is coupled to fluence rate (Chen et al. 2012). Voltage and $[Ca^{2+}]_i$ oscillations arise near the end of the daylight period, shortly before and during stomatal closure, as primary transport activity declines and the accumulated energy in the various ionic gradients is released. Much of the diurnal cycle is shown in Fig. 12.1 and the times late in the daylight period are expanded in Fig. 12.2. The overviews show oscillations in voltage as they arise, and they illustrate the gradual shift over a period of 3 h as these rapid and shallow cycles first develop, then contract through accelerating oscillatory cycles, culminating with a series of long membrane depolarizations, each of several minutes' duration.

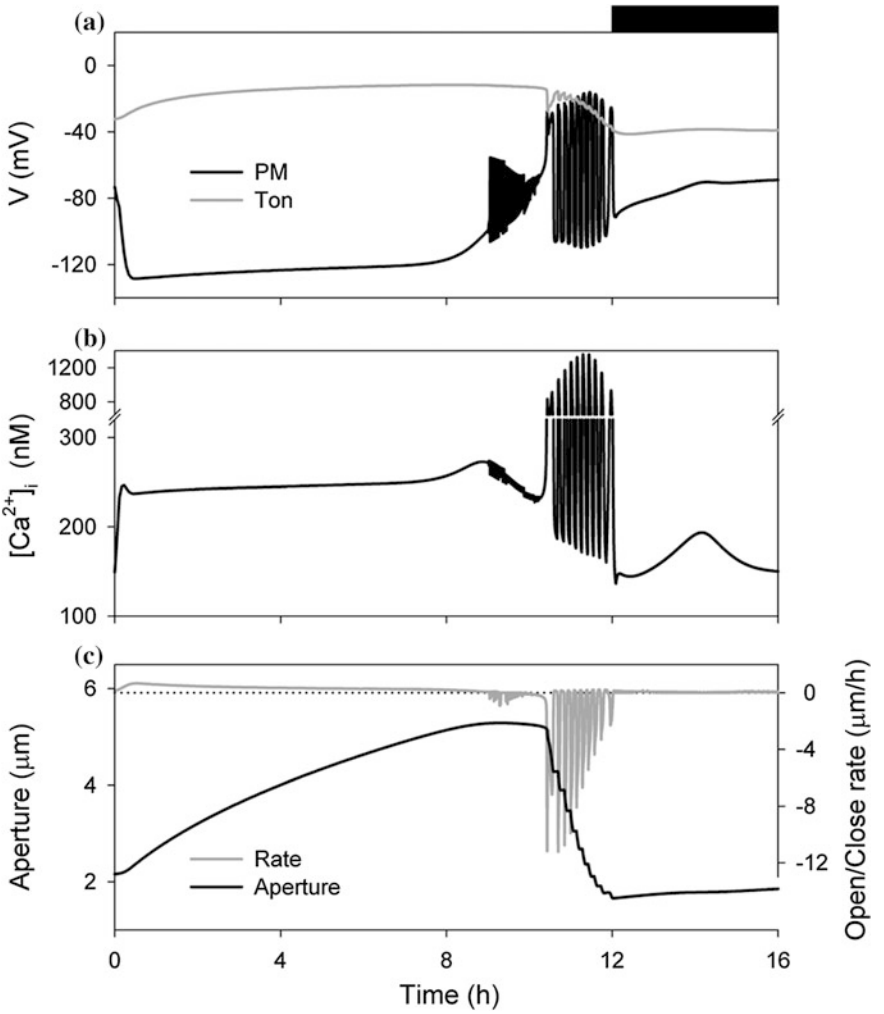

Fig. 12.1 Macrosocpic ouputs from the OnGuard model. Outputs resolved over a standard diurnal cycle (12 h light:12 h dark; *dark period* indicated by bar above) with 10 mM KCl, 1 mM $CaCl_2$ and pH 6.5 outside (Chen et al. 2012; Wang et al. 2012). Only the first 16 h of the diurnal cycle is shown. The full set of model parameters and initialising variables are listed in Wang et al. (2012) and are available with the OnGuard software at www.psrg.org.uk. Shown are **a** plasma membrane and tonoplast voltage, **b** cytosolic-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and **c** stomatal aperture and the rate of opening/closing in stomatal aperture (=ΔAperture/Δt). Note that, positive rates here indicate opening

Each of the oscillations in voltage is accompanied by comparably scaled cycles in $[Ca^{2+}]_i$. Voltage initially traverses a range of approximately 50 mV in cycles with periods near 0.3 min, these cycles decaying to nothing in ever shorter cycles over the next hour. Likewise, $[Ca^{2+}]_i$ cycles largely in phase throughout this time and its oscillations, too, relax to nothing in parallel. These oscillations and, in part those of

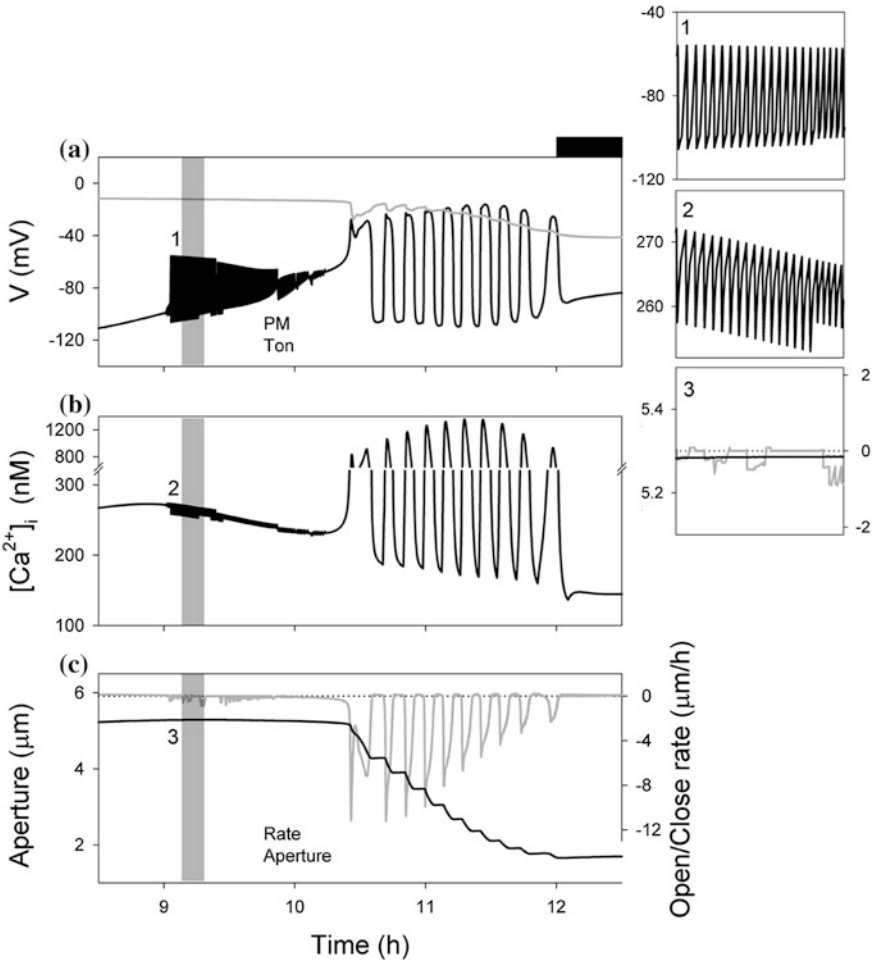

Fig. 12.2 Expanded view of the macrosocpic ouputs from the OnGuard model. Outputs as in Fig. 12.1 for the diurnal period of 8.5–12.5 h (*dark period* indicated by bar above). Shown are **a** plasma membrane and tonoplast voltage, **b** cytosolic-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and **c** stomatal aperture and the rate of opening/closing in stomatal aperture (=ΔAperture/Δt). Note that, positive rates here indicate opening. *Insets* (*right*) correspond to the period highlighted by the grey bars (9.1–9.3 h) and show the rapid cycles in voltage, $[Ca^{2+}]_i$, and the corresponding effects on the rates of opening/closing in expanded view (cross-referenced by numbers)

voltage, are clipped to give a saw-tooth pattern throughout. Similarly, the long voltage oscillations are closely matched by long cycles of $[Ca^{2+}]_i$ elevation and recovery. However, unlike the shorter cycles, the transitions in voltage appear to settle at each extreme. The accompanying $[Ca^{2+}]_i$ changes relax and, on elevation, rise and then decay as Ca^{2+} release is followed by its resequestration and export from the cytosol (Chen et al. 2012). Thus, these extended time periods are long enough to permit the rise and fall in $[Ca^{2+}]_i$ to 'catch up' with the voltage cycle.

Coupled with the long cycles in voltage and $[Ca^{2+}]_i$ are cyclic variations in the H^+-ATPase, anion and outward- and inward-rectifying K^+ currents (Chen et al. 2012). From the standpoint of total membrane current, these currents are evident in a bi-stable relationship with voltage—an N-shaped current–voltage curve with two positive inflection points on the voltage axis—and its displacement alternately above and below the voltage axis (Jack et al. 1983; Blatt and Thiel 1994). Indeed, all of the oscillatory behaviour is coupled to N-shapes in the current–voltage curve and corresponding alternations between solute uptake and efflux (Chen et al. 2012), but the effects on aperture are most obvious when the cycles are of sufficient duration. In this case, the oscillations are accompanied by substantial decreases in stomatal aperture separated by periods of little change in aperture. The effect is of stepwise decay from roughly 5.5 to 1.8 μm in aperture. What is of interest is the difference in rates of stomatal closure as the cycles in voltage and $[Ca^{2+}]_i$ expand. To illustrate this point, Fig. 12.3 summarizes the mean rates of stomatal closure as a function of oscillation period. The closure rates here are corrected for the decline in thermodynamic driving forces for K^+ and anion flux. Closure clearly follows a biphasic relation with oscillation period, showing a maximum rate near a period of 8.9 min and falling off steeply to either side of this value. Analysis (C. Minguet, Y. Wang and M.R. Blatt in preparation) shows that this maximum associates with a mean time fraction around 0.5–0.55 in the depolarized phase of the cycle—that is, closure is most rapid with a near-symmetrical cycle of elevated $[Ca^{2+}]_i$ (depolarized voltage) and resting $[Ca^{2+}]_i$ (hyperpolarized voltage)—and that the peak in closure rate corresponds directly with the maximum in the dynamic, $[Ca^{2+}]_i$-dependent suppression of the H^+-ATPase. By contrast, the same analysis shows no substantial association with the underlying activities of the Ca^{2+} channels, nor on that of the K^+ and anion channels at the plasma membrane.

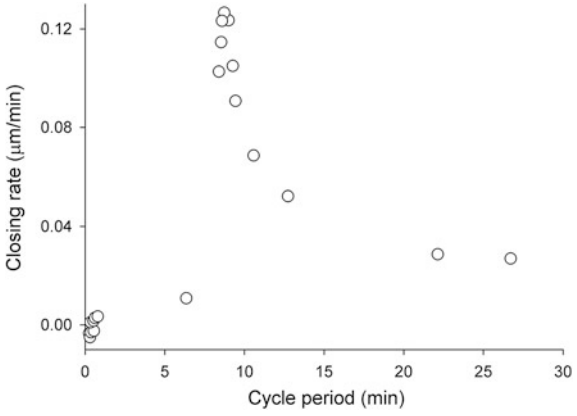

Fig. 12.3 Aperture closing rate as a function of the oscillation period in voltage and cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$). Mean aperture closing rates during each of the long oscillatory cycles and a selection of the initial rapid cycles are taken from the data of Figs. 12.1 and 12.2. Note the sharp maximum in closing rate at a period of 8.9 min (0.0019 Hz)

How, then, can we encapsulate these observations to define a role for $[Ca^{2+}]_i$ and voltage oscillations in the guard cell? Clearly, the simulations provide a close match to the behaviours observed in vivo and, thereby, support the initial perceptions of an interplay between voltage, the Ca^{2+} channels and endomembrane Ca^{2+} release in driving the $[Ca^{2+}]_i$ cycle (Blatt 2000). The OnGuard models give quantitative substance to these previous experimental findings of voltage and $[Ca^{2+}]_i$ oscillations (Grabov and Blatt 1998; Allen et al. 2001). They support the perception that the oscillations accelerate the rate of stomatal closure and that the most effective bandwidths fall within a narrow range of frequencies near 0.0019 Hz (= 8.9 min period). It is remarkable that the OnGuard models faithfully reproduce experimental data suggesting an optimal cycle period for accelerated stomatal closure. What is all the more important, therefore, is that the OnGuard analysis offers a new and truly mechanistic basis from which to understand the origins of this optimum cycle frequency. The analysis does not support the perception that this frequency is in any way unique, and this viewpoint is underscored by the range of oscillatory frequencies arising from the OnGuard models. All of these oscillations find their origins in the underlying N-shaped relationships between current and voltage which, in turn, arise from the component currents of the various charge-carrying transporters (Chen et al. 2012). Perhaps, more useful, then, is to recognize that these cycles of voltage and $[Ca^{2+}]_i$ simply reflect the several resonance frequencies that emerge from the balance of intrinsic transport activities of the guard cell. It happens that the slowest of these frequencies is sufficiently long-lived to permit substantial elevations in $[Ca^{2+}]_i$ and its recovery, and these oscillations in turn facilitate K^+, Cl^- and Mal^{2-} efflux from the guard cells.

12.7 Conclusion and Outlook

From the discussion and simulations presented here, two general conclusions can be drawn. The first—that transporters operating at one membrane will interact—is implicit to their situation, operating in parallel across a common membrane. We highlighted this point at the start of this chapter. It arises from the shared membrane voltage, as well as the common pools of ions on either side of the membrane, and it generates interactions, both direct and indirect, often between unrelated transport processes. The second observation similarly arises from the commonality of the pools of ionic species and the controls exerted by $[Ca^{2+}]_i$ and voltage. Both act on several key solute transporters at both the plasma membrane and the tonoplast, including the Ca^{2+} channels and ATPases that transport Ca^{2+} itself. The interactions of these interlinked regulatory loops, one mediated through the common voltage across the plasma membrane and the second through $[Ca^{2+}]_i$, give rise to resonance behaviours that emerge only when the system is treated as a whole. In short, we can conclude that there is nothing intrinsic to these resonance frequencies other than these interactions between these regulatory loops and their component transport processes.

What, then, can we learn that might be applied in experimentation? Clearly, one message is that 'the consequence of manipulating a single transporter at a membrane is rarely (if ever) restricted to this one process or solely to the distributions of the transported species' (Blatt et al. 2014). Inevitably, the challenge is to recognize the 'off-target' effects of experimental manipulations. These are almost always beyond intuitive grasp, and so their identification is best addressed through quantitative mathematical modelling. For work with the premier model of the guard cell, researchers and students have access to OnGuard and the models we have constructed. It is our expectation that these models will find applications in exploring many other fundamental problems that arise in studies of these cells.

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Chapter 13
Systems Biology Analysis of Changes in Potential Across Plasma Membrane: Physiological Implications

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Abstract Variation of the membrane potential difference (PD) across plasma membrane is considered in terms of one or more ion transporter populations changing their conductance and activation kinetics. Slow changes occurring over minutes can be investigated by the current voltage (*I*/*V*) technique. In some cases, data are sufficient to model electrical characteristics of each transporter population and their evolution with time. The proton pump at the plasma membrane of the salt-sensitive Characeae *Chara australis* provides an example of single transporter changing conductance against a steady background. The rise and fall in proton pump conductance may be prompted by circadian oscillations of indoleamines IAA and melatonin, measured in growing thalli of characean plants. In response to abiotic stress, two or more transporter populations change conductance and/or PD dependence. The voltage clamp to extreme negative PD levels transiently inhibits the proton pump in *C. australis*, activating H^+/OH^- channels, increasing the background conductance, and opening inward rectifier channels at more depolarized PDs. An increase in medium salinity (after pre-treatment with isotonic sorbitol medium) results in similar response, which is preceded by a typical noise in membrane PD. In salt-tolerant Characeae *Lamprothamnium* sp., increase in salinity (or osmolarity) provokes an increase in proton pumping as well as increase in background conductance and opening of the inward rectifier channels at more depolarized PDs to effect turgor regulation. The hypoosmotic turgor regulation also involves a complex interaction of several transporters, initiated by the increase of turgor pressure, $[Ca^{2+}]_{cyt}$ increase, and PD changes. A detailed modeling is in progress for most of these responses. The examples demonstrate the analytical and predictive power of the *I*/*V* methodology coupled with the systems biology modeling and monitoring of biochemical changes.

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13.1 Introduction

Ion transport across plant cell membranes is crucial in signaling, nutrient delivery, turgor pressure generation, and energy storage. Ion fluxes depend on concentration differences on each side of the membrane, the potential difference (PD) across the membrane, and the permeability of the ion channel. Ion fluxes responding to internal changes in biochemistry or external stressors often change the membrane PD. Modeling these changes reveals synergies of ion transporters that enable the plant to grow and to survive (or not) adverse conditions, such as salinity increase.

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. Channel blockers and metabolic inhibitors are often not specific, introducing artifacts. Integrative modeling deconstructs the total voltage clamp current into contributions by various transporter populations. 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.

Changes of the membrane PD imply two or more equilibrium points to which the membrane PD can be attracted. For instance, changes in a single membrane transporter can produce PD oscillations against some steady background PD and conductance level. The changes in rate of proton pumping may facilitate cell wall loosening and cell growth in response to circadian changes of auxin indole-3-acetic acid (IAA) and other indoleamines such as melatonin. In response to different types of abiotic stress, several transporters may be activated or deactivated through PD-dependent gating, $[Ca^{2+}]_{cyt}$ changes, or mechano-stimulation.

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 1986, 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.

Our experimental system is the characean plant, with comparatively simple morphology and extraordinarily large cells (Walker 1955; Hope and Walker 1975; Beilby and Casanova 2013). Phylogenetic studies (Karol et al. 2001) indicate that charophytes, containing the Characeae family, are the closest living relatives of the ancestors of all land plants. While Characeae are now thought to be more distantly related to land plants than another charophyte group Zygnematales (Wodniok et al. 2011; Timme et al. 2012), they are still positioned at the land plant origin. Consequently, the large body of electrophysiological and physiological data

provide valuable insights into many aspects of higher plants and into plant evolution (Beilby and Casanova 2013).

Our models of the proton pump under a range of conditions (Beilby 1984; Blatt et al. 1990; Beilby and Walker 1996), hyperosmotic and hypoosmotic regulation in salt-tolerant Characeae *Lamprothamnium* (Beilby and Shepherd 1996; Shepherd and Beilby 1999; Shepherd et al. 2001; Beilby and Shepherd 2001a, 2006a; Al Khazaaly and Beilby 2007), and salt stress pathology in salt-sensitive *Chara australis* (Shepherd et al. 2008; Beilby and Al Khazaaly 2009; Al Khazaaly et al. 2009; Al Khazaaly and Beilby 2012) have already provided useful analysis of mechanisms involved and feedback for experimental design.

13.2 Single Transporter Oscillations

13.2.1 Proton Pump Conductance Change

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 and export of toxic Na^+ at the time of salt stress.

The pump current, $I_{p,}$ dependence on membrane PD has been fitted by the HGSS (Hansen, Gradmann, Slayman, Sanders) model of cyclic enzyme-mediated transport (Hansen et al. 1981; Beilby 1984; Blatt et al. 1990 and references therein). In the simplest form, the number of carrier states can be reduced to two with a pair of PD-dependent constants, k_{io} and k_{oi}, across a symmetrical Eyring barrier, and PD-independent rate constants, κ_{io} and κ_{oi}. The constants κ_{io} and κ_{oi} subsume ATP, ADP, inorganic phosphate concentrations, binding and de-binding steps, and carrier recycling (Blatt et al. 1990).

$$I_p = zFN\frac{k_{io}\kappa_{oi} - k_{oi}\kappa_{io}}{k_{io} + k_{oi} + \kappa_{io} + \kappa_{oi}} \tag{13.1}$$

$$k_{io} = k_{io}^0 e^{\frac{zFV}{2RT}} \tag{13.2a}$$

$$k_{oi} = k_{oi}^0 e^{-\frac{zFV}{2RT}} \tag{13.2b}$$

F, R, and T are the Faraday constant, the gas constant, and temperature in degree Kelvin, respectively; z is the pump stoichiometry, which has been set to 1; N is a scaling factor (2×10^{-8}); k_{io}^0 and k_{oi}^0 are defined at 0 PD; and V is the PD across the membrane or membranes. The model I_p/V and G_p/V characteristics are shown in Fig. 13.1b, c.

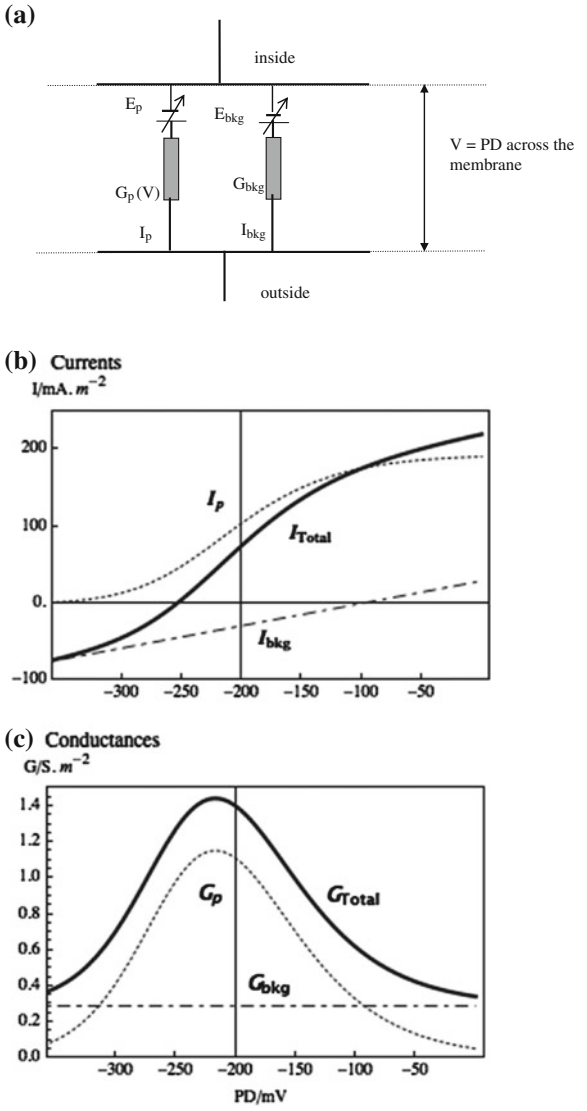


Fig. 13.1 **a** Simplified circuit of the membrane with only the background transporter and the H^+ pump. **b** Modeled total current (*continuous line*), I_p (*dotted line*) and I_{bkg} (*unequally dashed line*) as a function of the membrane PD. The parameters for the I_{bkg} are as follows: G_{bkg} = 0.29 S m^{-2} and E_{bkg} = −100 mV. The parameters for the I_p (in s^{-1}) are as follows: $k^0_{io} = 7000$, $k^0_{oi} = 0.5, \kappa_{io} = 0.5, \kappa_{oi} = 100$. **c** Conductances were obtained by differentiation of the currents (line types as in **b**)

When the pump activity is low, the PD attractor is the "background state" (see Fig. 13.1). This state exhibits reversal PD (E_{bkg}) close to −100 mV and linear current voltage (I_{bkg}/V) characteristics in the vicinity (±100 mV) of E_{bkg}. In our modeling, I_{bkg} is represented by an empirical equation with PD-independent conductance, G_{bkg}, and reversal PD, E_{bkg}, near −100 mV:

$$I_{bkg} = G_{bkg}\left(V - E_{bkg}\right) \tag{13.3}$$

There is extensive experimental evidence for the background membrane state in both *Chara* and *Lamprothamnium* spp. (Beilby 1984, 1985, 1986; Beilby and Shepherd 2001a, b). The E_{bkg} does not coincide with any ion abundant in the cell or the medium, suggesting that the channels are not very selective and may be the equivalents of NSCCs (non-selective cation channels) in higher plants (Maathuis and Sanders 2001; Tyerman 2002; Demidchik and Tester 2002, Demidchik and Maathuis 2007). Interestingly, the characean NSCCs show little permeability to K^+ (Beilby and Shepherd 2001b), but this property was also observed in some higher plant NSCCs (Demidchik and Maathuis 2007). In both salt-sensitive and salt-tolerant Characeae, the background conductance G_{bkg} increases with Na^+ concentration in the medium, while E_{bkg} remains close to −100 mV (Shepherd et al. 2008; Beilby and Shepherd 2001a, b). For non-plasmolyzing osmotic stress (for instance, 90 mM sorbitol medium, equivalent osmotically to 50 mM NaCl), G_{bkg} remains unchanged (Shepherd et al. 2008; Al Khazaaly and Beilby 2007). When salt-sensitive *C. australis* was exposed to plasmolyzing osmotic stress of 285 mM sorbitol medium, the G_{bkg} also increased and E_{bkg} often depolarized (Al Khazaaly 2011). Salt-tolerant *Lamprothamnium* cells did not show increase in G_{bkg} after osmolarity increase due to 167 mM sorbitol. Higher concentrations are yet to be tested.

In an unchanging environment, the I_{bkg}/V remains comparatively constant for each cell. The response to medium K^+ concentration or pH_o changes are also small (Beilby 1985, 1986). Further, as the linear characteristics are so different from the curved I_p/V, the current through the two types of transporters can be quite easily resolved by modeling. The changes in the rate of proton pumping are also quite distinct. The effect of the more influential parameters k^0_{io} and κ_{oi} is explored in Fig. 13.2a, c, respectively (for all the parameter values, see the captions of Figs. 13.1 and 13.2). The decrease in both parameters causes the membrane PD to depolarize toward E_{bkg}, but the shape of the I_p/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^0_{io} decrease, the conductance maximum moves to more depolarized PDs, its amplitude decreasing only slightly (Fig. 13.2b), whereas diminishing κ_{oi} leaves the conductance maximum close to the same PD level, while its amplitude becomes smaller (Fig. 13.2d). The decrease in pH_o is a good example of the k^0_{io} 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

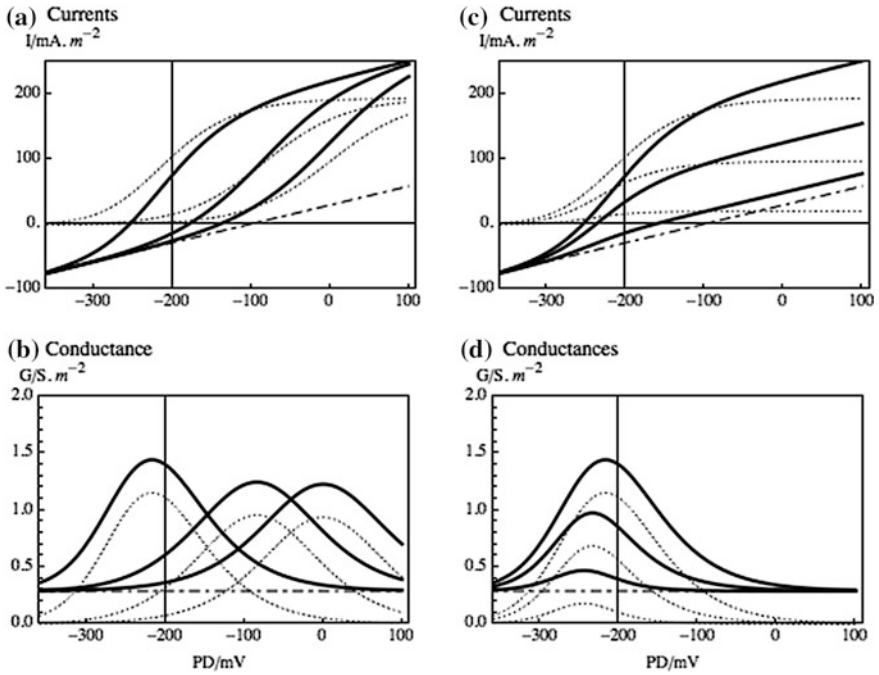


Fig. 13.2 Modeling changes in pump parameters that can produce depolarization. **a** Change in the k_{io}^{0} parameter from 7000 to 500 and to 100 s^{-1}, with I_{bkg}, the other pump parameters and the line types as in Fig. 13.1b. **b** Conductances calculated from the currents in **a**. Note the shift 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. 13.1b). **d** Conductances calculated from the currents in **c**. Note that the conductance maximum remains at the same PD level, but its magnitude diminishes

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.

13.2.2 Circadian Rhythm in Auxin IAA and Indoleamine Melatonin

In some Characeae, such as *Chara inflata* (now reclassified as *Lamprothamnium inflatum*—Casanova 2013), the pump activity increases in the daylight hours (Coleman and Findlay 1985). There might be also a seasonal switch, as *C. australis* cells in the winter months sometimes exhibit quite depolarized resting PDs (Beilby,

unpublished data). Changes in cell biochemistry probably underlay these low-frequency oscillations in resting PD.

C. australis plants, grown in the laboratory on day/night cycle of 12/12 h, exhibited a distinct peak in concentrations of auxin indole-3-acetic acid (IAA) and indoleamine melatonin (see Fig. 13.3a). The concentration changes persisted in cut explants, pretreated for 3 days in darkness (Fig. 13.3b), confirming that they are not just a reaction to light-on, but true circadian rhythms.

Circadian changes in IAA concentration were observed in higher plants (Pavlova and Krekule 1984; Jouve et al. 1999; Novakova et al. 2005). However, the concentration peak (or peaks) occurred at different times of the *day* depending on plant, tissue, or entrainment cues. Recent genetic profiling established intimate entanglement between circadian clock and auxin signaling pathways (for review, see Covington and Harmer 2007). The observed differences in concentration peak times can be explained by the evidence that plants may have independent oscillators in different parts of roots and shoots, which are out of phase and lack extensive cross talk (Cuin 2007).

Melatonin is synthesized from tryptophan via a pathway that also produces IAA in plants (Murch and Saxena 2002). Genes encoding four consecutive enzymes in the pathway for melatonin biosynthesis, namely tryptophan decarboxylase (TDC), tryptamine 5-hydroxylase (T5H), serotonin N-acetyl transferase (SNAT), and *N*-acetyl serotonin methyl transferase (ASMT), have recently been characterized (Kang et al. 2007, 2011, 2012; Park et al. 2011). In animal kingdom, melatonin is involved in setting or stabilizing circadian rhythms, signaling darkness by an increase in concentration (Brzezinski 1997). In plants, melatonin concentration was also found to peak at different times of the circadian cycle (Poeggeler et al. 1991; Wolf et al. 2001; Van Tassel et al. 2001; Boccalandro et al. 2011; Tan et al. 2007). The relative ratios of melatonin to other indoleamines are implicated in regulation of photoperiod responses and light/dark cycles, regulation of plant growth and developmental pathways, root branching and secondary root growth, detoxification of reactive oxygen species, and reduction of the physiological impact of environmental stresses such as heavy metals, UV radiation, temperature fluctuations, and drought in plants (reviewed in: Murch et al. 2001; Paredes et al. 2009; Posmyk and Janas 2009; Zhang et al. 2013; Feng et al. 2014). The finding of synchronous IAA, serotonin, and melatonin concentration peaks is interesting and suggests that melatonin is also involved in circadian mechanisms in plants.

While melatonin concentration has not been related to the activity of the proton pump, there are studies connecting exogenous IAA and the increase in proton pumping. Felle et al. (1986) added IAA to medium bathing corn coleoptile tissue and observed acidification of the cytoplasm and membrane PD hyperpolarization, which increased with IAA concentration (from 0.01 μM to 1 mM). The authors suggested that acidification of the cytoplasm stimulates proton pumping. Senn and Goldsmith (1988) measured external acidification in oat coleoptiles upon addition of 10 μM IAA to the medium. They also observed hyperpolarization of membrane PD and increase in growth, supporting the acid growth hypothesis. Felle et al. (1991) exposed corn coleoptiles to several growth-promoting auxins and some

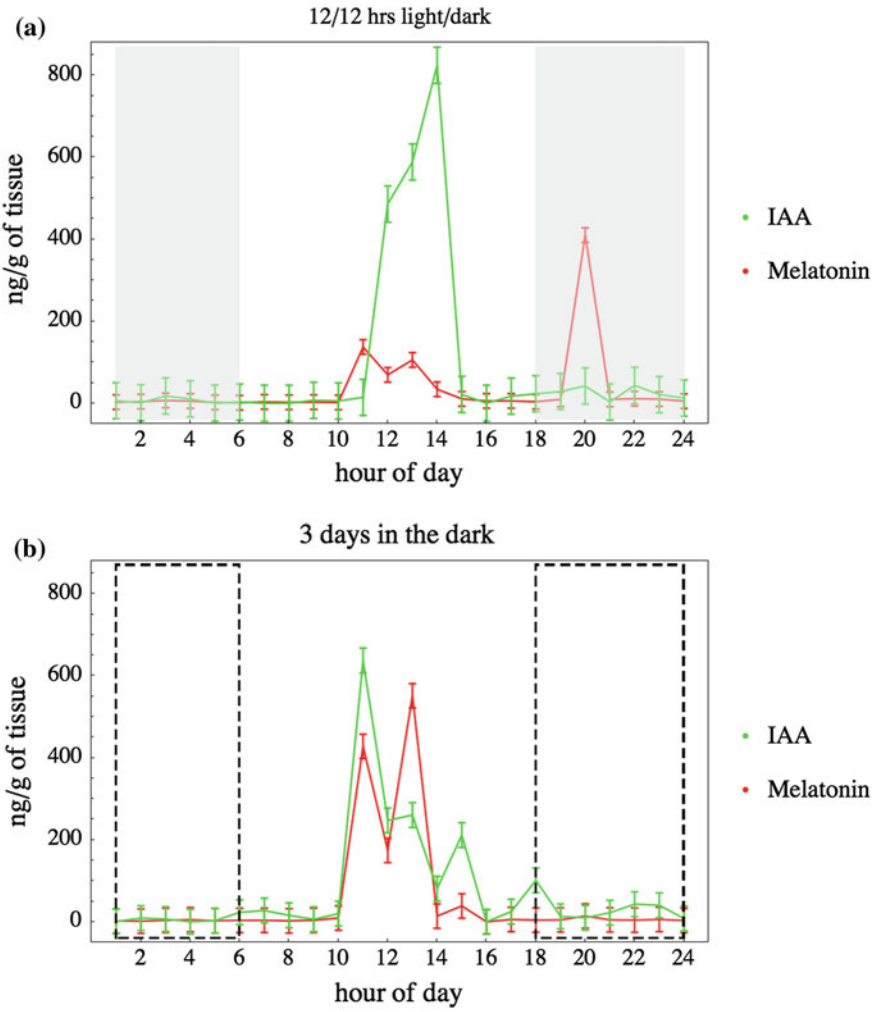


Fig. 13.3 Diel assay of concentrations of IAA and melatonin in growing tops of plants of *Chara australis*. **a** The samples were cut on the hour for 24 h from the tank exposed to 12/12 day/night cycle. The explants contained about 50 large cells (not counting the small nodal cells). The explants were manipulated into pre-weighed labeled Eppendorf tubes and weighed again to record the amount of wet tissue. The sample tubes were then snap frozen by plunging into liquid nitrogen and stored in dry ice and later in −80 °C freezer, prior to dry ice shipment for analysis. IAA and melatonin concentrations were measured in Murch laboratory in Kelowna, Canada, using well-established methods (Murch et al. 2009, 2010). **b** Some explants were cut and stored in continuous darkness for three days prior to sampling. The sampling for both groups of plants was done at the same time

structural analogues. Their results suggested that IAA^- is symported with $2H^+$ into the cells and that proton extrusion is necessary, but not sufficient, to produce elongation growth. Experiments are planned to correlate the membrane PD and *I/V* characteristics with the circadian changes in endogenous IAA concentration.

13.3 Transporter Interaction in Response to Abiotic Stress

13.3.1 *Clamp to Negative PD Levels: H^+ Pump, H^+/OH^- Channel, Inward Rectifier Channel, and Non-selective Channel in* Chara Australis

The invention of voltage clamp made it possible to force cell membrane/membranes to extreme PDs not normally measured in plant cells. What can we learn about ion transporters by taking the membrane to very negative PDs, close to postulated reversal PD of the $1H^+/1ATP$ pump? Exposure to extreme conditions (perturbation technique) can reveal signaling pathways that may be difficult to resolve under smaller step in the investigated stress parameter. If the *Chara* membrane was voltage-clamped to very negative levels (−400 to −500 mV) for more than 10 s, the subsequent free-running membrane PD became strongly depolarized. The pre-clamp negative resting PD took 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 arise from the inhibition of the proton pump and activation of the H^+/OH^- channels (Beilby and Casanova 2013, Sect. 3.2.3). 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. 13.4, the cell initially exhibited a normal pump state (Fig. 13.4a; Beilby 1984). After being voltage-clamped to −490 mV for 12 s, *I/V* curves were data-logged at 2, 3, 5, and 10 min after the end of the clamp. First two sets of data were fitted by OH^- current (Fig. 13.4b). The H^+/OH^- channel current was modeled by Goldmann–Hodgkin–Katz (GHK) equation (Eq. 13.4), multiplied by Boltzmann distributions of open probabilities P_{o+} and P_{o-} (Eqs. 13.5a, 13.5b) 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). We chose to use OH^- rather than H^+ (Beilby and Al Khazaaly 2009).

$$I_X = \frac{P_{o+}P_{o-}N_X P_X (z_X F)^2 V \left([X]_i - [X]_o \mathrm{e}^{-\frac{z_X FV}{RT}} \right)}{RT \left(1 - \mathrm{e}^{-\frac{z_X FV}{RT}} \right)} \tag{13.4}$$

$$P_{o-} = 1 - \frac{1}{1 + \mathrm{e}^{\frac{z_g F (V - V_{50-})}{RT}}} \tag{13.5a}$$

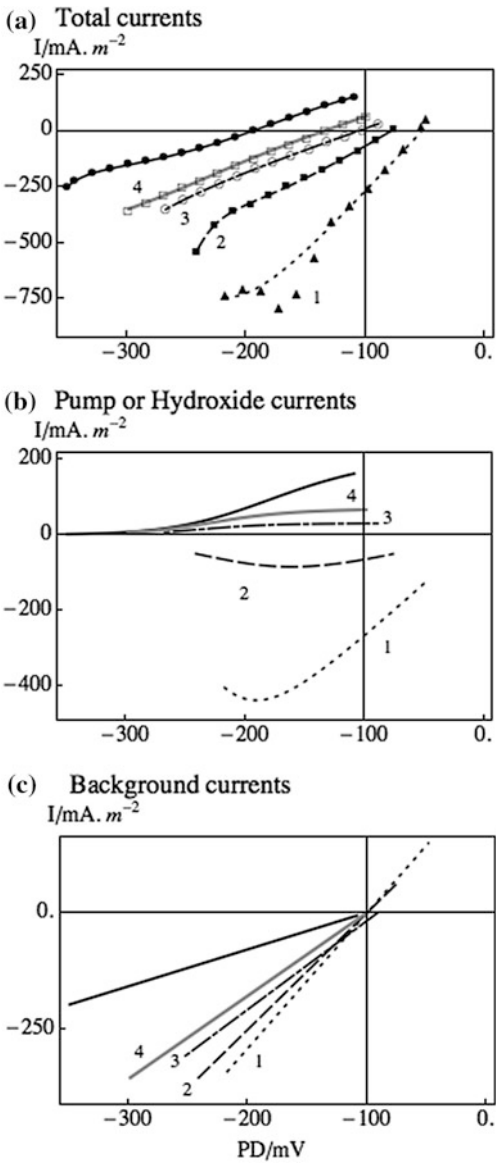


Fig. 13.4 Inactivation of the H^+ pump and activation of the H^+/OH^- channel by extreme hyperpolarization. **a** Total currents: *I/V* characteristics prior to the hyperpolarizing voltage clamp (*black continuous line*, data as *filled circles*); *I/V* characteristics at 2 min after 12 s voltage clamp to −490 mV (*curve 1*, *short dashed line*, data as *filled triangles*); at 3 min (*curve 2*, *dashed line*, data as *filled squares*); at 5 min (*curve 3*, *unequally dashed line*, data as *empty circles*); and at 10 min (*curve 4*, *thick gray line*, data as *empty squares*). **b** The pump or OH^- currents with same numbers and line types as in **a**. **c** The background currents with same numbers and line types as in **a** and **b**. The modeling parameters can be found in Table 13.1

$$P_{o+} = \frac{1}{1 + e^{\frac{z_g F(V - V_{50+})}{RT}}} \tag{13.5b}$$

F, R, and T have been defined above (see Eqs. 13.2a, 13.2b); z_X is the valence of the transported ion (OH^-); V is the PD across the plasma membrane or across both membranes; and $[X]_o$ and $[X]_i$ are the ion concentrations in the medium and the

cytoplasm, respectively (in this case [OH^-]). $N_X P_X$ denotes the number of X (OH^-) 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 PDs of channel closure, respectively.

The inward rectifier was modeled by Eqs. (13.4) and (13.5a), with P_{o+} set to 1.0 and K^+ being the transported ion (Beilby and Shepherd 2001a). As only a small numbers of data points are fitted, the model may not be very accurate, but allows us to quantify the rate of current increase with membrane hyperpolarization and changes of the half-activation parameter V_{50-} (see Table 13.1). The transient feature on the *I/V* curve at 2 min was not modeled. The pump recovery, observed at 5 min post clamp, shows an initial increase in k_{io}^0 parameter and decrease in κ_{io}^0 parameter. As the pump current increased at 10 min post clamp, both parameters move toward their initial steady-state values (see Table 13.1a). The background conductance exhibited transient post clamp increase (Table 13.1a, b). The simultaneous inactivation of the proton pump and activation of the H^+/OH^- channel is interesting and hints at some level of interdependence between these transporters. This is also suggested by the responses to some inhibitors (Bisson 1986). Similar switch from the pump to H^+/OH^- channels can be observed at the time of salinity stress (see next section).

13.3.2 *Saline Stress: H^+ Pump, H^+/OH^- Channel, Inward Rectifier Channel, and Non-selective Channel in Salt-Sensitive* Chara australis

The response to salinity stress can also be studied by the perturbation technique: a step increase in salinity. The components of salinity stress are resolved by an increase in osmolarity (e.g., by sorbitol), followed by isotonic saline solution. Such experiments reveal short-term defensive and stress responses to each component in dose-dependent manner.

The proton pump in *Chara* cells does not respond to non-plasmolyzing decrease in turgor (Shepherd et al. 2008). When sorbitol APW (artificial pond water) was changed to isotonic 50 mM NaCl APW, *Chara* exhibited salinity-induced noise in the membrane PD (see Fig. 13.5a). While there is always some noise in the membrane PD, the degree of "noisiness" was quantified by fitting a running average (n set between 50 and 200), which was then subtracted (Fig. 13.5b, Beilby et al. 2014). The classical Fourier analysis identified this increased noise as "red" noise. Such noise is ubiquitous in many microscopic and macroscopic systems and did not help in identification of the mechanism (Al Khazaaly et al. 2009). The time domain shows a continuous but random series of small depolarizations followed by a recovery (Fig. 13.5c, d). In this case, the period is too short to apply *I/V* analysis. As the PD noise was not affected by exchanging NaCl for Na_2SO_4, initially we hypothesized that high Na^+ concentration transiently activates groups of H^+/OH^- channels (Al Khazaaly et al. 2009).

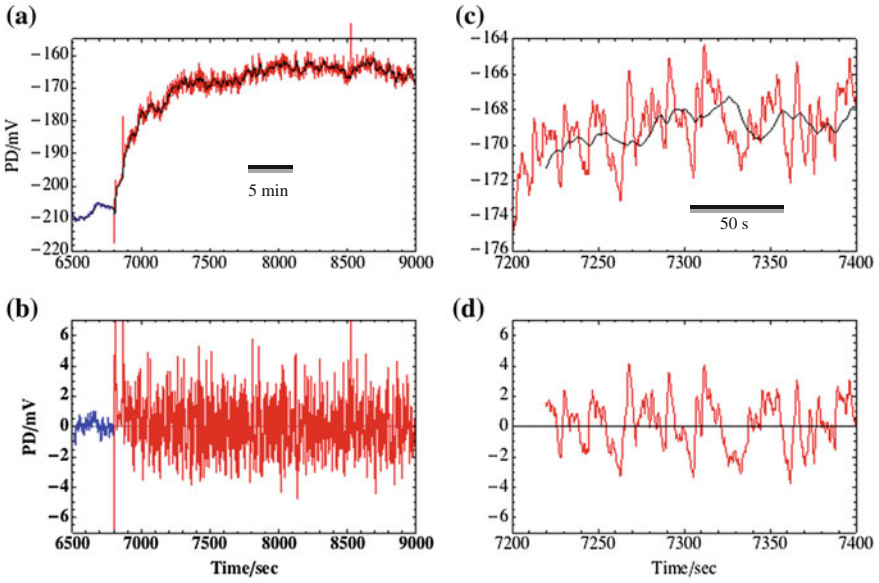


Fig. 13.5 Salinity-induced noise in membrane PD of salt-sensitive *Chara australis*. **a** Transition from sorbitol APW (*blue* trace) to 50 mM NaCl saline APW (*red* trace) resulted in depolarization and noisy membrane PD. The membrane PD was data-logged at 1 point/0.1 s. **b** The higher frequency noise was isolated by subtracting the running average (fitted with $n = 150$—*black line* in **a**). **c** Same data on expanded timescale fitted with running average ($n = 200$—*black line*). **d** The isolated noise retains its "shape"

With longer exposure to high salinity, the membrane PD continued to depolarize toward zero, the noise diminished, and the shape of the *I*/*V* characteristics changed and could be modeled by H^+/OH^- channels (Fig. 13.6; Beilby and Al Khazaaly 2009). The global opening of H^+/OH^- channels leads to the loss of the negative membrane potential and electromotive force necessary for the cell survival—an important element of stress pathology.

Can we show conclusively that both the salinity-induced noise and the later depolarization and change of *I*/*V* characteristics are due to global H^+/OH^- channel opening? Zinc ions are the most potent inhibitors of animal proton channels. While the permeating ion in Characeae is more likely OH^-, the channel proteins may still be closely related, as replacement of aspartate 112 by a neutral amino acid facilitated anion conduction in animal "proton" channels (DeCoursey 2013). At the time of salt stress, the depolarization to PD levels above −100 could be reversed by including 1.0 mM $ZnCl_2$ in the saline APW. Even the function of the proton pump was temporarily restored (Al Khazaaly and Beilby 2012). The saline noise was also inhibited by zinc ion. However, as zinc has many functions in plant tissues, further proof of H^+/OH^- channel involvement in salt stress pathology is needed.

Recent experiments of Eremin et al. (2013) suggest that reactive oxygen species (ROS) can open the H^+/OH^- channels by oxidizing either histidine or SH

Table 13.1 Modeling parameters for Fig. 13.4

(a) Pump and background parameters

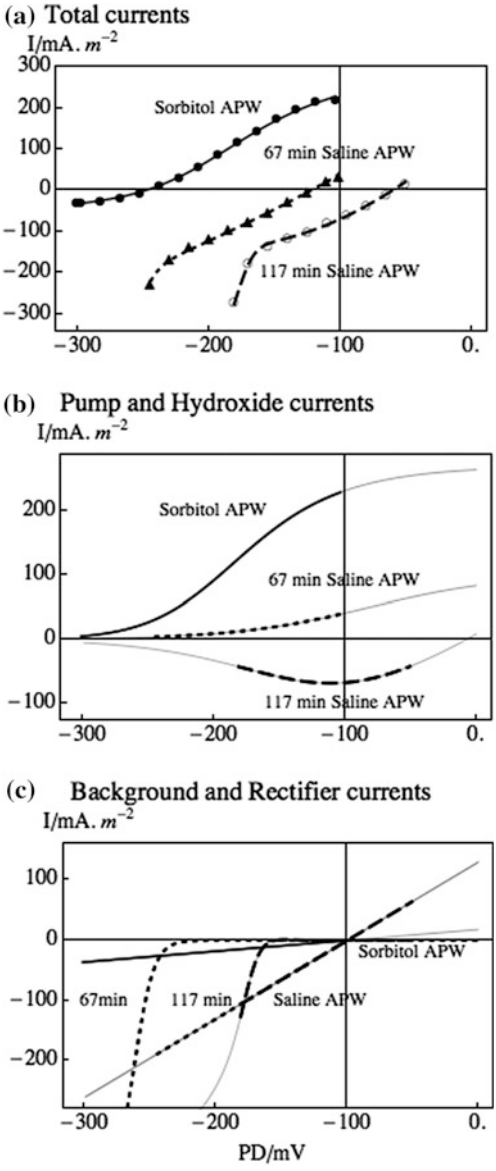
Conditions	$k_{io}^{0}(s^{-1})$	$\kappa_{oi}(s^{-1})$	G_{bkg} S m^{-2}	E_{bkg} mV	Resting PD mV
Before hyper polarizing step	3600	105	0.79	−100	−194
5 min after hyperpolarizing step	6800	15	1.9	−91	−106
10 min after hyperpolarizing step	6500	35	1.78	−100	−135

(b) H^+/OH^- channel parameters

Conditions	$N_{OH}P_{OH} \times 10^{-4}$ m s^{-1}	V_{50-} mV	z_g	G_{bkg} S m^{-2}	Resting PD mV
2 min after hyperpolarizing step	28	−240	0.9	2.9	−53
3 min after hyperpolarizing step	7.5	−205	0.6	2.5	−79

(a) The steady-state *I/V* characteristics, before the application of hyperpolarizing voltage, were modeled with the pump current, background current, and inward rectifier current ($N_KP_K = 60 \times 10^{-7}$ m s^{-1}, $V_{-50} = -385$ mV and $z_g = 1.9$). The pump recovery started after 5 min post hyperpolarizing step and was also modeled with the pump current, background current, and inward rectifier current ($N_KP_K = 60 \times 10^{-7}$ m s^{-1}, $V_{50-} = -350$ mV and $z_g = 1.0$). The *I/V* profile, 10 min post hyperpolarizing step, was modeled by background current and pump current only. The other two pump parameters, k_{oi}^{0} and $\kappa_{io,}$ were kept at 0.5 (s^{-1}) each

(b) The H^+/OH^- channels were modeled as passing OH^- current. The *I/V* characteristics 2 min post hyperpolarizing step included background current only, while the *I/V* characteristics at 3 min also included inward rectifier ($N_KP_K = 60 \times 10^{-7}$ m s^{-1}, $V_{50-} = -255$ mV and $z_g = 2.0$)


(a) Total currents
I/mA. m^{-2}
300
200
100
0
−100
−200
−300
Sorbitol APW
67 min Saline APW
117 min Saline APW
−300
−200
−100
0.
(b) Pump and Hydroxide currents
I/mA. m^{-2}
200
100
0
−100
Sorbitol APW
67 min Saline APW
117 min Saline APW
−300
−200
−100
0.
(c) Background and Rectifier currents
I/mA. m^{-2}
100
0
−100
−200
Sorbitol APW
67min
117 min
Saline APW
−300
−200
−100
0.
PD/mV

◀ **Fig. 13.6** **a** Trend in the *I/V* characteristics from the pump-dominated profile (*filled circles*) in steady state in the sorbitol APW to background current-dominated profile after 67 min of saline APW (*filled triangles*). After 117 min of saline APW, the membrane PD depolarized above −100 mV with a typical upward bend (*empty circles*). The experimental data were modeled by background current and pump (*continuous line*, model parameters: background current: G_{bkg} = 0.18 S m^{-2}, E_{bkg} = −100 mV, pump parameters: $k^0_{io} = 6700\,s^{-1}, k^0_{oi} = 2.7\,s^{-1}$, $\kappa_{io} = 0.5\,s^{-1}$, $\kappa_{oi} = 140\,s^{-1}$, by background current, declining pump, and inward rectifier current (*short dashed line*, model parameters: background current: G_{bkg} = 1.3 S m^{-2}, E_{bkg} = −100 mV, pump parameters: $k^0_{io} = 250\,s^{-1}, k^0_{oi} = 0.1\,s^{-1}, \kappa_{io} = 0.5\,s^{-1}, \kappa_{oi} = 52\,s^{-1}$, inward rectifier parameters: $N_K P_K = 40 \times 10^{-7}$m s^{-1}, V_{50-} = −261 mV, z_g = 3.5) and background current, OH^- channels and inward rectifier current (*long dashed line*, model parameters: background current: G_{bkg} = 1.3 S m^{-2}, E_{bkg} = −100 mV, OH^- channel parameters: $N_{OH}P_{OH} = 29 \times 10^{-4}$ m s^{-1}, V_{50-} = −130 mV, z_g = 0.6, inward rectifier parameters: $N_K P_K = 40 \times 10^{-7}$m s^{-1}, V_{50-} = −175 mV, z_g = 4, pH_i = 6.98). The modeled pump currents and the OH^- current are shown in **b**, and the background currents and inward rectifier currents are displayed in **c**, using the same line types. The *gray solid lines* in **b** and **c** show extrapolation of the model currents beyond the PD window delimited by the data. The figure was adapted from earlier version (Beilby and Al Khazaaly 2009)

(sulfhydryl) groups on transport proteins. In the animal kingdom, the voltage-gated H^+ channels in the brain microglia are activated by H_2O_2 (Wu 2014). Beilby et al. (2014) were able to inhibit or postpone the saline-induced noise by pre-treating the *Chara* cells in strong antioxidant melatonin prior to saline stress.

The other two transporters changed by exposure to saline (but not sorbitol) were the background channels and the inward rectifier channels. G_{bkg} increased from 0.18 to 1.3 S m^{-2} (Fig. 13.6), while E_{bkg} remained constant. The inward rectifier appeared at progressively more depolarized PDs for longer saline exposures, and the current increased faster with negative PD (Fig. 13.6).

13.3.3 *Saline Stress: H^+ Pump, Inward Rectifier Channel, and Non-selective Channel in Hyperosmotic Regulation in Salt-Tolerant* Lamprothamnium *sp.*

The salt-tolerant Characeae *Lamprothamnium* sp. 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 as in salt-sensitive Characeae (Beilby and Shepherd 2001a; Shepherd et al. 2008), so that the pump has to work even harder (see Fig. 13.7). Note that in this case, k^0_{oi}, k^0_{io} and κ_{oi} all increase (see the parameter values in Table 13.2), changing the I_p/V shape as combination of the trends shown in Fig. 13.2. Note also that while the changes in membrane PD are small, 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, I_{bkg}, and I_{irc}.

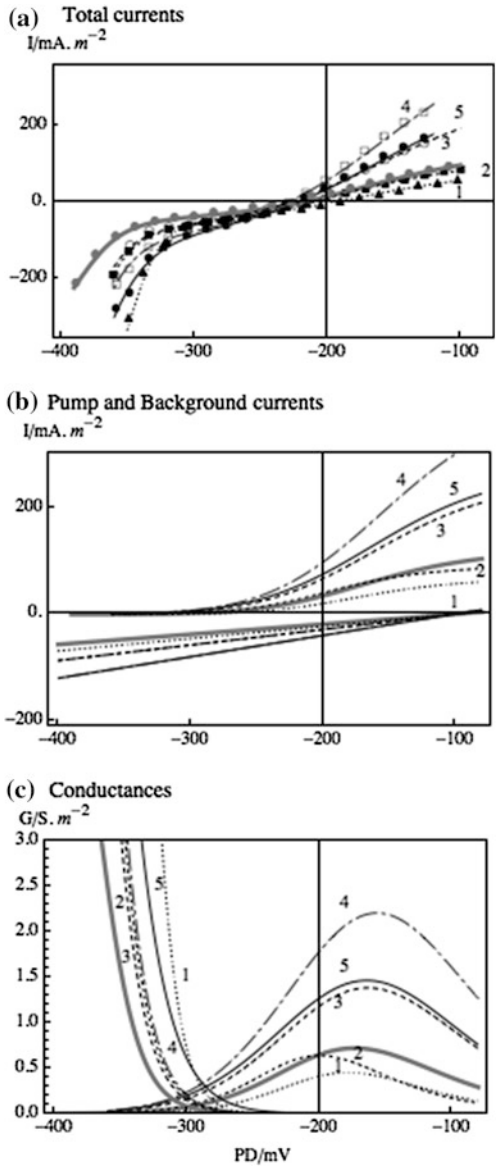


Fig. 13.7 Response of salt-tolerant Characeae *Lamprothamnium* to hyperosmotic medium step from 0.2 to 0.4 artificial seawater (Beilby and Shepherd 2001a). **a** The steady-state *I/V* profile is depicted by a *thick gray line* and *filled circles*. The successive profiles in hyperosmotic medium: 5 min (*curve 1*, *filled triangles*, *dotted line*), 21 min (*curve 2*, *filled squares*, *short dashed line*), 41 min (*curve 3*, *empty circles*, *long dashed line*), 2 h 34 min (*curve 4*, *empty squares*, *unequally dashed line*), and 3 h 30 min (*curve 5*, *gray filled circles*, *black line*). **b** The pump and background currents, depicted by same types of lines as in **a**. The fit parameters are listed in Table 13.2. **c** Pump and inward rectifier conductances. The fit parameters for inward rectifier currents can also be found in Table 13.2. Same types of lines are used as in **a** and **b**. The figure was adapted from earlier version (Beilby and Shepherd 2001a)

Table 13.2 Modeling parameters for Fig. 13.7

Conditions	Pump current			I_{irc}			G_{bkg} S m^{-2}	V_{m} mV
	$k^0_{io}(s^{-1})$	$k^0_{oi}(s^{-1})$	$\kappa_{oi}(s^{-1})$	$N_K P_K \times 10^{-7}$ m s^{-1}	V_{50-} mV	z_g		
Steady state in 0.2 SW	2000	0.6	62	1.5	−397	1.45	0.19	−219
0.4 SW curve 1: 5 min	1500	0.6	35	1.5	−364	1.5	0.23	−195
curve 2: 21 min	3500	0.7	47	1.5	−392	1.5	0.29	−211
curve 3: 41 min	3200	0.7	130	1.5	−395	1.5	0.29	−228
curve 4: 2 h 34 min	4500	1.1	210	1.5	−390	1.5	0.40	−230
curve 5: 3 h 30 min	3500	0.6	140	1.5	−385	1.2	0.40	−222

The parameter values are slightly different to original fit (Beilby and Shepherd 2001a). The data were refitted, so comparisons can be made to other examples discussed in this chapter

K^+ is probably imported through the inward rectifier. In the data shown in Fig. 13.7, the inward rectifier was fitted with the GHK model (see Sect. 13.3.1). While the fitting procedure is not very reliable due to small number of available experimental points, the activation of this conductance at more depolarized PDs is clearly visible (Fig. 13.7c). The increase in salinity seems to affect two I_{irc} parameters: number of gating charges and the half-activation PD (see Table 13.2).

The Cl^- is probably imported through the $2H^+/Cl^-$ symporter (Beilby and Walker 1981), which will be investigated by the removal of Cl^- from the outside medium. The modeling of the hyperosmotic regulation demonstrates how the model can be built up gradually, while providing feedback for further experimental design.

13.3.4 Saline Stress: MS Channel, Ca^{2+}-Activated Cl^- Channel, and K^+ Channel in Hypoosmotic Regulation in Salt-Tolerant Lamprothamnium *sp.*

The hypoosmotic regulation involves a complex signal cascade, and the accompanying changes in membrane PD show a great variability (see Fig. 13.8 and review by Beilby et al. 2006). In this process, the *Lamprothamnium* cell has to get rid of ions from the vacuole to balance increased water potential in the medium.

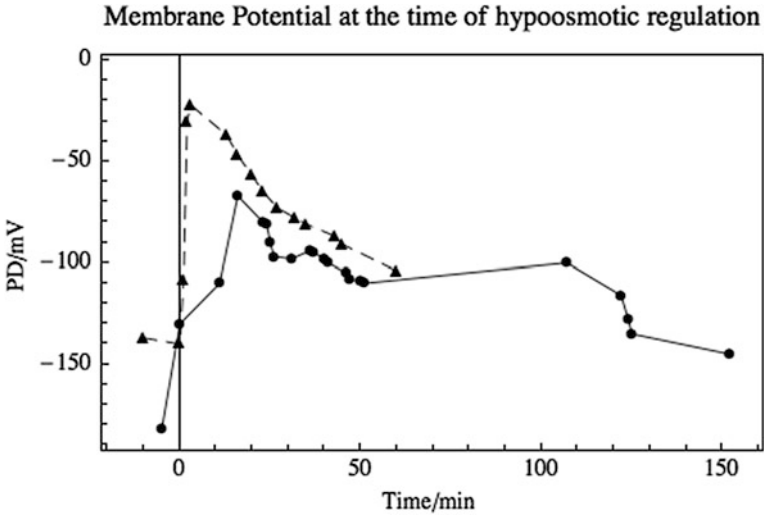


Fig. 13.8 The PD of two young *Lamprothamnium* cells (cell 1: *filled circles* and *continuous line*, cell 2: *filled triangles*, *dashed line*) with minimal mucilage, undergoing hypotonic regulation after step from 1/3 to 1/6 artificial seawater (Beilby and Shepherd 1996). The hypotonic step was imposed at time 0. Note the PD hyperpolarization at 100 min, which indicates the reactivation of the proton pump

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 (mechano-sensitive) channels as E_{bkg} depolarizes and G_{bkg} increases (Fig. 13.9d). The depolarization opens Ca^{++} channels either on plasma membrane, internal stores (Wacke and Thiel 2001), or both. The Ca^{++}-activated Cl^- channels dominate the first 20 min after the hypoosmotic exposure (Beilby and Shepherd 1996). The Cl^- channel *I/V* characteristics are inwardly rectifying (Fig. 13.9b; see also Beilby and Shepherd 2006a). If the cell is not in K^+ channel-dominated state prior to the hypoosmotic exposure, then the K^+ channels activate after about 10 min of the hypotonic stress (see Fig. 13.9c). 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).

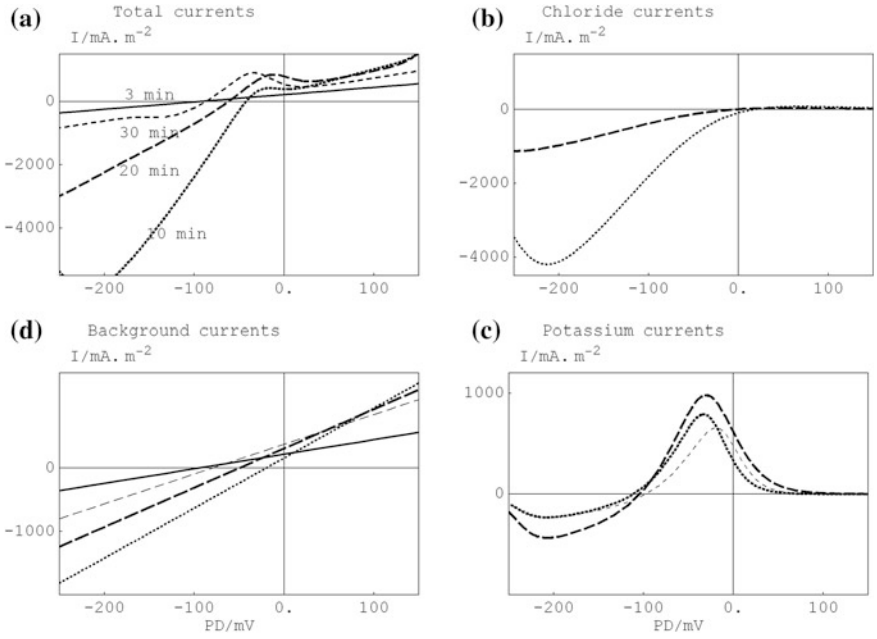


Fig. 13.9 **a** Synthesis of data from fitting hypoosmotic regulation under a range of conditions. All the data came from cells with minimal mucilage. The *continuous line* simulates the current at about 3 min after the hypotonic step, the *dotted line* 10 min, the *long dashed line* 20 min, and the *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. (13.4) and (13.5a, 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. (13.4) and (13.5a, 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

The hypoosmotic response in *Lamprothamnium* is modulated by extracellular polysaccharide mucilage (Shepherd and Beilby 1999; Shepherd et al. 1999). 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 formed thicker mucilage (Torn et al. 2014). 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 hypoosmotic 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. 13.1a. At the time of hypoosmotic 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. 13.9b; Beilby and Shepherd 2006a). G_K is PD dependent, and E_K also changes as the regulation progresses (Fig. 13.9c; Beilby and Shepherd 2001b). Both G_{bkg} and E_{bkg} change, presumably as functions of the turgor pressure (Fig. 13.9d; 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. 13.8).

The Cl^- and K^+ currents were modeled by Goldmann–Hodgkin–Katz (GHK) equation (Eq. 13.4), multiplied by Boltzmann distributions of open probabilities P_{o+} and P_{o-} (Eqs. 13.5a, 13.5b). 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 plasma membrane 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.

13.4 Conclusions

The responses of salt-sensitive *Chara* to extreme hyperpolarization and to saline stress exhibit similar elements: H^+ pump inactivation, H^+/OH^- channel activation, an increase in G_{bkg}, and activation of the inward rectifier channels at more depolarized PDs. A transient burst of ROS could be the link between these two rather different challenges, activating the H^+/OH^- channels (Beilby et al. 2014). The depolarization

of the inward rectifier channel activation could also be affected by ROS, as it is transient (upon hyperosmotic exposure) in salt-tolerant *Lamprothamnium*. G_{bkg} rises permanently in *Lamprothamnium*, as the plants are acclimated to higher salinities (Beilby and Shepherd 2001a), so the cause is most likely Na^+ concentration increase.

The response of salt-tolerant *Lamprothamnium* to hyperosmotic step lacks the inactivation of the H^+ pump and the global opening of the H^+/OH^- channels. Further, the H^+ pump current increases whether the cell is exposed to sorbitol or saline APW (Al Khazaaly and Beilby 2007). The increase consists of a transient change to energize turgor regulation and permanent increase to keep the membrane PD sufficiently negative against the shunt of the greater G_{bkg} (Beilby and Shepherd 2001a). The lack of salinity-induced noise and activation of H^+/OH^- channels need further investigation, as *Lamprothamnium* exhibits pH banding (Beilby, unpublished data) and H^+/OH^- channel-dominated state (Kirst and Bisson 1982). Detailed measurements of ROS concentration as function of time after exposure to salinity in both *Chara* and *Lamprothamnium* will provide more information on ROS function in channel kinetics and consequently in salt tolerance or sensitivity.

In general, changes in membrane PD reveal 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 hypoosmotic 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. Recent biochemical measurements (Beilby et al. 2014; Lazar et al. 2013) add another dimension to the research.

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Chapter 14
Rhythms, Clocks and Deterministic Chaos in Unicellular Organisms

David Lloyd, Miguel A. Aon and Sonia Cortassa

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 organisation has been the subject of a great deal of speculation, and many models have been suggested. This process is highly temperature dependent, and like all chemical and biochemical reactions, its rate approximately doubles for every 10 °C rise in temperature over a certain range of growth temperatures (i.e. the $Q_{10} \triangleq 2$). Clock-controlled biological processes on the other hand are temperature-compensated, so that $Q_{10} \triangleq 1$; two well-established examples are the circadian ($\tau \triangleq 24$ h) and ultradian clocks ($\tau \triangleq 40$ min in *Saccharomyces cerevisiae*). Other biological processes proceeding in faster time domains often show reactive 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 reactions (enzyme-catalysed), as well as chemical reactions. It has also 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 spatio-temporal coherence (order) arises in unicells as an output from a tuneable multi-oscillator (controlled chaos). Self-similarity of oscillatory properties across multiple time domains indicates temporal coherence described by an inverse power

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law proportional to $1/f^{\beta}$ and long-term temporal correlations. Clocks, rhythms, oscillations, deterministic chaos and scale-free coherence are fundamental hallmarks of life on every time scale.

14.1 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, as driven by ineluctable thermodynamic forces, islands of self-organisation 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 proceeds apace and in a truly robust manner. Explanations require more than biochemical understanding. For the details of the molecular structures of components, their synthesis, inter-conversion 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 levels of isolated tissues and organs rather than as an organismic 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 now "systems biology" (Lloyd 2005, actually not a new concept, but one made ever more feasible by the latest 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). 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 discoveries of intracellular oscillations in the concentrations of coenzymes and metabolites during the utilisation of glucose (Chance et al. 1964, 1973) by yeast and mammalian cells gave impetus to these activities (Higgins 1963).

Time scales (Fig. 14.1) 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, although, in plants, infradian processes (seasonal changes and annual rhythms) are even more obvious. In population ecological and on evolutionary time scales, oscillatory and complex non-linear behaviour is also well documented.

Thus, life is characterised by complexity on spatial scales that range from the infinitesimal to the global and over 30 decades of time measured in seconds (Lloyd

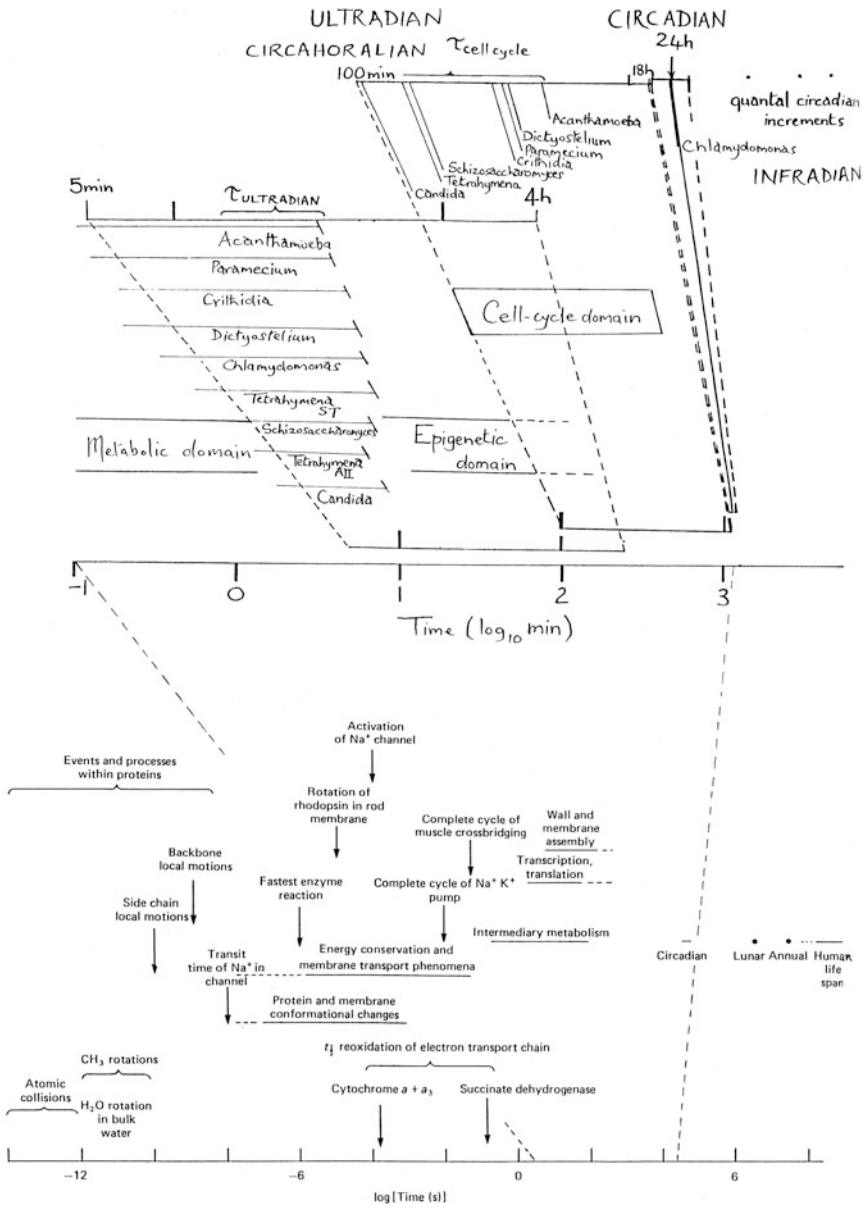

Fig. 14.1 The time domains of living systems. Approximate relaxation times (to a return of 1/e following perturbation) are plotted against time in seconds (log scale)

1974; Lloyd et al. 1982b; Aon and Cortassa 1997). Methods for studies on these different time scales (Lloyd et al. 1982b), now supplemented by rapid intracellular super-resolution optical imaging, enable a deep appreciation of dynamics, although

the time spans corresponding to our "spectrum of ignorance" (Kamen 1963) have not been completely elucidated.

14.2 Circadian Rhythms

The Earth has rotated on its axis more than a million million times since the dawn of life. It has been suggested that evolutionary advantage may have accrued in an organism that could change period by changes in just one or a few genes as day length changed from 4 h in the prebiotic earth, through 8 h during the expansion of photoautotrophs, to the present 24 h (Klevecz and Li 2007). 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 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 time scales (Fig. 14.1), and on each of these time scales, 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 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 to the daily circadian clock that allusion is being made. However, accumulating evidence (Lloyd and Rossi 1992, 2008) strongly suggests that the ultradian clock that still dominates the temporal control of intracellular coherence is both the core evolutionary precursor and conserved bellwether of present-day cellular oscillatory performance.

14.2.1 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 these claims are still a matter of dispute (Halberg et al. 2005). Several examples of eukaryotic unicellular organisms (*Chlamydomonas, Tetrahymena, Paramecium, Gonyaulax* (*Lingulodinium)* and yeasts) have provided excellent systems for study, as has also the mycelial fungus, *Neurospora* (Hurley et al. 2014). Many of the fundamental advances in understanding have come from studies of these organisms and have resulted in an enormous literature (Lloyd 1998; Lakin-Thomas 2000; Lakin-Thomas and Brody 2004; Gillette and Sejnowski 2005). The mechanism of

temperature compensation, a key characteristic of circadian periodicity, has been intensively studied in *Lingulodinium* (Hastings and Sweeney 1957) and modelled specifically in *Neurospora* (Tseng et al. 2012), as well as in more general terms (Ruoff et al. 2007; Thorsen et al. 2014). Transcriptomics and functional proteomics enable a rich diversity of components involved in the expression of circadian systems and reveal evolutionary exploitation of very many distinct scenarios (Wagner et al. 2005). Interaction with environmental cues, not only via photic pathways, but also by nutrient input (especially researched in animal systems), can act on chromatin remodelling (Oike et al. 2011, 2014; Sahar and Sassone-Corsi 2012, 2013). This increases rhythmic complexity and disturbs circadian regularity, sometimes leading to patho-physiological states.

14.2.2 Cyanobacterial Circadian Rhythms

The prokaryotic, *Synechococcus elongatus*, has three clock genes *KaiA, KaiB* and *KaiC*, with the latter 2 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, poly-3-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 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 (Goda et al. 2014). The phosphorylation state of *KaiC* oscillates with a circa 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 reconstruct in vitro this 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 the protein encoded by *LdpA* (Ivleva et al. 2005). This protein contains two $4_{Fe}4_{S}$ 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 believed to be derived from an ancestral cyanobacterium.

14.3 Glycolytic Oscillations

Although studied for almost 60 years (Duysens and Amesz 1957; Chance et al. 1964, 1973), and still yielding new information on the control mechanisms governing the principle pathway of glucose utilisation (Olsen et al. 2009; Kloster and Olsen 2012), a clear function for oscillations in the intracellular concentrations of glycolytic intermediates and cofactors is yet to be uncovered. Potential relevance to the maintenance of cellular synchronisation in yeast using acetaldehyde as a key coupling agent (Pye 1969) does not fully explain the kinetics of fast synchronisation dynamics provoked by glucose only when the substrate transporter is not fully saturated (Dano et al. 2007). Disappearance of oscillations in all the population together with the synchrony at low cell counts suggests a quorum-sensing mechanism (De Monte et al. 2007). Techniques for studying glycolytic oscillations in single ischaemic cardiomyocytes (Ganitkevich et al. 2010) and in isolated yeast organisms held in "laser tweezers" (Gustavsson et al. 2012, 2014) provide new insights into metabolic control mechanisms as well as possible functions. The recent observation that the aqueous intracellular phase shows relaxation properties (as revealed by fluorescent emission from Acdan, Prodan or Laurdan probes) tightly coupled with glycolytic oscillations opens up new vistas for investigation (Thoke et al. 2015).

14.4 Ultradian Respiratory Rhythms: The 40-Min Clock in Yeast

In addition to 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. Time scales 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, 2014). Time domains here cover many decades when measured in seconds on a logarithmic scale (Fig. 14.1); from attoseconds (in the trapping of photons in the photosynthetic light absorption events), through picoseconds (ligation of CO to the haem of haemoglobin), nanoseconds (in fluorescence energy transfer phenomena), microseconds (in conformational state changes in membranes), milliseconds (for electron transport steps), seconds (for metabolic inter-conversions), minutes for transcription, to hours for cell divisions. On and between all these levels of the temporal hierarchy, there is a necessity for timekeeping, so that convergence of parallel processing is ensured and that the whole system is coherent (Lloyd and Murray 2005). The analogy with a computer is apt but only partial. The magnificent miraculous machine also creates itself as it performs, 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. But we stress that this phenomenon is not a special case, not a mere curiosity. The strict rhythmic self-organisation evident here is evident in another yeast (Kippert and Lloyd 1995), in mammalian cells (Brodsky 1975, 2006, 2014; Lloyd and Gilbert 1998; Klevecz and Braly 1987) and probably in every living organism (Klevecz and Li 2007): its pervasive ubiquity emphasises its conserved central importance. In other yeasts, its period is different, as it is in protozoa (Table 14.1), in plants and in humans; the time base that the ultradian clock provides is a necessity for the synchrony and hence coherence of all life processes.

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 (Pirt 1975; Harrison 1973). Initially, cells are grown in a batch culture where 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 wave form; its trough-to-peak amplitude is about half that of the highest levels of dissolved O_2 attained, and its period is about 40 min (Fig. 14.2) (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 was 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 synchronised, and as the oscillations of dissolved O_2 are of such large amplitude, this synchrony must have involved a majority of the 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 oxidised 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

Table 14.1 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]. Adenine nucleotides. Intracellular pH. Majority of metabolites
		H_2S evolution
		Redox state [NADPH, GSH]
		Mitochondrial structure, inner membrane potential
		Transcription products
		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		Respiration [O_2]
	69	Adenine nucleotides [ATP, ADP, AMP]
		Total protein, RNA
		Flavin fluorescence
		Enzyme activities
		Cytochromes
Crithidia fasciulata	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
	50	Motility

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.

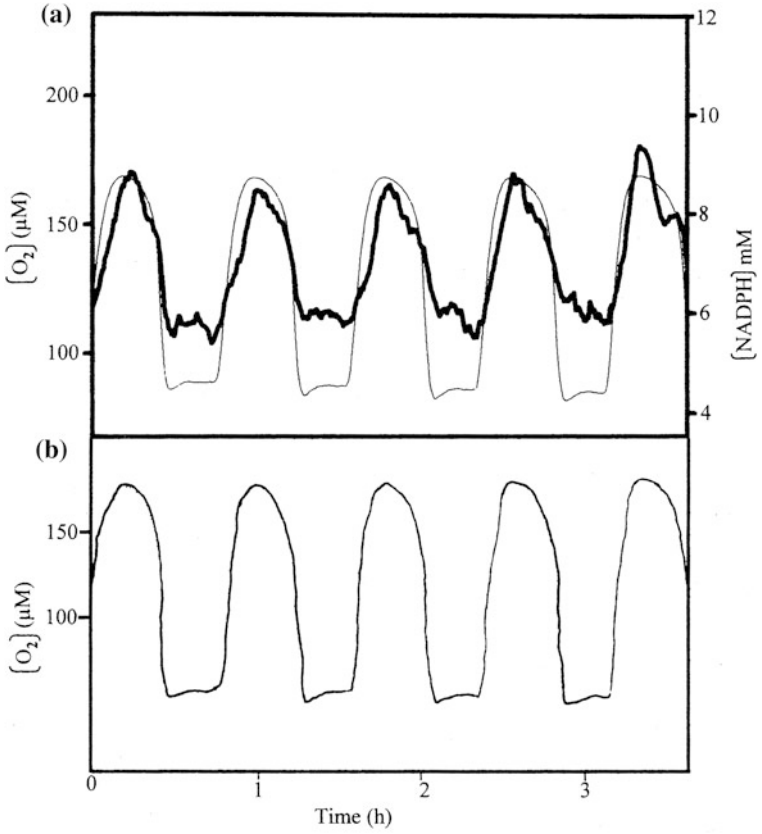

Fig. 14.2 Dissolved O_2 and reduced nicotinamide nucleotide fluorescence in a continuous culture of *S. cerevisiae*. **a** Simultaneous O_2 electrode and fluorometric monitoring (366 → 450 nm) in the same culture: fluorescence emission was calibrated by nicotinamide nucleotide extraction from the culture. **b** Control culture (no UV irradiation)

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 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 respirations was more rapid, and there was a slower increase in 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.

In addition to 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 characterised by redox switching. Further evidence for this comes from the perturbative effects of 5-nitro-2-furaldehyde (a GSH synthesis inhibitor) and DL-buthionine (*S*,*R*)-sulphoximine (a γ-glutamyl cysteine inhibitor). Furthermore, 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. 1998a, b).

Mitochondria undergo energisation to produce the accelerated respiratory rates observed in the 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 further supplemented by adding ADP. That massive changes in ionic balances across the inner mitochondrial membrane accompany this transition (and its reversal to restore the initial state) which occur on the completion of ADP phosphorylation are well documented. Mitochondrial events that accompany respiratory oscillations include change in cytochrome redox states, inner mitochondrial membrane potential changes (as measured by fluorimetric assay using rhodamine 123, a cationic dye internalised by mitochondria) and 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 the time of protonophore addition was at times 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 is no longer evident. Recovery occurred as the protonophore became diluted by the flowing medium, and after about 10 h, the respiratory oscillation had returned to almost 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 ($0.085\ h^{-1}$), this interval approximated to the cycle time of budding. Similar events were observed with CCCP as the protonophore. When a culture that has spontaneously become synchronised 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 energisation 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 the biosynthetic to degradative modes that accompany cycling ATP/ADP ratios as first demonstrated in *Acanthamoeba castellanii* (Edwards and Lloyd 1978, 1980).

Analysis of the genome-wide transcriptional activity during respiratory oscillations (Klevecz et al. 2004) showed that of the 5329 expressed transcripts, only 161 gave trough–peak differences of greater than 3-fold. However, the transcripts could be assigned into two blocks or redox super-clusters: those 650 that gave maximal expression during the phase of active respiration or oxidative phase of the cycle and 4679 that were maximally observed during the "reductive" phase of the cycle. Furthermore, a sub-population of organisms (5–10 %) is gated in the transition between G_1 and S-phase every oscillation cycle, during the onset of the low respiration phase. Gene transcripts are coordinated both with more than metabolic function and the cell cycle (Chin et al. 2012). Most of the DNA synthesis and RNA synthesis occur when the cells are in a reductive state. Further analyses reveal a global feedback between 7 co-expression clusters that consolidate into two super-clusters providing the elements of a global feedback between metabolism and chromatin dynamics (Machné and Murray 2012).

Temporal compartmentation may represent an evolutionary strategy to avoid oxidative damage to DNA and RNA, and to nascent metabolic products of translation. The concentrations of the majority of metabolites oscillate in a defined order on the 40-min period time scale (Murray et al. 2007). 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-oxidising proto-mitochondrion using such a timekeeper, so that the separation of oxidative and reductive states is 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. Instead, 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. Nested synchrony, as proposed for neuronal dynamics (Monto 2012), whereby cross-scale integration of global interactions involves the phase of a slower oscillator modulating synchrony in a higher frequency band, also presents an attractive hypothesis for the yeast system.

Recent contributions show that mitochondria oscillate as coupled oscillators either during respiratory oscillations in yeast (Aon et al. 2007a, b, c) or in cardiac mitochondria (Aon et al. 2006).

Analysis of 96,000 discrete time points of O2 and CO2 time series obtained by mass spectrometry from yeast continuous cultures revealed a broad range of temporal scales among multi-oscillatory states comprising periods of 13 h, 40 min and

4 min (Roussel and Lloyd 2007). Further analytical work, involving power spectral and relative dispersional analyses, revealed that the multi-oscillatory frequencies exhibit self-similar scaling, described by an inverse power law proportional to $1/f^{\beta}$ and long-term temporal correlations (Lloyd 2008; Aon et al. 2008): both of these traits in yeast physiology imply that a change on one time scale will be felt across the frequency range from the intracellular to the intercellular (Murray et al. 2013). These recent findings of scale-free behaviour in yeast physiology (Lloyd et al. 2012) and mitochondrial network dynamics imply the modulation of intracellular timekeeping on several time scales simultaneously. Moreover, striking similarities between the cardiac redox control systems and those in the evolutionarily distant yeast, *S. cerevisiae*, indicate an ancient commonality of central core metabolic mechanisms. The core mechanism generating the rhythmic dynamics in yeast and cardiac myocytes entails the cycling of cytoplasmic and mitochondrial proteins between their oxidised and reduced states, mainly driven by reactive oxygen species (ROS) and NAD(P)H, as reflected in the redox potentials of the thiol pools (Murray et al. 1998a, b, 1999; Lloyd and Murray 2000, 2006, 2007; Slodzinsky et al. 2008), and especially as a function of the glutathione redox thiol status, resulting in the control of mitochondrial inner membrane anion channels (Aon et al. 2007a, b, c). The implications of this mechanism, and the pivotal role of mitochondria both as a source and as a victim of redox stress (Park et al. 2011), triggering the early events leading to necrotic or programmed cell death both in yeast and in cardiac cells have been extensively documented (Lemar et al. 2005, 2007; Aon et al. 2007a, b, c; Lloyd et al. 2012) and simulated (Nivala et al. 2011, 2013; Selivanov et al. 2012; Cortassa et al. 2014; Kembro et al. 2014). In single mitochondria of *Arabidopsis thaliana*, pulsing of inner membrane potential linked to Ca^{2+} influx provides a stress-induced mechanism for the regulation of bioenergetics (Schwartzländer et al. 2012). In plants as in the mammalian heart, care must be taken in not attempting to predict the molecular mechanisms governing homeodynamics of an intact multi-cellular organism from results obtained with isolated organelles, single cells (Aon et al. 2007a), tissues or organs, as the emergence of qualitative novel interactions (Aon et al. 2007b) and overall behaviour may appear at every level of integration (Weiss and Yang 2010; Yang et al. 2010; Weiss et al. 2011).

In conclusion, the oscillation that characterises the ultradian clock (Fig. 14.3) percolates not only throughout the population of organisms, but also the entire cellular network, including organelles, transcriptome, metabolome and proteome. Recent refinements in rapid sampling and quenching of metabolite, DNA, RNA and protein concentrations (Sasidharan et al. 2012a, b, c) provide greater precision of the their dynamic trajectories. Parallel enhancement of waveform analysis using a Fourier-based measure of de-noised multiple significant frequencies (Amariel et al. 2014) provides oscillation waveform and multi-periods metrics. The complex oscillatory network thereby revealed (Amariel et al. 2013), and coordinated by the ultradian clock, links mitochondrial energetics and the control of redox state to transcriptional regulation and organelle remodelling, DNA replication and cell division events. A relatively simple feedback ATP loop on chromatin architecture

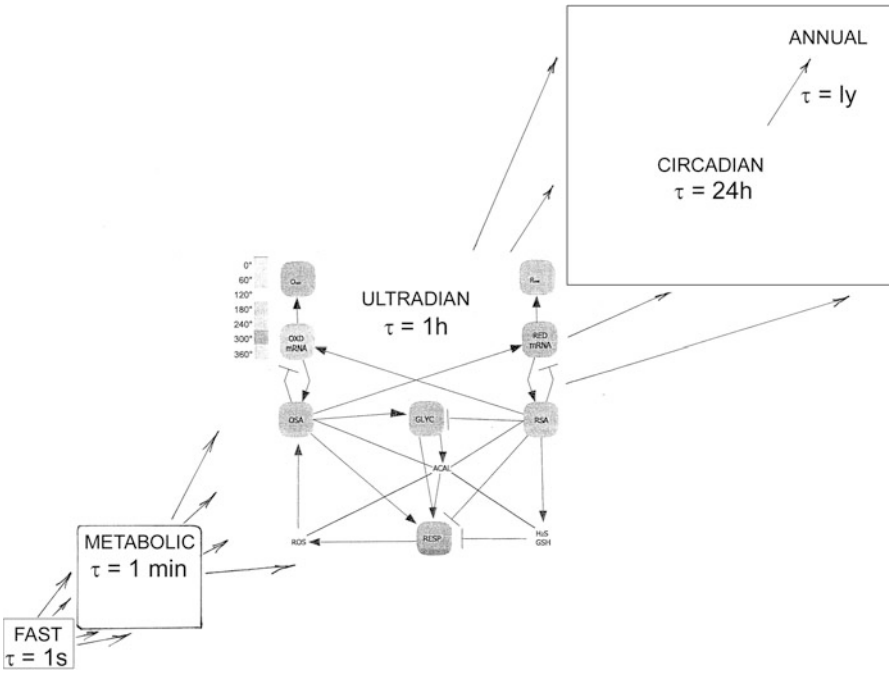

Fig. 14.3 The ultradian clock (composed of a massive array of metabolic and transcriptional elements) self-optimises to produce an output characteristic of the biological species in which it is studied. Its core is a redox-sensing network (for details, see Lloyd and Murray 2006). Cycling between oxidative 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 is still responsible for circadian organisation and that on longer-time scales (seasonal, annual)

(Machné and Murray 2012) leads to global partitioning of anabolism and catabolism and the synthesis and expression of enzymes involved.

The temporal partitioning first described in yeast (Satroutdinov et al. 1992), and intensively studied ever since (Lloyd et al. 2008), is not an exceptional curiosity found only in a peculiar system, but rather an universal trait, necessary for the maintenance of the robust but flexible metabolic auto-dynamic state, characteristic of normally healthy cells. Further consequences include the quantisation of cell division cycle times (cell doubling times) as integral values of ultradian clock cycle times. We have previously obtained ultradian clock cycle times for 8 different yeast and protozoal species (Lloyd 1992), and for the algae *Chlamydomonas reinhardtii* (Jenkins et al. 1989, 1990) and *Euglena gracilis* (Balzer et al. 1989; Adams 1990) (Table 14.1) (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 O_2 species-produced damage to cellular components, especially lipids and

proteins. As oxidative stress varies during the mitochondrial energisation cycles, this leads to the intriguing hypothesis that cell aging can be quantitatively measured in ultradian cycles (Lloyd et al. 2003). Similar ideas have been expressed for circadian rhythms (Hardeland et al. 2003).

14.5 Oscillatory Behaviour During the Cell Division Cycles of Lower Organisms

Earlier work has been reviewed (Lloyd et al. 1982a, b; 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), even under the most strictly controlled conditions, operate in pseudo-steady states, and the literature is vast. Many examples are typified by reactions to triggering events (transient anaerobiosis, pulsatile pH control by acid or alkaline titration, dropwise nutrient addition, etc.). In other cases, synchronised cell growth and division (budding) as characterised by oscillatory O_2 consumption, partly as a consequence of changing trehalose utilisation (Kuenzi and Fiechter 1969), leads to oscillatory states (Strässle et al. 1988; Parulekar et al. 1986; Duboc et al. 1996; Chen and McDonald 1990; Münch et al. 1992). 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 of 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 emphasised that the overall cell cycle and 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), many theoreticians have suggested candidates for the molecular components of cell division control machinery and the mechanisms that drive cell division cycles (Norel and Agur 1991). Now that molecular genetics has provided many details, key control points have been revealed, and many recently discovered oscillators described. Modelling proceeds apace (Mackey 1985; Mackey et al. 1986; Grasman 1990; Tyson et al. 2003; Battogtokh and Tyson 2004a, b, 2006; Ball et al. 2013) and, provided that a full and realistic consideration of physiologically scenarios are fulfilled, new understanding of the variables important for biological phenomena (duration of cell cycle stages, identity of checkpoints, transcriptional noise) 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 1992). Nuclear regulation of the chloroplast circadian expression has been confirmed (Matsuo et al. 2006).

14.6 Ultradian Gating of the Cell Division Cycle

14.6.1 Experimental Systems

We have observed in some protozoa (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 quantised. Using an established mathematical model of the cell cycle (Mustafin and Volkov 1977; Chernavskii et al. 1977), it is possible to see how quantisation 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 latter model (Lloyd et al. 1992) can explain the dispersion of cell cycle times through a population in strictly deterministic terms, whereas previous models had involved stochastic effects (Brooks 1985) which necessitated the provision of a "noise" term in the equations.

As an example of interacting oscillatory systems, we may take one that provides timekeeping for cell division. Here, recent experiments with yeast provide experimental validation of these theoretical predictions.

14.6.2 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):

$$\begin{aligned} \tau_L \frac{dL}{dt} &= \eta - 2LR - DL \\ \tau_R \frac{dR}{dt} &= K + LR - R^2 - \frac{\gamma R}{R + \delta} \end{aligned} \tag{14.1}$$

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:

$$\tau_L \frac{dL}{dt} = \eta - 2LR - DL + C \sin \Omega t \tag{14.2}$$

where

$$T_{UR} = 2\pi/\Omega \ll T_{\text{cell cycle}}$$

The auto-oscillating solution considers that cells divide when $L(t)$ reaches a threshold to initiate a rapid phase of the cycle.

14.6.3 Computer Simulations

Dynamic behaviour of the equations was studied numerically.

The bifurcation diagram, a plot that indicates the dynamic state of the system as it is further and further into its non-linear region (L_{max} vs. η, Fig. 14.4), shows that a chaotic region begins at η = 4.65 and gives way to simple periodic oscillation at

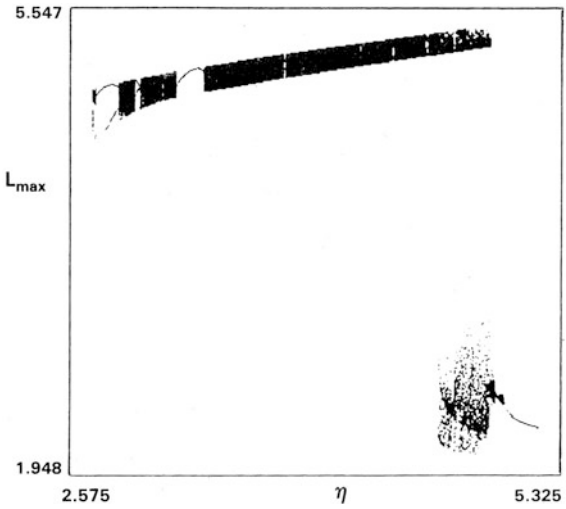

Fig. 14.4 Bifurcation diagram for the model described in Eqs. (14.1) and (14.2) as η varies between 2.5 and 5.2. For each value of η, 100 maxima of L are plotted following a transient of 100 time units. Other parameters were as follows: D = 0.4, K = 0.15, γ = 1.5, δ = 0.15, C = 0.45, Ω = 5.0, τ_L = 1 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 quasiperiodic regime. (Reproduced with permission from Lloyd et al. 1992)

$\eta = 5.0$: 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.

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 $\lambda_1 = 0.21$, $\lambda_2 = 0$ and $\lambda_3 = -6.32$, and the Lyapunov dimension was of $D_L = 2.03$. The positive value of λ_1 and finite value for D_L confirm chaotic dynamics. A relative frequency plot of cell cycle times for $\eta = 4.95$ shows a trimodal distribution with negatively skewed dispersions (Fig. 14.5c): for comparison, we also present (Fig. 14.5a) experimental data obtained by precise measurement of individual cell division times for the protozoon *Paramecium tetraurelia* (Lloyd and Kippert 1987) and (Fig. 14.5b) computer-simulated results for a noisy relaxation oscillator (Lloyd and Volkov 1990).

In the model presented here, dispersion and quantisation 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; Novak et al. 2001), 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). It results in a frequency distribution of cell division times which closely resembles those observed for animal cells (Klevecz 1976) and single-celled organisms (Lloyd and Kippert 1987). It remains to be established that evolutionary selection of chaotic cell division dynamics (Klevecz and Li 2007) 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 (Restrepo et al. 2005) or, in a multi-cellular 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 optimisation of water regulatory transport processes (Prytz 2001).

14.7 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, 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 non-linear van de Pol oscillator (Pyragiene and Pyragus 2005), and this principle can be utilised to stabilise the unstable periodic orbits of chaotic systems (Pyragas 2002, Fig. 14.6). Anticipated chaos is an unexpected consequence of a time delay between master and slave oscillators, where the latter

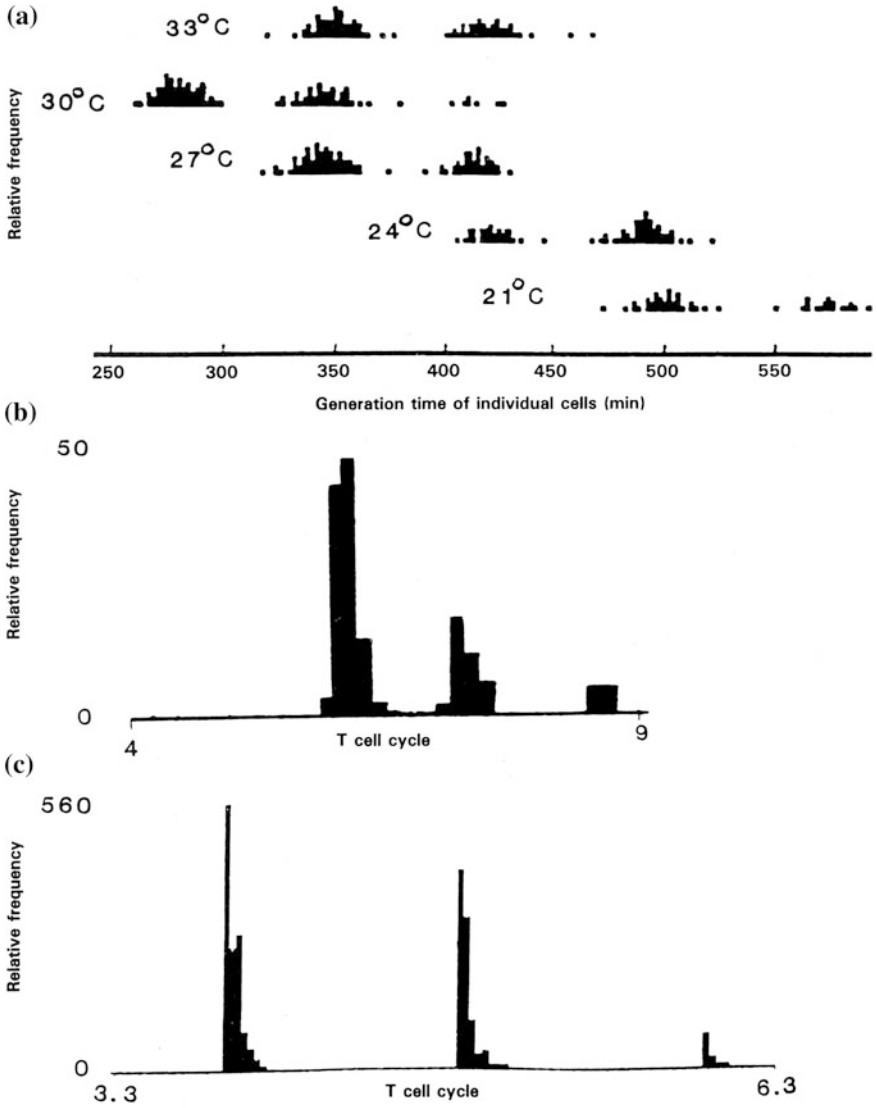

Fig. 14.5 Quantised cell cycle times. **a** Experimentally observed distributions of generation times of individual *Paramecium tetraurelia* cells at various steady-state growth temperatures. Each square represents timing of division of an isolated organism. (Reproduced with permission.) **b** Generation time distributions from the model system incorporating noise (Eq. 3) when $\tau_L = 1$; $\tau_R = 0.1$; $K = 0.15$; $D = 0.4$; $\gamma = 1.5$; $\delta = 0.15$; $\Delta_\eta = 0.3$; ξ = random numbers (0,1); $C = 0.45$, $T_{\mathrm{UR}} = 1.25$ and $\eta = 5.55$. **c** Frequency distribution of cell cycle times ($T_{\mathrm{cell\ cycle}}$) for parameter values as follows: $\eta = 4.95$, $D = 0.4$; $K = 0.15$, $\gamma = 1.5$, $\delta = 0.15$, $C = 0.45$, $\Omega = 5.0$, $r_L = 1$ and $\tau_R = 0.1$ for Eq. 14.2 (in the absence of a noise term). (Reproduced with permission from Lloyd and Rossi 1992)

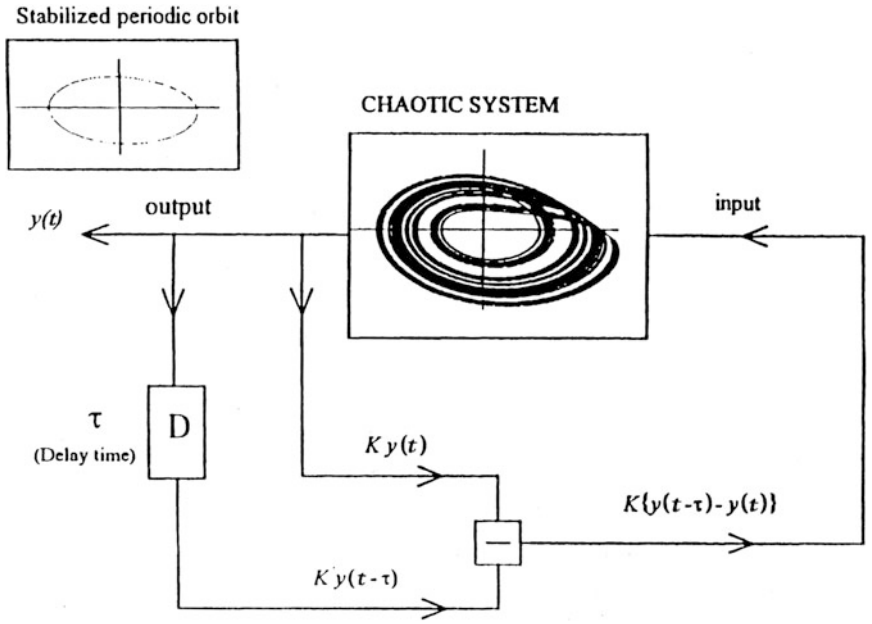

Fig. 14.6 Control of a chaotic system by feedback with delay to select a stable periodic orbit of required frequency. (Modified from Pyragas 2002)

can anticipate the former in a non-symmetric coupled external-cavity-laser system (Rees et al. 2003). It seems likely that in 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; Lloyd and Lloyd 1995; Aon et al. 2000). This is because the identification 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).

The recent discovery of scaling, criticality and coloured noise behaviour in mitochondrial networks has enabled new insights into the understanding of complexity in biology (West 1999; Aon et al. 2011). Under critical conditions, complex systems of disparate nature may collapse, rupture or crash under stressful conditions (Sornette 2000). 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). Chaos can be induced in glycolysis in cell-free systems prepared from yeast by periodic forcing with glucose

(Marcus et al. 1985; Nielsen et al. 1997). In vivo, a chaotic growth regime has been demonstrated experimentally in a continuous culture of yeast (Davey et al. 1996) by using a variety of time series analyses (Fourier transformations, Hurst and Lyapunov exponents, the determination of embedding dimension and the Sugihara-May methodology for non-linear time series predictions).

The presence of a strange attractor is strongly suggested as a feature of yeast dynamics in a continuous culture (Murray and Lloyd 2007). Lowering the pH of the culture from the value of 3.4 (as 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 (Fig. 14.7).

Roussel and Lloyd (2007) used a membrane-inlet mass spectrometric probe measuring dissolved gases in a self-synchronous continuous culture of *S. cerevisiae* to provide further conclusive evidence for chaotic operation. Semi-continuous (data points obtained every 12 s) monitoring of dissolved O_2, CO_2 and H_2S over a period of 2 months gave 40,000 data points, which revealed multi-oscillatory output with periodicities of 13 h, 36 min and 4 min. The latter two modes were not evident at all times, but returned at regular intervals. The data were used to visualise a strange attractor (Fig. 14.8): computation of the leading Lyapounov exponent, a measure of the mean rate of divergence of neighbouring points on the attractor, revealed the dynamics on this attractor to be chaotic. Its value was 0.752 ± 0.004 (95 % confidence): that it was positive indicates sensitive dependence on initial conditions, and that it was not zero indicates that the dynamics was chaotic rather than quasi-periodic. Thus, the culture dynamics was chaotic in the region studied in this experiment.

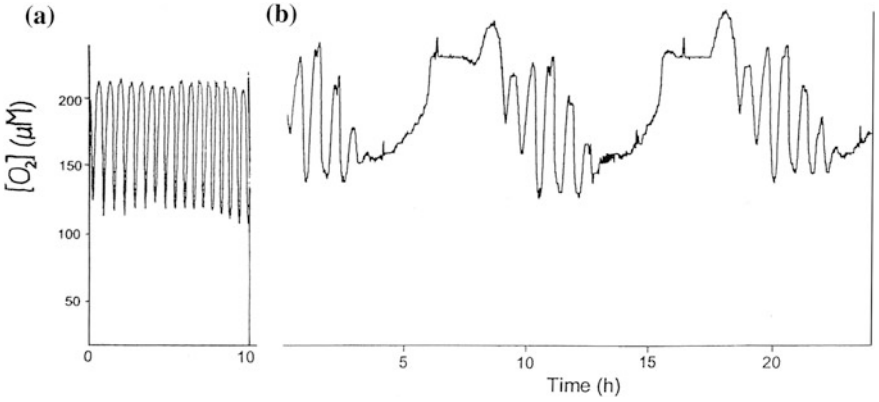

Fig. 14.7 Respiratory oscillations in a continuous culture of yeast: various outputs observed experimentally. In **a**, the 40-min ultradian clock is observed. In **b**, lowering the pH of the culture in steps of 0.1 pH unit drives the culture into a state where a low-dimensional chaotic attractor can be revealed (Murray and Lloyd 2007)

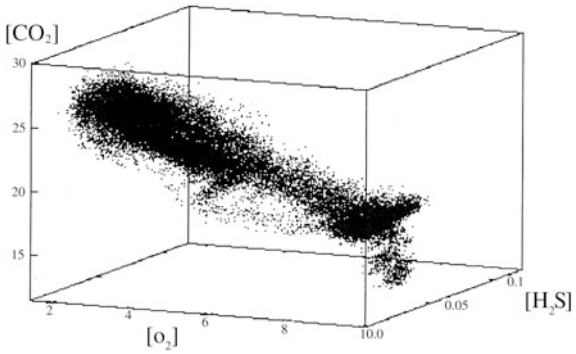

Fig. 14.8 A strange attractor in the dynamics of gas evolution/consumption in a continuously monitored continuous culture of *S. cerevisiae* as revealed by membrane-inlet mass spectrometry. Values of m/z 32, 34 and 44 (O_2, H_2S, CO_2, respectively) were measured every 15 s, compared with m/z 40 (argon), The experiment lasted two months and 40,000 data points were collected

14.8 Functions of Rhythms

Table 14.2 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 spatio-temporal 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

Table 14.2 Functions of oscillations/rhythms/clocks

1. Increased energy efficiency? (e.g. in glycolysis)
2. Signalling (e.g. Ca2 + oscillator, superoxide radical ions) (Amplitude modulation or frequency modulation)
3. Spatial organisation (e.g. in segmentation, the somite "clock")
4. Temporal organisation
(a) Matching intracellular/inter-organism events with the environmental cycles (e.g. circadian rhythms, seasonal rhythm)
(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)
(d) Synchrony of populations and in tissues by cell–cell communication
5. Entrainment by a. phase or b. frequency
6. Prediction (e.g. dawn or dusk by circadian rhythms)
7. Responsiveness
8. Biological "fitness"

been consistently underestimated. Homeodynamics ensures an apparent constancy of the structure–function continuum (Lloyd et al. 2001; Aon et al. 2000).

14.9 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 headings under which this topic has been considered: these include innovation, preservation of diversity, prevention of entrainment and dissipation of disturbance (A. Lloyd and D. Lloyd 1995). Lloyd and Lloyd (1993) have further suggested that a controlled chaotic system may have a further central and fundamental use: as the tuneable oscillator responsible for coordinated, integrated and coherent intracellular physiology (Lloyd et al. 2004).

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 stabilise each of these as required using control algorithms (Fig. 14.6; Pyragas 1992), and it becomes clear that a highly adaptable and flexible dynamic system follows as a natural consequence. Such a system could be of evolutionary advantage. A controlled chaotic attractor can provide for rapid switching between states and thereby flexibility of response to changing conditions. Ultradian–circadian switching provides one such example (Lloyd and Lloyd 1993, 1995). Self-optimisation of dynamic behaviour and a robust defence of the status quo provide homeodynamics rather than homeostasis (Yates 1992a, b, 1993; Lloyd 1994; Lloyd et al. 2001). The rich dynamic behaviour of life processes (relaxation times from *f*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 of respiration (Figs. 14.7 and 14.8) under controlled conditions of cell multiplication (Lloyd and Murray 2005; Lloyd et al. 2003; Murray et al. 2003).

Future work includes further analysis of transcriptome expression and proteomic changes during the 40-min cycle to further characterise the details of the oscillatory response and time-keeping functions that underlie cellular integrity. Interrogation of higher frequency temporal coordination will require work with single organisms, rather than in coherent populations, as faster time resolution will be necessary. This information will be crucial to a valid system analysis approach to the temporal coherence of the organism.

14.10 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 14.2) and that a more precise and robust performance gradually evolved. Oscillations became rhythms, and the development of temperature compensation permitted timekeeping. This would have occurred on and between every time scale in the heterarchy of spatio-temporal organisation, so that the massively interconnected network self-optimised 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 just of the outputs of a small number of "canonical circadian genes" but 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-oxidising α-proteobacterial symbiont by an archaeal host that produced H_2S (Searcy 2003). The ultradian organisation of the clock in yeast suggests that accommodation to this inter-relationship involved separation in time of the reductive and oxidative stages thereby required (Lloyd and Murray 2006). We suggest that the ultradian clock preceded the circadian clock and 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 where 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 circa 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) and Lara-Aparicio et al. (2014) with crayfish and of Morré et al. (2002a, b) with plant and (Brodsky 1975, 2006, 2014) with 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 currently studied in flies, moulds, mice and humans (Lloyd and Rossi 2008). Implications of these concepts for the differentiated organisation 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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Index

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