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Minireview

# Osmoregulation of leaf motor cells

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**Abstract "Osmotic Motors" – the best-documented explanation for plant leaf movements – frequently reside in specialized motor leaf organs, pulvini. The movements result from dissimilar volume and turgor changes in two oppositely positioned parts of the pulvinus. This Osmotic Motor is powered by a plasma membrane proton ATPase, which drives KCl fluxes and, consequently, water, across the pulvinus into swelling cells and out of shrinking cells. Light signals and signals from the endogenous biological clock converge on the channels through which these fluxes occur. These channels and their regulatory pathways in the pulvinus are the topic of this review.**


*Keywords:* Pulvinus; Leaf movement; Stretch-activated channels; Potassium channels; Aquaporins; Biological clock

## 1. Introduction

### 1.1. Leaf movements – the 'hands' of an endogenous clock

Some plants move their leaves upon sudden shaking or touch (seismonastic and thigmonastic movements) and many do so in synchrony with diurnal alternations between light and dark (rhythmic, nyctinastic movements). The French astronomer, De Mairan, is credited with the first attempt to resolve experimentally the origin of rhythm in the leaf movements of the "sensitive plant" – probably *Mimosa pudica* – a rhythm which continued even when the plant was locked in a dark cellar. These "sleep movements" of leaves, a name coined by Linnaeus, led to the gradual emergence of the concept of an internal oscillator – for which leaf movements serve as "clock hands". However, although leaf movements have been noted since antiquity, the concept of the "osmotic motor" as the underlying mechanism of these movements arose only in the 19th century (reviewed by [1]).

### 1.2. The osmotic motor

The osmotic motor often resides in specialized leaf organs, pulvini, at a base of leaves and leaflets (Fig. 1). Towards morning, when the upper, "flexor" part of the pulvinus shrinks and its lower, "extensor" part swells, the bent pulvinus straightens, extending the leaves. The movement reverses towards evening. The motor cell in the swelling part of the pulvinus accumulates solutes, mainly $K^+$ and $Cl^-$, and in $Cl^-$ absence – malate, a processed powered by $H^+$ extrusion, and the lowered water potential drives water influx. In the shrinking part, the motor cell loses passively solutes and, consequently, water. Thus, the rhythmic, reversible movements of the pulvinus stem from coordinated volume and turgor changes of the extensor and flexor motor cells, reflecting fluxes of water and KCl between the two parts of the pulvinus, and across the motor cell membranes. Ion channels are believed to be the conduits for the influx and efflux of $K^+$ and the efflux of $Cl^-$ (reviewed by [2–5]). Water channels (aquaporins) are believed to serve as water conduits through the pulvinar motor cell membranes [6,7].

The currently accepted model for the osmotic volume changes of pulvinar cells, which evolved from Pfeffer's "Osmotic Analyses" of leaf movements [8], does not differ in principle from that accepted for the stomata guard cells (reviewed by [9]). The flux exchanges between extensors and flexors possibly parallels the exchanges between guard cells and their subsidiary cells (e.g. [10]). In a contrast to guard cells, in the intact pulvinus the solute and water fluxes may occur to some extent also via plasmodesmata interconnecting the pulvinar motor cells. An additional proposed exception to the guard-cell model addresses specifically the driving forces for pulvinar cell shrinking during the very fast seismonastic response of *M. pudica*; here, leaflets fold in seconds, rather than in tens of minutes, as in rhythmic movements. This particularly rapid movement is thought to result from (a) sudden sucrose unloading from phloem into the extensor apoplast, increasing the osmotic drive for water efflux from cells [11], and/or (b) ultrafiltration of water out of the cells due to sudden squeezing action exerted by activated cytoskeletal elements [12]. The touch-provoked clasping-shut of the leaves of *Dionea*

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*Abbreviations:* ABA, abscissic acid; AMP-PNP, 5′-adenylyl-imidodiphosphate; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BL, blue light; CRY, cryptochrome; DAG, diacylglycerol; H7, 1-(5-isoquinolinesulpho-nyl)-2-methylpiperazin; GLR, glutamate receptor channel; $IP_3$, inositol 1,4,5-trisphosphate; $K_{D(H)}$ channels, depolarization (hyperpolarization)-dependent K channels; MscS, mechanosensitive channel small; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; OA, okadaic acid; $P_f$, osmotic water permeability; PHOT, phototropin; PHYA(E), phytochrome A(or E); PIs, phosphatidylinositides; PKA, cAMP-dependent protein kinase; $PLA_2$, phospholipase $A_2$; PLCδ, phospholipase C-δ; Pr and Pfr, the red and the far-red absorbing forms of phytochrome; PRC, phase response curve; PtdCh, phosphatidylcholine; PtdInsPs, phosphatidyl-inositol phosphates; R, red light; SAC, stretch-activated channel; SV, slow vacuolar [channel]; TEA, tetraethyl ammonium; TMB-8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate; TPK, tandem-pore K [channel]; TRP, transient receptor potential [channel]

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Fig. 1. The movement of *Samanea* leaf. Noon and evening photos of the same leaf, in an open and folded state. Note the straight and bent shapes of the pair of secondary terminal pulvini within the circles.

*muscipula* (Venus fly trap) – occurring in an astonishing 100 ms range – is yet another exception: here the osmotic motor performs a relatively minor function: only that of shifting the setting point of the elastically unstable leaf, already inherently poised for snapping [13].

A wide range of plants exhibit diurnal and signal-induced leaf movements, including growth movements, but the best studied are probably the pulvinar motor cells of *Samanea*, *Mimosa* and *Phaseolus*. These cells serve not only as models for studying leaf movements, but also, along with guard cells, as models for the regulation of plant ion and water channels. This review focuses on the regulation of channels in the pulvinar motor cells by light and the biological clock, and in particular, on findings reported since the reviews compiled by Satter et al. [4]. Additional details may be found in a recent review [14].

## 2. Pulvinar K and water channels

Much of the more recent work on pulvinar channels employed protoplasts isolated from the motor cells. With minor exceptions [15], such protoplasts appear to represent rather well the physiological properties of the intact pulvinar motor tissues. They are the site of the rhythm generator, containing both the ''oscillator'' and the ''motor'', and, in addition, photoreceptors with links to the ''motor'' [15–18]. Although it is obvious that the fluxes of $K^+$, $Cl^-$ and water occur between the vacuole and the apoplast, i.e. across two membranes, as elaborated recently with regard to the guard cell [19], there is little information about the tonoplast channels of the pulvinar motor cells. Admittedly, even among the plasma membrane channels only a few have been observed in situ and partially characterized.

### 2.1. $K^+$-release channels in the plasma membrane

These channels are presumed to mediate $K^+$ efflux from pulvinar motor cells during their shrinking. Patch-clamp studies revealed depolarization-dependent, $K^+$-release ($K_D$) channels in the plasma membrane of pulvinar cell protoplasts in *Samanea* [20], *Mimosa* [21] and in *Phaseolus* [22], resembling those of the guard cells [23,24].

*2.1.1. Ion selectivity and sensitivity.* The selectivity for $K^+$ of the *Samanea* $K_D$ channel was somewhat higher than for $RB^+$, and much higher than for $Na^+$ and $Li^+$, and the channel was blocked by external $Cs^+$, $Ba^{2+}$, $Cd^{2+}$ and $Gd^{3+}$ [25], and also by tetraethyl ammonium (TEA) [20]. $K_D$ channels in extensors were slightly less $K^+$ selective than in flexors [26] and while extensors and flexors differed also in the details of the cytosolic $Ca^{2+}$ sensitivity of the $K_D$ channels gating, the overall effect of cytosolic $Ca^{2+}$ on these channels was rather minor [26]. By contrast, the *Mimosa* $K_D$ channel currents, although generally similar in their voltage dependence and similarly blockable by external $Ba^{2+}$ and TEA [21], were severely attenuated – they ''ran down'' – by treatments presumed to increase cytosolic $Ca^{2+}$ [27]. The emerging differences between these seemingly identical channels invite seeking differences between their functions in these pulvini.

*2.1.2. Molecular identity.* Among the four putative K channel genes cloned from the *Samanea saman* pulvinar cDNA library [28], which possess the universal K channel-specific pore signature, TXXTXGYG, the *Samanea*-predicted protein sequence of SPORK1 is similar to SKOR and GORK, the only *Arabidopsis* outward-rectifying *Shaker*-like K channels. *SPORK1* was expressed in all parts of the pulvinus and also in the leaf blades, mainly in mesophyll, as demonstrated in Northern blots of total mRNA, and *SPORK1* expression was regulated diurnally in all these tissues. However, circadian regulation of *SPORK1* expression was evident only in extensor and flexor and not in the vascular bundle in the rachis or in the leaflet blades [28]. Thus, it is conceivable that the pulvinar rhythm requires SPORK1 expression and, by extrapolation, that SPORK1 is the molecular entity underlying the pulvinar $K_D$ channels. However, the functional expression of SPORK1 has yet to be achieved.

### 2.2. $K^+$-influx channels in the plasma membrane

$K^+$-influx currents were reportedly observed in extensor protoplasts from pulvini of *Phaseolus*, using patch-clamp in the

whole-cell configuration [22], but they were not characterized. However, hyperpolarizing pulses failed to activate such currents in protoplasts from the primary pulvini of *Mimosa* [21].

*2.2.1. Voltage-dependence.* In *Samanea* extensor and flexor protoplasts, Yu et al. [29] described inward $K^+$ currents through hyperpolarization gated K ($K_H$) channels in the plasma membrane, accompanied by relatively large, leak-like, voltage-*in*dependent $K^+$ currents. The $K_H$ channels, but not the "leak", were blocked by external protons [29]. This was unexpected of channels presumed to mediate the $K^+$ influx during cell swelling which is known to occur concurrently with external acidification [2], and was also in stark contrast to the promotion by protons of the guard cell $K_H$ channels – KAT1/KAT2, or their homologues (e.g. [30,31]). Whether or not the inward "leaks" in *Samanea* protoplasts are just another, phosphorylated form of the $K_H$ channels, as shown for the *Arabidopsis* AKT2 channels [32], they probably contribute to $K^+$ influx during pulvinar cell swelling [29].

*2.2.2. Molecular identity.* *KAT1* (or *KAT2*), the genes of two of the $K^+$-influx ($K_H$) channels of the *Arabidopsis* guard cells, were not detected in the pulvinar cDNA library in several repeated trials [28]. Hence, the choice of the candidate channel genes for the *Samanea* $K_H$ channel fell on two of the *Shaker* K-channel-like genes cloned from the *Samanea* cDNA pulvinar library, *SPICK1* and *SPICK2*, with predicted protein sequences homologous to AKT2, a weakly inward-rectifying *Shaker*-like *Arabidopsis* K channel [28]. This choice was motivated also, as in the case of SPORK1, by the demonstration of a diurnal and circadian rhythm in their transcript levels. In particular, the circadian rhythm seen at three levels seems to link *SPICK1* and *SPICK2* to their putative role in the pulvinus as the $K^+$-influx channel(s): (i) in the pulvinar movement, (ii) in the mRNA level, (iii) in the resting permeability of the pulvinar $K^+$-influx channels, and the susceptibility of this permeability to blue light stimulation [17].

### 2.3. A tonoplast K channel

$K^+$ ions traversing the tonoplast during the volume changes of the *Samanea* pulvinar motor cells, as described for guard cells [19], may flow through SPOCK1, the putative *Samanea* tandem-pore K (TPK)-like channel [28]. SPOCK1 shares a 59% identity with the *Arabidopsis* TPK1 – the KCO1 renamed. KCO1 has been recently relegated from the role of the slow vacuolar (SV) channel, which participates in signaling, and for which no homologue in pulvini has been found, so far, to that of a $Ca^{2+}$-dependent $K^+$-selective and $K^+$-conducting voltage-*in*dependent vacuolar channel [33]. Alternatively – though less likely – SPOCK could be a plasma membrane channel, like AtTPK4 [34], with which it shares only 34% identity. Whatever its localization, SPOCK1 is probably important for the diurnal rhythm of the movement, as its mRNA level in the *Samanea* extensor and flexor fluctuated under diurnal control, although not in constant darkness [28].

### 2.4. Anion channels

Plasma membrane anion channels, quite extensively studied in the guard cells (e.g., [35], and references therein), are the presumed $Cl^-$ efflux pathway during the motor cell shrinkage. There is practically no information about anion channels in the pulvinar plasma membrane. Pharmacological evidence that Cl channels mediate ABA-induced shrinking of protoplasts isolated from a laminar pulvinus of *Phaseolus vulgaris* [36] is not conclusive, as 5-nitro-2-(3- phenylpropylamino)benzoic acid (NPPB), the inhibitor used in the study, has been shown also to inhibit plant $K^+$-release channels with an even higher affinity [37].

### 2.5. Water channels (aquaporins)

*2.5.1. Water permeability in situ.* Osmotic water permeability ($P_f$) of *Samanea* motor cell protoplasts, determined from their swelling rates in hypotonic solution, peaked in a diurnal rhythm, at periods of most pronounced volume changes, i.e. the periods of highest water fluxes: in the morning, in extensor and flexor, and in the evening, in extensor. Inhibition of the $P_f$ peaks by $HgCl_2$ (50 μM) and by phloretin (250 μM), both non-specific transport inhibitors shown to inhibit aquaporins in some systems, and by 2 mM cycloheximide, an inhibitor of protein synthesis, has been interpreted as evidence for the function of plasma membrane aquaporins [7].

*2.5.2. PIPs in Samanea – molecular identity and function.* Two plasma membrane intrinsic protein homologue genes, *SsAQP1* and *SsAQP2*, representing two separate subfamilies of aquaporins, PIP1 and PIP2, were cloned from the *Samanea* pulvinar cDNA library, and, according to their predicted protein homologies to the *Arabidopsis* aquaporins, are renamed here, respectively, *SsPIP1;5* and *SsPIP2;2*. They were characterized in *Xenopus laevis* oocytes, respectively, as a glyceroporin, and a very efficient aquaporin, the latter sensitive to $HgCl_2$ (0.5 mM) and phloretin (1 mM). The transcript levels of both in the pulvinus varied diurnally in phase with leaflet movements. Additionally, *SsPIP2;2* mRNA level was under circadian control. These results linked SsPIP2;2 to the physiological function of rhythmic cell volume changes [7].

*2.5.3. PIPs and TIPs in Mimosa: molecular identity and function.* As in *Samanea*, two plasma membrane aquaporins *MpPIP1;1* and *MpPIP2;1*, representing PIP1 and PIP2, were isolated from a *M. pudica* (Mp) cDNA library and characterized in heterologous expression systems, the frog oocytes and mammalian Cos cells. MpPIP1;1 by itself 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 [38]. However, the relation of the *Mimosa* MpPIP1;1 and MpPIP2;1 to the rhythmic movement of the pulvinus has yet to be demonstrated.

The aqueous, colloidal, vacuoles of *Mimosa* primary pulvinus harbor γ-TIP (tonoplast intrinsic protein). γ-TIP abundance, detected immunologically, increased during the maturation of the pulvinus, in parallel to the increase in the pulvinus responsiveness to stimulation – implicating this aquaporin in the increased rate of response [6]. A single TIP aquaporin gene, *TIP1;1*, was cloned from *Mimosa* cDNA library, and its product, expressed in frog oocytes, conducted water [38]. It is yet to be established, however, whether TIP1;1 is identical with the *Mimosa* γ-TIP protein, which was identified immunologically by an antibody raised against a radish γ-TIP, and was implicated in the pulvinus movements through a correlation between its increasing abundance and increasing movement capability of the pulvinus during its maturation [6].

## 3. Signaling

The membrane transporters of the Osmotic Motor – the proton ATPase and the K and Cl channels are the end points in the signalling cascades regulating pulvinus movements. The cascades originate in the circadian clock, in stimuli of light, hormones and temperature. This regulation occurs at both transcriptional and posttranslational levels. Explicit regulation by the clock of the transcript level of the candidate K channels and water channels of *Samanea*, has been described above under the respective "molecular identity".

Light has a profound effect on leaf movement, both rhythmic movement and stimulated ("acute") responses. Red, far-red and blue light have different effects (reviewed by [1,2]). The specific effects of light on the $H^+$-pump and the plasma membrane K channels are described below.

### 3.1. Regulation at the level of the Osmotic Motor elements – the cascade end

*3.1.1. Regulation of the $K_D$ channel by light.* Suh et al. [39] demonstrated, using patch-clamp, an increase in the $K_D$-channels activity in cell-attached membrane patches of intact *Samanea* flexor protoplasts within a few-minutes illumination with blue light (BL). In the same membrane patches $K_D$-channel activity subsided within a few minutes of darkness preceded by a brief red-light (R) pulse [39]. This BL effect, reversible by R + dark (Fig. 2A), appeared to consist independently of two processes: (1) $K_D$ channel activation due to membrane depolarization; this resembled depolarization measured with microelectrodes in flexor cells in intact tissues in response to white light, which also unfolds leaflets, as does BL [40]; the depolarization resulted, in turn, at least partially, from a blue-light-induced arrest of the proton pump [39], and, (2) increase of $K_D$-channel activity *in*dependently of depolarization [39].

*3.1.2. Regulation of the $K^+$-influx channel by light.* In continuous darkness, the activity of the $K^+$-influx channel in *Samanea* protoplasts waxed and waned in a circadian rhythm, such that in extensors it was one-half of a cycle out of phase with that in flexors; this was inferred from changes of plasma membrane potential in response to pulses of elevated external $K^+$ concentration, as reported by a fluorescent probe (DiS-C3(5)). Depolarization reported by the dye signified high $K^+$ permeability, [16]. During a 21 h-long period in the dark, elevated channel activity coincided with "anticipation" of a "swelling" signal, and with the entry of $K^+$; in extensors, this occurred towards morning and in flexors – towards evening [17]. Throughout the normal day/night cycles, dark opened flexor $K^+$-influx channels only when preceded by R pulse, but a subsequent far-R stimulus abolished opening in the dark (Fig. 2C). In extensors, dark closed and white or BL opened $K^+$-influx channels, and R had no effect (Fig. 2D, F).

Very interestingly, during normal day/night cycles, the *responsiveness* of $K^+$-influx channels of flexors to BL stimuli changed rhythmically. During hours 10–12 of the day, only white light, but not BL, closed $K^+$-influx channels (Fig. 2C). However, during the second half of the night of the normal day/night cycle, the closure of $K^+$-influx channels by BL became "enabled" (Fig. 2E). This intriguing behaviour invites hypotheses about the nature of the photoreceptors and also about clock-channel coupling.

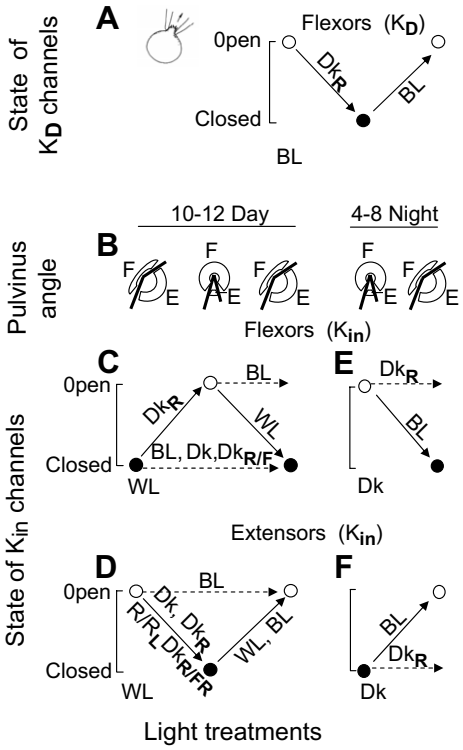


Fig. 2. The responsiveness of *Samanea* pulvinar $K_D$ and $K_{in}$ channels to light. (A) The activity of $K^+$-release ($K_D$) channels measured directly by patch-clamp in a cell-attached configuration (inset) from flexor protoplasts. BL: blue light, WL: white light, Dk: dark, $Dk_R$: dark preceded by R, $Dk_{R/FR}$: dark preceded by red and far-red light, $R/R_L$: R followed by low R. Symbols: channel states ("open" = active). Arrows indicate direction of change, dashed arrows – no change (based on Fig. 7C, Suh et al. [59]). (B) The state of the pulvinus: open or closed; note the angle between the rachis and rachilla (e.g., as in [28]); incidentally, the *Mimosa* primary pulvinus bends down, not up, upon BL stimulus; note also the relative volumes of pulvinar flexor (F) and extensor (E). (C–F) $K^+$-influx ($K_{in}$)-channel activity inferred from changes of membrane potential in flexor and extensor protoplasts. (C, D) during hours 10–12 of daylight (based on Table 1, Kim et al. [17]). (E, F) during hours 4–8 of night (dark) (based on Table 1, Kim et al. [68]). All abbreviations and symbols as in A. Note the similarity in light-sensitivity between A and D or A and F.

Also, notably, the BL promotion of flexor $K^+$-release ($K_D$)-channel activity in patch-clamp experiments (Fig. 2A) resembled the late-night BL promotion of extensor $K^+$-influx ($K_{in}$) channels (Fig. 2D, F). This resemblance may stem from the same signaling cascade coupled to different channels in the oppositely functioning motor cells.

*3.1.3. An "identity question".* It is not clear whether $K^+$-influx channels of Fig. 2 are equivalent to the voltage-dependent $K_H$ channels, or the voltage-*in*dependent 'leak' pathways of Yu et al. [29], or to both. That a voltage-*in*dependent $K^+$-pathway – in addition to $K_H$ channels – could be activated by BL, is hinted in the induction of $K^+$ permeability by BL, even when BL could not hyperpolarize the cell through the $H^+$-ATPase activity because the pump was inhibited by vanadate [16].

### 3.2. Light photoreceptors – the initiation of osmotic changes

Light, perceived by plant pigments, is the most obvious and easily quantifiable stimulus for osmotic changes.

*3.2.1. Phytochrome and phytochrome-mediated responses.* A hallmark for phytochrome-mediated signaling is the mutual

reversibility between R – or, sometimes, BL – and far-R, manifesting the two inter-converting forms of phytochrome, Pr and Pfr (recently reviewed by [41]). Phytochrome mediates the efficient phase-shifting of leaf-movement rhythms in various plants, e.g. in *Samanea* and *Albizzia* (reviewed by [42]). In *Samanea*, phytochrome mediated alternations between hyperpolarization and depolarization of flexor cells, measured with microelectrodes, in response to alternating illumination of a whole darkened flexor (respectively) with red and far-red light [40]. Phytochrome in its Pfr form enhanced leaf closure – and the underlying swelling of flexor cells in the pulvinus as well as of isolated flexor protoplasts (reviewed by [2], and see also [18]). Moreover, the shrinking of pulvinar extensors was thought to require Pfr [2], but in isolated extensor protoplast Pfr appeared to be unnecessary [16,17].

In the pulvinar protoplasts of *P. vulgaris*, Pfr had to be present for the shrinking response to be induced by BL, reminiscent of the interaction between PHYB and cryptochrome (CRY2), a blue-light receptor, documented in *Arabidopsis* [43]. Far-red light abolished the blue-light responsiveness, R, preceding the blue, restored it [44].

*3.2.2. Molecular identity of phytochrome.* In contrast to the five types of *Arabidopsis* phytochrome – phytochrome A (PHYA) through phytochrome E (PHYE) – only two have been suggested so far in pulvini. There is only one direct experimental hint – immunological evidence from *Robinia* – in favour of PHYA [45]. On the other hand, the range of low-fluence irradiance effective in evoking responses in the pulvinar cells (1–1000 fmol $m^{-2}$ $s^{-1}$ of light, e.g. [17]) classifies the pulvinar phytochrome as PHYB [41].

*3.2.3. Phytochrome localization.* In *Robinia* pulvinus, mustard (*Sinapis alba* L.) anti-PHYA antibody specifically localized phytochrome to cortical motor cells in both extensors and flexors [45]. This is in contrast to the reported lack of red-light responsiveness in *Samanea* extensor protoplasts, but supported with regard to flexors, by demonstrating red/far-red responsiveness in isolated *Samanea* flexor motor cell [17]. The subcellular distribution of phytochrome in both motor cell types did not appear to be confined to (or excluded from) any specific compartment [45].

*3.2.4. Blue light photoreception.* BL, perceived by an unknown photoreceptor in pulvini, causes the shrinking of flexor cells and swelling of extensor cells, causing nastic leaf unfolding in *Samanea* and *Albizzia* (Fig. 2B), and it can also shift the rhythm of leaf movement, although this requires illumination of a few hours (reviewed by [42]).

In contrast to *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 as seen in solar tracking in *Phaseolus* (reviewed by [5]). Consistent with this, in protoplasts isolated from the *P. vulgaris* laminar pulvinus, BL evoked shrinking, without distinction between the extensor and flexor cells, but, reportedly, this required the presence of the far-R-absorbing form of phytochrome, i.e., R pre-illumination [44].

The strict requirement for phytochrome for the blue-light responsiveness [44] needs to be reconciled with the lack of "red peaks" in the action spectra of (a) the depolarization recorded in the *P. vulgaris* laminar pulvinus, concomitant with the initiation of the "shrinking cascade", and (b) the diaheliotropic and paraheliotropic movements of the pulvini of unifoliolate leaves of greenhouse-grown soybean (*Glycine max*) seedlings; the first contained the characteristic three "blue peaks" at 380, 420 and 460 nm [46], and the second, "blue peaks" between 410 and 440 nm and between 470 and 490 nm [47]. Thus, spectroscopic studies, with the above-noted reservations, suggest that the pulvinar blue-light receptor is similar to the receptor involved in the general phototropic responses (as reviewed by [48]).

*3.2.5. Molecular identity of blue light photoreceptors.* Recently, three genes of phototropins, *PvPHOT1a*, *PvPHOT1b* and *PvPHOT2*, have been cloned from the *P. vulgaris* pulvinus, and their protein products demonstrated to be the pulvinar blue-light receptor(s) for the acute responses [49]. They are probably expressed in the plasma membrane, as are their *Arabidopsis* homologues, PHOT1 and PHOT2 [50].

*3.2.6. Phosphorylation of the proton pump.* The three isophototropins of the *P. vulgaris* pulvinus were identified as the first element in the phototransduction cascade of shrinking pulvinar motor cells [49]. Upon the perception of a 30-s pulse of BL they underwent autophosphorylation and caused dephosphorylation of the plasma membrane $H^+$-ATPase. The dephosphorylation of the $H^+$-ATPase upon BL stimulation in the *P. vulgaris* pulvinus was precisely the reverse of that occurring in the guard cell, where BL stimulated $H^+$-ATPase phosphorylation and activated the $H^+$-ATPase (see references in [49]). This is in accordance with the opposite effects BL has in these two systems: a shrinking signal and a swelling signal, respectively. Such contrast – at the level of $H^+$-ATPase activity – was manifested also in the reversed reactions to BL illumination in flexors and extensors of *Samanea*: activation of $H^+$ secretion in *Samanea* extensors and a decrease of $H^+$ secretion in *Samanea* flexor, albeit, after a transient increase in activity [51].

*3.2.7. Questions about pulvinar photoreceptors.* It is not clear whether other blue-light photoreceptors, such as CRY, or a flavin-binding PIPl-type aquaporins [52] are also involved in pulvinar blue-light responses. While green light has recently been shown to affect stomatal movement [53], it has so far been considered a neutral "safe light" in studies of pulvini. Similarly obscure is the involvement of the pulvinar photoreceptors in regulating the rhythmic movements through the clock input vs. mediating the directly light-stimulated responses. These links may, or may not be identical to those currently emerging in *Arabidopsis* research (e.g., [54]) and in particular, in research on stomatal guard cells [55]. Elucidating these links in pulvinar motor cells depends critically on the availability of molecular and genetic resources for plants with reversibly moving leaves, similar to the genome sequence that has made rapid progress possible in *Arabidopsis*.

### 3.3. Intermediate steps – channel phosphorylation

*3.3.1. In-situ phosphorylation of the $K_D$ channel.* Suh et al. [39] suggested phosphorylation as the voltage-*in*dependent mediator of the above blue-light effect. That a certain degree of phosphorylation is indeed required to support $K_D$- channels activity has been already inferred from earlier patch-clamp experiments [56]. In these experiments, $K_D$-channels activity required the simultaneous presence of $Mg^{2+}$ and ATP – or its hydrolysable analogue, ATP-γ-S, but not the non-hydrolysable ATP analogue, AMP-PNP (5′-adenylylimidodiphosphate) – at the cytoplasmic surface of the plasma membrane.

Furthermore, $K_D$-channels activity was blocked reversibly by H7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine), a broad-range kinase inhibitor [56]. Later experiments – autophosphorylation of plasma-membrane-enriched-vesicles originating in *Samanea* pulvinar cells – supported directly this early notion of membrane-delimited phosphorylation [57]. Interestingly, enhancing phosphorylation further, e.g., by okadaic acid, an inhibitor of serine/threonine phosphatases types 1 and 2A, acted oppositely, diminishing $K_D$-channel activity [58]. It is yet to be seen whether the $Ca^{2+}$-induced rundown of the $K_D$-like channel in *Mimosa* pulvini [27] involves similar phosphorylation.

The targets of the phosphorylation which governs $K_D$-channel activity in *Samanea* pulvini could be proteins, perhaps the channel itself, or membrane lipids. In support of the latter, SKOR, the *Arabidopsis* "candidate-homolog" of the *Samanea* $K_D$ channel (see above), was shown to require the phosphorylation of phosphatidylinositol phosphates (PtdInsPs) for its activity, when expressed heterologously in a frog oocyte [59].

*3.3.2. In situ phosphorylation of the $K_H$ channel.* In *Samanea* pulvinar protoplasts monitored by whole-cell patch-clamp, the $K_H$-channel activity, but not the "leak", was inhibited by the phosphorylation-promoting okadaic acid, OA [57]. Low levels of phosphorylation (5 nM of OA) promoted channel activity in flexors but had no effect in extensors, whereas high levels of phosphorylation (300 nM of OA) inhibited $K_H$-channel activity in both cell types [57]. This sensitivity of $K_H$-channel activity to low-level phosphorylation, peculiar only to flexor, could be the mechanism by which the clock "gates" the blue-light sensitivity of $K^+$-influx channels, a phenomenon also limited only to flexor (Fig. 2).

*The recombinant SPICK2 protein*, raised in cultured insect cells, Sf9, could be phosphorylated *in vitro* by the catalytic subunit of the broad-range cyclic-AMP (cAMP)-dependent protein kinase (PKA [57]). This finding hints that SPICK2 undergoes phosphorylation also endogenously, though not necessarily by PKA. Thus, the reported effects of OA on $K_H$ channels could be the reflection of SPICK2 phosphorylation *in situ*.

*3.3.3. Aquaporins.* The water permeability of the heterologously expressed MpPIP1;1/MpPIP2;1 complex of *Mimosa* (see above Section 2.5.3.) increased in parallel to its phosphorylation [38].

### 3.4. Intermediate steps – $Ca^{2+}$ and Ca channels

*3.4.1. Responses to pharmacological manipulation of calcium.* Cytosolic $Ca^{2+}$ has been the central focus in most studies of signalling in the pulvinus, with attempts to confirm it as part of the phosphatidylinositol (PtdIns) signalling pathway. The possible target effectors of $Ca^{2+}$ may be calmodulin, actin and annexins; these have only begun to be examined in pulvini.

Applying effectors of $Ca^{2+}$, such as EGTA, or calmodulin, or calcium mobilization antagonists, to pulvini interfered with their movement rhythms as well as with their acute movement responses to illumination [60,61]. All these antagonists produced phase response curves (PRCs), i.e., advances and delays in the rhythm phase at different phases of the cycle. These PRCs were somewhat similar in shape to the PRC produced by 2-h pulses of BL [60,61]. Interestingly, applying agents presumed to *increase* the internal $Ca^{2+}$ concentration, such as calcium ionophore A23187 and, separately, 2-h pulses of 10 mM $CaCl_2$, created PRCs almost identical to the PRC of 15 min of R, opposite to blue-light PRC [60,61].

In isolated extensor protoplasts of *Phaseolus coccineus*, swelling and shrinking in a regime of 9 h light/15 h dark, which paralleled their expected behaviour in the intact pulvinus [62], light-induced swelling required $Ca^{2+}$ influx from the surrounding medium, and evoking $Ca^{2+}$ influx in the dark mimicked the "light-on" signal. In contrast, dark-induced shrinking required $Ca^{2+}$ release from internal stores, but evoking rise in internal $Ca^{2+}$ in the light did not substitute for a "light-off" signal [62].

*3.4.2. Phytochrome and $Ca^{2+}$.* Phytochrome has been shown directly to increase cytosolic $Ca^{2+}$ in other systems, such as in etiolated wheat leaf protoplast, where this was associated with protoplast swelling [63]. However, such evidence has yet to be obtained for pulvinar cells.

*3.4.3. Phototropins and $Ca^{2+}$.* 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 modified significantly when protoplasts were incubated in a nominally calcium-free medium and was not inhibited by calcium influx blockers ($LaCl_3$ and nifedipine), arguing for a mobilization from intracellular stores [64]. This resembles the phospholipase C (i.e., PtdIns-pathway)-mediated response initiated at phot2 in de-etiolated *Arabidopsis* seedlings [50].

*Circadian $Ca^{2+}$* oscillations were documented, so far, only in tobacco *Nicotiana plumbaginifolia* and in *Arabidopsis* seedlings, most likely in synchrony with the growth movements of the cotyledons (see review by [65]). They need yet to be demonstrated in the mature pulvinar cells, where they would seem inevitable, in view of the strong evidence for the involvement of $Ca^{2+}$ in the rhythmic movements (see above).

*3.4.4. PIs (phosphatidylinositides) in the leaf-moving motor cells.* $Ca^{2+}$ mobilization from internal stores has been often attributed to the activation of the PtdIns pathway, in particular to the hydrolysis of $PtdInsP_2$ (phosphatidylinositol 4,5-bisphosphate) by phospholipase C-δ (PLCδ) into diacylglycerol (DAG) and $IP_3$ (inositol 1,4,5-trisphosphate, $Ins(1,4,5)P_3$). In the *Samanea* pulvinus, this has been confirmed by pharmacological agents, such as PLC inhibitors, and also in direct lipid assays (reviewed by [18,66]).

Fifteen seconds of white-light accelerated the turnover of phoshphoinositides in the *Samanea* pulvinar tissues [67]. Furthermore, in *Samanea* pulvinar protoplasts, cell-shrinking stimuli applied at the appropriate circadian time (see Fig. 2B–F) increased $IP_3$ levels, paralleling closure of the $K^+$-influx channels (Fig. 2 and [17,68]). These "shrinking signal" effects were inhibited by neomycin (10 μM), an inhibitor of $PtdInsP_2$ hydrolysis [18,62,68], and mimicked by mastoparan, a G-protein activator [18,68]. This suggested that a phospholipase C-catalyzed hydrolysis of phosphoinositides, possibly activated by a G protein, was an early step in the signal-transduction pathway by which BL and darkness closed $K^+$-influx channels in *Samanea* flexors and extensors, respectively [68]. Preventing the dark-induced shrinking by 8-(*N*,*N*-diethylamino)octyl-3,4,5- trimethoxybenzoate (TMB-8), inhibitor of $IP_3$-receptor binding in animals, supported $IP_3$ involvement as a mediator of cell-shrinking stimuli also in cycling *P. coccineus* extensors [62].

*3.4.5. $Ca^{2+}$-permeable channels in the plasma membrane.* While the plasma membrane K and anion channels are presumed to conduct the major fluxes of osmoticum

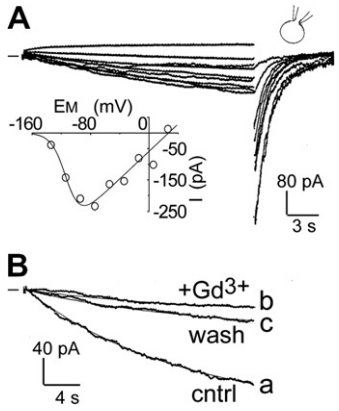


Fig. 3. $Ca^{2+}$-permeable channels in *Samanea* extensor. (A) Membrane currents (superimposed traces) recorded from *Samanea* extensor protoplasts by patch-clamp in a whole-cell configuration. The currents were evoked by depolarizing voltage steps from a holding potential of −154 mV to between −134 and 26 mV, in 20 mV intervals. (Inset) symbols: Current–voltage ($I$–$E_M$) relationship of the asymptotic values of the current, calculated separately from each trace by a single-exponential fit; the resulting steady-state $I$–$E_M$ was further fitted between −154 and −40 mV with the following equation:

$$I = G_{max}(E_M - E_{rev})/(1 + e^{(E_{1/2}-E_M)zF/RT}), \quad (1)$$

and with the following parameter values: $G_{max}$ (maximum conductance) = 2.2 nS, $E_{rev}$ (reversal potential) = 26.7 mV, $E_{1/2}$ (half-activation potential) = −112 mV, and $z$ (the effective gating charge) = 2.6. Solutions (mM), bath: $K^+$ = 6,$CaCl_2$, MES = 10, pH 6.0, osmolarity adjusted with sorbitol to approx. 600 mOsm; pipette: NMG-MES: 125, HEPES: 20, $K^+$: 4, MgATP: 1, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA): 1, $CaCl_2$ 0.25 (final free [$Ca^{2+}$]: 30 nM), pH 7.2, sorbitol, to final osmolarity of approx. 650 mOsm (Expt. S950715, representative of over 20 similar experiments, N. Moran, unpublished). (B) Current block by $Gd^{3+}$. Traces of current (superimposed) elicited by repeated (10 min apart) depolarizing voltage steps from −148 mV to −78 mV, were recorded before (a, cntrl), after (b) a gentle puff from an adjacent pipette with 200 mM $GdCl_3$ in bath solution briefly lowered into the bath, and after (c, wash) pipette removal. The block and the partial recovery are superimposed on a strong, characteristic currents rundown, roughly 60% in 10 min. Lines superimposed on the traces: fitted exponentials with identical parameters:

$$-24 - 184f(1 - e^{-t/18}) \quad (2)$$

with a different scaling factor for each trace (accounting for the rundown) $f$(a) = 1, $f$(b) = 0.19 and $f$(c) = 0.34. Solutions as in A, except, in the pipette, $MgCl_2$ 2mM, $CaCl_2$ 0.45mM (free [$Ca^{2+}$]cyt = 110 nM), and $IP_3$ 12.5 μM). (Expt. S9608c03-5, N. Moran, unpublished).

(KCl) across the plasma membrane, other channels, such as $Ca^{2+}$-permeable channels, are likely to participate in signaling. Using the rundown of $K_D$-like channels as indirect evidence for the influx of $Ca^{2+}$, Stoeckel et al. demonstrated the function of hyperpolarization-activated $Ca^{2+}$-permeable, and $La^{3+}$- and $Gd^{3+}$-blockable pathways (channels?) in the plasma membrane of extensor protoplasts from the primary pulvinus of *Mimosa* [27]. Interestingly, $Gd^{3+}$-blockable inward currents, likely carried by $Ca^{2+}$, and possibly also by $K^+$, were observed in *Samanea* extensor protoplasts at a range of hyperpolarizing membrane potentials (Fig. 3; Moran, unpublished). The still rather enigmatic fast-running-down channels underlying these currents, observed in a non-negligible fraction of extensor protoplasts, but not in flexors, were activated, in fact, by *de*polarizations *above* −160 mV, with a maximum influx of $Ca^{2+}$ expected to occur around −180 mV (Fig. 3), in accordance with the presumed absolute voltage range of inward $Ca^{2+}$currents in *Mimosa* [27].

*3.4.6. Molecular candidates.* Annexins are $Ca^{2+}$-, phospholipid- and protein-binding proteins, conserved evolutionarily between plants and animals, with an increasingly broad range of signalling functions revealed to date, including extracellular reception ([69], and references therein), and nucleotide-induced oligo- (possibly tri-) merization to form active ion channels of unspecified selectivity [70]. Annexins could be the molecular counterparts of the hyperpolarization-activated Ca channels. Annexins may also mediate $Ca^{2+}$ effects. Annexin protein isolated from *Mimosa* was found to bind *in vitro* to a phospholipid and to F-actin in the presence of calcium, its abundance being developmentally regulated. In the primary pulvinus during daytime, the amount of annexin increased with ABA concentration between 1 and 75 μM, but was not affected by cold nor by mechanical stimuli. Annexin abundance increased also at night, and its distribution changed from the cell periphery during the daytime to cytoplasmic at night [71]. It is thus interesting that, while actin, which binds to annexin, is thought to be involved in the seismonastic function of this pulvinus, annexin appears to be associated rather with nyctinastic transitions.

*3.4.7. Glutamate receptor channels (GLRs).* The *Samanea* pulvinar cDNA library yielded a clone of *SPIRL1*, a homologue of glutamate receptor-channels with a predicted sequence about 60% identical to several genes of the *Arabidopsis* GLR2 gene family [72]. Since glutamate invoked influx of $Ca^{2+}$ into roots of *Arabidopsis*, and an even greater $Ca^{2+}$ influx into *Arabidopsis* expressing a GLR from small radish [73], SPIRL1 too is a plausible candidate for a $Ca^{2+}$-permeable channel in pulvinar cells. Moreover, since a homologous glutamate-gated animal channel is also mechanosensitive [74], and since mechanical stimulation (touch) upregulated the expression of an *Arabidopsis* GLR (AtGLR3.4, [75]), the *Samanea* putative GLR-channel, SPIRL1, might be involved also in transduction of mechanical stimuli.

*3.4.8. Stretch-activated (mechano-sensitive) ion channels (SACs) in the plasma membrane.* Stretch-activated channels (SACs), discovered by patch-clamp in cell membranes in both pro- and eukaryotes (reviewed by [76]), were observed also in *Samanea* flexor and extensor protoplasts, upon suction via the patch-pipette, during and after the formation of a giga-seal, and also in excised membrane patches. Membrane-stretching pressure pulses less than 30 mm Hg, well within the physiological range of estimated turgor values occurring in pulvini [77] activated reversibly channels of undefined selectivity in outside-out patches: $Ca^{2+}$-permeable or anion-selective, but not specifically $K^+$-selective [18].

*3.4.9. SACs identity.* In animals, SACs appear among glutamate-gated channels, tandem-pore-domain K (TPK), transient receptor potential (TRP) and the epithelial Na channels (reviewed by [78]). Of these, only the first two have been found in plants: GLRs, mentioned above, and TPK channels (e.g. [34]). A TPK4 homologue, SPOCK1 is expressed in *Samanea* pulvini [28]. Because of this homology, and because the *Arabidopsis* pollen TPK4 channel homologue is expressed in the plasma membrane [34], SPOCK1 might be considered a candidate SAC.

Lastly, since a bacterial mechanosensitive channel small (MscS)-like protein has been identified in *Arabidopsis* – albeit, so far, only in plastids [79] – a similar protein might

perhaps function as a SAC in the pulvinar cells plasma membrane.

*3.4.10. Selective SACs inhibition.* $Gd^{3+}$ has been often used as a selective SACs blocker, although its mode of action is unclear, and although in some TRP-type animal channels it has been shown to have dual effects, activating the channels at low micromolar concentrations (<100 μM), but blocking at higher concentrations (>300 μM [78]). The block by $Gd^{3+}$ of at least two types of channels in *Samanea* motor cells – the $K_D$ channel [25] and the $Ca^{2+}$ - permeable channel – seems to suggest they are mechanosensitive. This question awaits better SACs-specific reagents, such as SACs activator, Maitotoxin (a highly potent marine poison from a dinoflagellate), or GsMTx-4 (a 34-amino acid tarantula venom peptide), the most specific SACs blocker identified to date [78].

At present, one can only speculate about the physiological function of SACs in the motor cells. The prototypic, best characterized bacterial stretch-activated channels act as burst-preventing osmoticum-releasing "safety valves" activated upon cell volume increase (reviewed by [76,78]). A role in $Ca^{2+}$ signaling may be envisaged for the pulvinar SACs, whether they are cation ($Ca^{2+}$)-permeable or anion-permeable. They might be activated in a turgid pulvinar motor cell by local distortions in the plasma membrane appressed against the cell-wall microfibrils in the course of swelling, or due to a touch, and initiate a "shrinking cascade" by raising internal [$Ca^{2+}$], or by depolarization, or both.

*Cytoskeletal involvement* – a possible mediation of $Ca^{2+}$ effect on leaf movement – has been suggested to explain the rapid seismonastic response of the primary pulvinus of *Mimosa*. Pharmacological tools differentiating between actin and tubulin, in combination with physiological assays and immunocytochemistry helped to single out actin as important for this movement [80]. Actin may regulate pulvinar ion channels as in guard cells (e.g. [81]).

## 4. A mosaic to complete

The light-signalling transduction steps converging on the "osmotic motor" of a pulvinar flexor cell have been outlined schematically in Fig. 4. In the known details, extensor cells seem to conform quite well to guard cell models, although the paucity of information about the extensors is particularly striking in view of the wealth of details about the workings of the guard cell (e.g. [55], and references therein). The factual and the hypothetical processes have been linked in a following scenario of the flexor cell volume changes (Fig. 4):

(i) Stretch-activated channels (SACs) initiate shrinking by causing $Ca^{2+}$ influx (increasing cytosolic [$Ca^{2+}$]) and/or $Cl^-$ efflux [18]; either one leads to depolarization, thus opening $K_D$ channels [20–22]; (ii) BL initiates the PI cascade (reviewed by [18,66]), possibly via a G-protein, causing $Ca^{2+}$ mobilization from internal stores, e.g., in flexor-like *Mimosa* pulvinar protoplasts [64]. (iii) BL, acting via phototropins, dephosphorylates the proton pump [49], possibly by a $Ca^{2+}$-dependent phosphatase, and inhibits it [39,51,82]. (iv) Elevated cytosolic [$Ca^{2+}$] activates Cl channels in the plasma membrane, enhances activity of PLC-δ, and its own release from $Ca^{2+}$ stores, (v) Both Cl and $K_D$ channels allow sustained KCl efflux (reviewed by [3,5,18]), (vi) leading to water efflux via the membrane matrix and via aquaporins [7], and, consequently, to shrinking. (vii) The PI cascade may exert its effect on channels also directly via $PtdInsP_2$, as seen elsewhere.

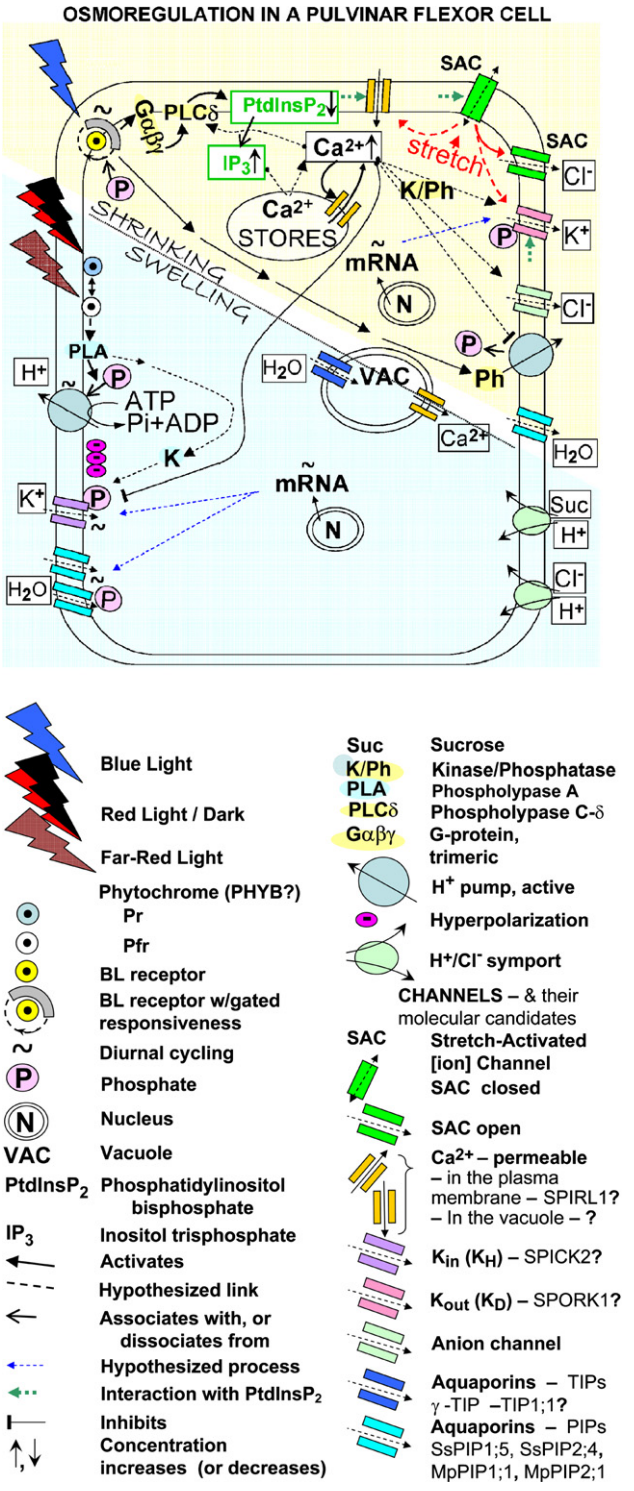


Fig. 4. Pulvinar flexor osmoregulation – a model. Top: the shrinking phase during leaf unfolding, bottom: the swelling phase, during leaf folding. $H^+$/sucrose symport is suggested to explain documented sucrose and mannitol uptake into pulvinar motor cells [87]. Below: The key for symbols and abbreviations. See text for details.

### 4.1. The swelling phase

(i) Red light, acting via a phytochrome (PHYB?) activates a vanadate-sensitive P-type $H^+$-ATPase, which hyperpolarizes the cell [40]. This, in turn (ii) activates $K^+$-influx channels [29] and (iii) creates the electrochemical gradient for the influx of $K^+$ via these channels [16,17] and (iv) the proton-motive force for the uphill uptake of $Cl^-$, possibly via a proton-anion symporters (reviewed by [3]). Eventually, $K^+$ and $Cl^-$ accumulate in the cell vacuole. In the absence of external $Cl^-$, the malate content of the swelling tissues increases [3,83]. (v) The accumulation of osmoticum drives water influx via the membrane matrix and via aquaporins [7]. In contrast to shrinking, the "swelling cascade" is not known in pulvini, apart from (vi) a requirement for calcium [62], observed also in guard cells [55], but not shown in Fig. 4. (vii) LysoPtdCh and free fatty acids – the products of phosphatidylcholine (PtdCh) lysis by phospholipase $A_2$ ($PLA_2$) – may constitute the second messengers for motor cell swelling [84]. Indeed, exogenous $PLA_2$ caused premature swelling of *Samanea* flexor protoplasts in the light, whereas the same $PLA_2$ inactivated by a short preincubation with its inhibitor, manoalide, was inactive [84].

*The circadian clock* appears to dictate the timing of responsiveness of the blue-light receptors (phototropins?), or of their coupling to the closing of $K^+$-influx channels in *Samanea* flexor protoplasts [17]. Without light cues, it regulates the activity of these $K^+$-influx channels [17], the activity of the proton pump [40], and the levels of transcripts of a few putative ion and water channel genes [7,28].

*Chemically signaling molecules* added exogenously, such as hormones (auxin (indole-3-acetic acid, IAA), gibberellin ($GA_3$), ethylene, abscisic acid (ABA) and jasmonic acid) and turgorin (sulfonated gallic acid glucoside), evoked different responses in various pulvini, such as gravistimulated leaf sheath pulvini of grasses, non-growing pulvini of legumes. In particular, auxin caused the swelling and ABA the shrinking of *Phaseolus* pulvinar protoplasts [36], but no receptors for the hormones or turgorin action are known in pulvini (reviewed in [14]).

### 4.2. Feedback into the circadian oscillator

Membrane potential and ion fluxes were formerly considered to be a part of the internal clock oscillator (e.g., see [85]). With the cloning of clock genes and the advent of molecular genetic studies of circadian rhythmicity, membrane events were relegated to roles of the Osmotic Motor, or transduction, rather than overt participation in circadian pacemaking. However, in animals, hyperpolarization of the pacemaker cells, achieved (a) in *Drosophila* putative circadian pacemaker neurons by expressing the constitutively open mutant $K^+$ channel dORK, which likely hyperpolarizes and silences spiking, and (b) in rat suprachiasmatic nucleus slices by low-$K^+$ medium, disrupted clock gene rhythm. This suggests that rather than being simple output messengers, membrane potential rhythms may feed back onto and help sustain the intracellular core clock mechanism ([86], and references therein). Careful dissection of the function and regulation of ion and water channels in the pulvinar motor cells is likely to lead to similar insights into the osmoregulation of leaf movements.

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