Chapter 4
Rhythmic Leaf Movements: Physiological and Molecular Aspects

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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
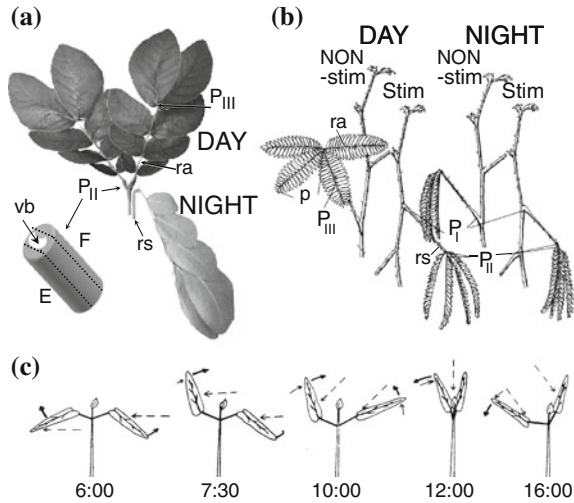
Fig. 4.1 Types of leaf movements. **a** Nyctinastic movement of the terminal pinnae of the compound leaf of *Samanea saman* (Jacq.) Merrill, between open during the DAY and folded during the NIGHT. *Inset:* a schematic drawing of a pulvinus: *E* extensor, *F* flexor, *vb* vascular bundle, P_{II}–P_{III} 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
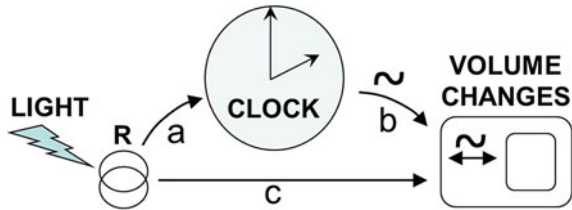
Fig. 4.2 Light stimulates cell volume changes. A model of clock-mediated (circadian) and clock-independent (acute) pathways. **a** Light, perceived by one or more light receptor(s), *R*, affects the clock. **b** The clock governs volume changes, imparting fluctuations ($\sim$) in activity or abundance 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° 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.
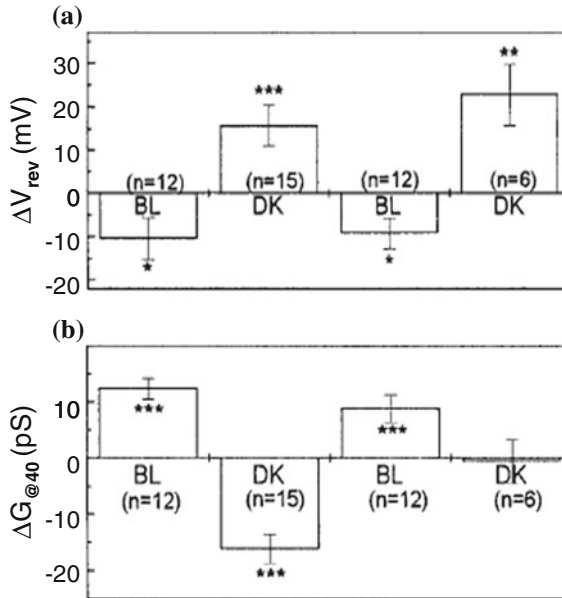
Fig. 4.3 Blue light enhances the activity of the K_D *Samanea* channels in flexor protoplasts. **a** Light-induced shift of the membrane potential, manifested as shifts of the reversal potential, V_{rev} of K_D-channel currents in single cell-attached membrane patches during alternating between blue 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 Ca²⁺ 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 Ca²⁺ 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).
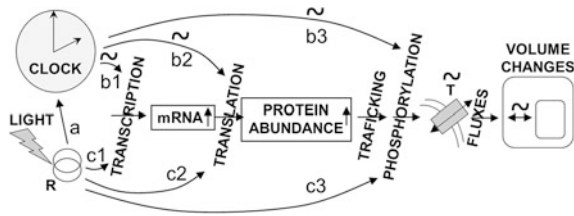
Fig. 4.4 Regulation of membrane transporters in the pulvinus at the levels of transcription, translation and protein modification (a schematic model). *bn* are clock output signalling pathways, and *cn* are signalling pathways from the light-activated receptor, *R. T* is the transporter protein in the 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 flavo-protein 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.
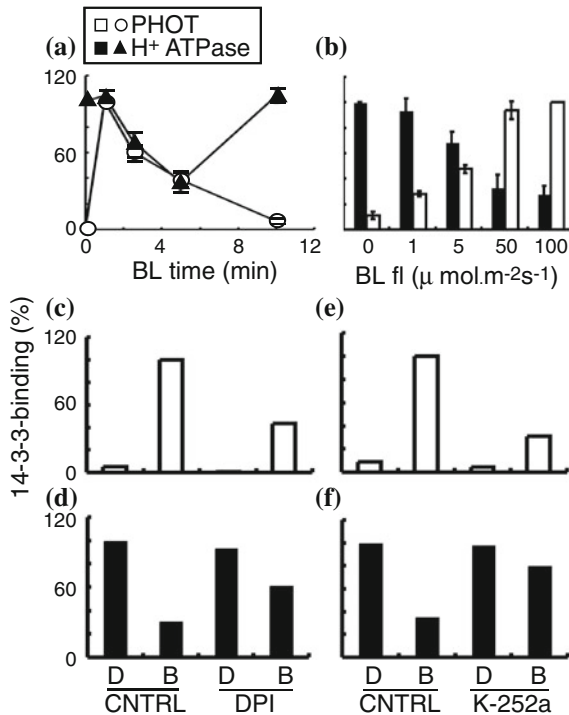
Fig. 4.5 Pulvinar phototropins mediate the dephosphorylation of the plasmalemmal $H^+ATPase$ by blue light. **a** Time courses of recombinant 14-3-3 protein binding to phototropin and to H^+-ATPase (as a measure of their phosphorylation status) in pulvinar microsomal membranes in response to a blue-light 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, *PvPHOT1a*, *PvPHOT1b* and *PvPHOT2*, 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).
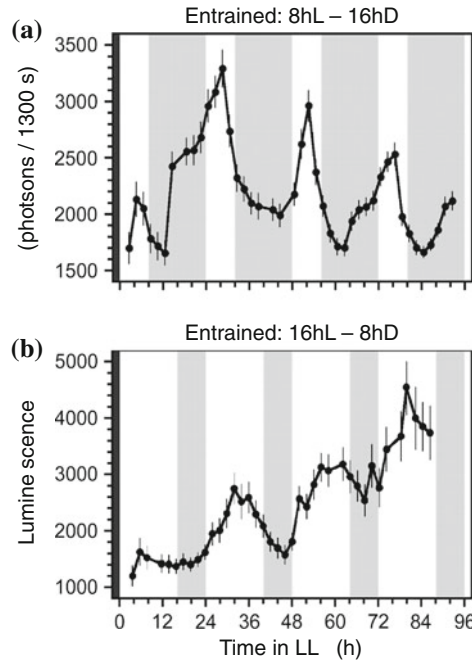
Fig. 4.6 Circadian oscillations of $[Ca^{2+}]_{cyt}$ in *Arabidopsis* seedlings entrained to different photoperiods. Aequorin luminescence emitted by seedlings kept under 110 µmol m^{-2} s^{-1} constant light (LL). Shown are measurements from seedlings entrained in 8L/16D (**a**) and 16L/8D (**b**) for 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$_3$PIP, 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;
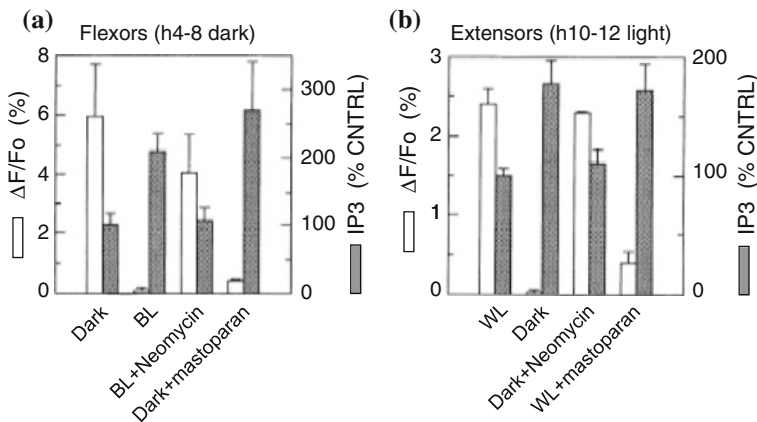

Fig. 4.7 Inositol 4,5 trisphosphate ($InsP_3$) and K^+ permeability of protoplasts in response to shrinking signals. Protoplasts were isolated during light period and transferred to growth chamber at time of normal "light off" (means ± SD of 3 or 4 separate experiments, each done in duplicate). **a** 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-methyl-tetrahydropteroyl-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.
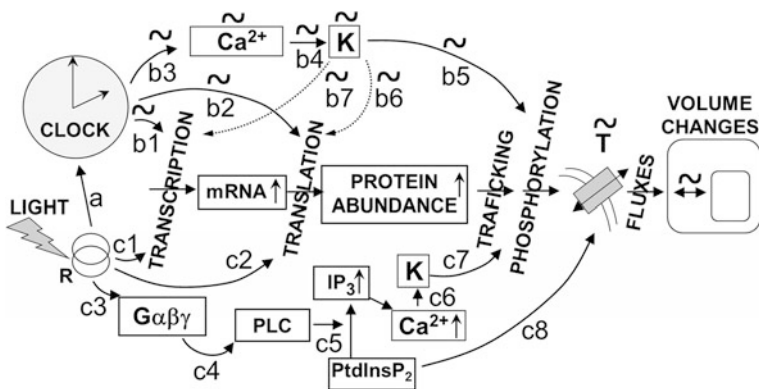

Fig. 4.8 Ca^{2+} involvement in the volume changes (a [partial] schematic model). *bn* are clock output signalling pathways, and *cn* are the "acute" signalling pathways from the light-activated receptor, *R*. The acute "shrinking signalling" includes the PtdIns pathway, starting with the activation (*c*3) of a trimeric G protein (G$\alpha\beta\gamma$), 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 ($\uparrow$) 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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