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Update on Signal Transduction

Signal Transduction in Leaf Movement[1]

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PLANT MOVEMENT

Plants are rooted and immobile and must accept whatever fate brings their way. They are not, however, static. Flowers open and close. Leaves extend to the daylight and fold up by night or, in shade-loving species, turn away from direct sunlight or, in *Mimosa pudica*, collapse suddenly and dramatically when touched. Modified leaves of the Venus' flytrap snap shut on doomed insect prey.

All of these macroscopic movements depend on hydraulics. Water moving into cells increases their internal pressure, or turgor, and leads to changes in cell shape and cell volume. Water moving out of cells decreases their turgor, leading to opposite changes in shape and volume. Together these changes can move structures as large as leaves, petals, and flytraps. The movement of water into or out of cells is driven by fluxes of ions. Turgor-mediated movements throughout the plant kingdom, both microscopic and macroscopic, are likely driven by identical, or at least similar, ionic fluxes (for review, see Coté et al., 1995). The best-studied macroscopic turgor-driven movement in plants is the movement of leaves in legumes. The best-studied microscopic turgor-driven movement is the opening and closing of leaf stomata—the pores through which carbon dioxide is taken up into leaves—by stomatal guard cells. Evidence suggests that the regulation of turgor changes in leaf movement is similar to the regulation of turgor changes in stomatal guard cells. As we shall see, studies of guard cells may provide clues to understanding turgor regulation in leaf movement.

The leaf-moving organ is the pulvinus, a swelling at the bases of the stalks of leaves and leaflets (Fig. 1). Two groups of cells in the pulvinus, flexor and extensor cells, are arrayed, respectively, above and below the central vascular tissue (Fig. 1). Flexor cells swell and extensor cells shrink to bend the pulvinus and flex or fold the leaf or leaflet, whereas flexor cells shrink and extensor cells swell to straighten the pulvinus and extend the leaf or leaflet to the light. These two groups of cells, visibly indistinguishable under the microscope, behave oppositely and in concert to produce leaf movement.

Ruth Satter was one of the pioneers in the study of the physiology of leaf movement, focusing her efforts on *Samanea saman*, a tropical tree of the legume family (Fig. 1). Since her untimely death from leukemia in 1989 her work has been continued by many of her colleagues and former students. Recently some of this work has provided clues to an understanding of the signaling pathways that control leaf movement.

ION FLUXES CONTROLLING LEAF MOVEMENT

The hydraulic movement of water in *S. saman* pulvini is driven by fluxes of K^+ salts (reviewed by Satter et al., 1988; Lee, 1990; Fig. 2). Pulvinar cells lose K^+ when shrinking and actively take up K^+ when swelling; as much as 60% of the total K^+ within the pulvinus moves from the flexor side to the extensor side and back again during a complete cycle of leaf folding and unfolding (Satter et al., 1982). The main counterion to K^+ is Cl^-, although malate synthesized from starch breakdown may also play a role.

K^+ enters and leaves plant cells by different channels, which are differently regulated (reviewed by Lee, 1990; Coté et al., 1995). K^+-selective channels activated by membrane hyperpolarization allow K^+ to enter cells. Active extrusion of protons by the plasma membrane ATPase hyperpolarizes cells, activating the channels and also energizing net K^+ influx. K^+-selective channels activated by membrane depolarization allow K^+ to leave cells. In guard cells, these channels are also activated by low external K^+ concentrations, and they open only when the membrane potential is more positive than the K^+ equilibrium potential (Blatt, 1988) so that K^+ moves out of the cells. Opening of anion-specific channels is believed to produce the depolarization that activates the K^+ channels and triggers K^+ efflux, since the principal cellular anion, Cl^-, is always present at higher concentrations inside plant cells than outside and therefore will flow out through anion-specific channels, depolarizing the cell. Both hyperpolarization-activated and depolarization-activated channels have been demonstrated in *S. saman* pulvinar cells by patch-clamp techniques (Moran, 1990).

TIMING LEAF MOVEMENT

Leaf movement in *S. saman* continues day after day, the leaves folding up at night and unfolding by day, but the plants are not merely reacting to light and darkness. In constant light or constant darkness they continue to move

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Abbreviations: $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate.

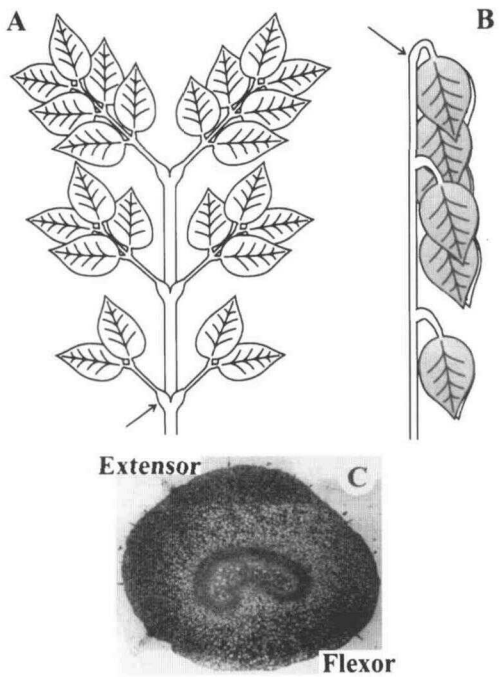

Figure 1. Leaf movement in *S. saman*. The compound leaves of the legume *S. saman* extend to the sun in the daytime (A) and fold at night (B) in a classic example of turgor-mediated plant movement. The leaf-moving organs, pulvini, are found at the base of the stalk of each leaf and each leaflet. A primary pulvinus moves the entire leaf, secondary pulvini (arrows) move the major leaflets, and tertiary pulvini move the ultimate leaflets. C, Cross-section of a secondary pulvinus. Movement is caused by alternate swelling and shrinking of flexor and extensor cells on either side of the pulvinus. Flexor cells swell and extensor cells shrink to lower the leaf or leaflet, whereas the opposite changes raise the leaf. The central U-shaped cluster of cells is the vascular tissue; it does not take part in leaf movement.

their leaves rhythmically, with a period of about 1 d (Satter and Morse, 1990). Thus, leaflet movement follows a circadian rhythm, an endogenous rhythm with a period of about 1 d. The first circadian rhythm ever described scientifically was a leaf movement rhythm of a legume, probably *M. pudica* (de Mairan, 1729). Circadian rhythms have since been found in all eukaryotes examined and in some prokaryotes; they are believed to follow an internal biochemical oscillator, often known as the biological clock.

In *S. saman* there must be at least one operating biological clock within each pulvinus, since pulvini removed from the plant and stripped of their leaves continue to bend and straighten rhythmically in constant darkness with a period of about 24 h (Satter and Morse, 1990). In fact, flexor protoplasts, released from the pulvinus by digestion of their cell walls and suspended in buffer, continue to swell and shrink with a 24-h period (Moran et al., 1995). This indicates that each individual cell within the pulvinus has its own controlling oscillator.

How does the biological clock control leaf movement? Kim et al. (1992, 1993) have taken this question down to the molecular level, asking whether the clock controls the state of the K^+ channels that let K^+ into the cell. They monitored the state of these K^+ channels, using a membrane-permeable dye sensitive to membrane potential to report depolarization produced by added K^+ ions entering the protoplasts through inward-directed K^+ channels. Although this method monitors the channels indirectly, these authors used a combination of studies of K^+ concentration dependence, ionic specificity, and inhibitor sensitivity to show that the dye was, in fact, responding to a depolarization dependent on inward-directed K^+ channels (Kim et al., 1992).

Kim et al. (1992) found that the inward-directed K^+ channels were open in flexor protoplasts and closed in extensor protoplasts during the normal dark period. This is consistent with the expected behavior of these cells in the intact plant; during the dark period flexor cells are swollen from the uptake of K^+ and water and extensor cells are shrunken, having lost K^+ and water. If the protoplasts were then maintained in constant darkness, at the time the lights would have normally come on, channels in flexor protoplasts spontaneously closed, whereas those in extensor protoplasts spontaneously opened (Kim et al., 1993). Thus, the channels were open in extensor cells and closed in flexor cells during what would have been the normal light period had the lights been turned on, consistent with the expected states of the channels in the cells of the intact plant in the daytime.

In every organism studied, the circadian clock is sensitive to light, which resets the phase of the rhythm. In particular, delaying the transition from light to darkness generally delays the rhythm by the same amount of time, as if light halted the clock at the end of the light period and darkness released it. Kim et al. (1993) showed that a 2-h extension of the light period before the protoplasts were

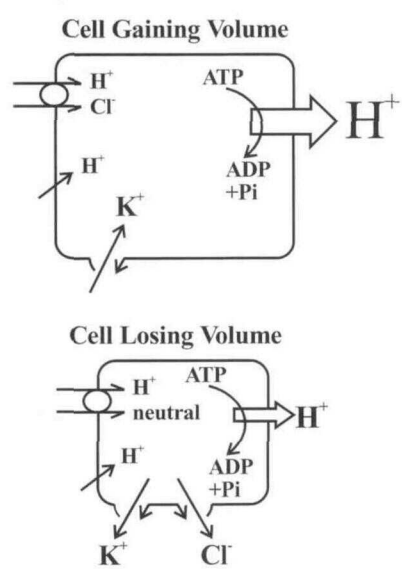

Figure 2. Ion fluxes that mediate leaf movement. K^+ and Cl^- fluxes mediate leaf movement by triggering osmotic movement of water. In a cell gaining volume the energy-dependent pumping of protons out of the cell drives K^+ uptake through specific inward-directed K^+ channels. In a cell losing volume the flux of Cl^- out of the cell down its concentration gradient drives K^+ efflux through specific outward-directed K^+ channels.

transferred to darkness delayed the subsequent channel opening and closing in constant darkness by exactly 2 h, confirming that a circadian clock was controlling the channels.

The biochemical mechanism of the endogenous clock remains unknown, and the signals by which it might control overt rhythms, such as K^+ channel state, are equally unknown. Demonstration that the inward-directed K^+ channels are rhythmically controlled by the clock opens up the possibility of tracing the signal transduction pathway backward from channel opening and closing to discover the endogenous controlling oscillator.

A COMPLEX WEB OF SIGNAL TRANSDUCTION

Leaf movements may follow an internal clock, but they are also directly sensitive to environmental signals (Satter et al., 1981). *S. saman* leaves fold up if the plant is moved prematurely from light into darkness and unfold if the plant is moved prematurely from darkness into light. This response continues in pulvini removed from the plants. Pulvini in darkness straighten when stimulated with blue light or white light, although red light has no effect. Pulvini in the light similarly bend when transferred to darkness, and a pulse of red light, simulating sunset, potentiates this effect.

Isolated pulvinar protoplasts are also responsive to light signals (Kim et al., 1992, 1993). In the dark period, the closed inward-directed K^+ channels of extensor cells are opened within 3 min by blue light, as demonstrated by a K^+-induced depolarization reported by the added fluorescent dye (Kim et al., 1992). Conversely, the channels of flexor cells, open in the darkness, are closed by blue light (Kim et al., 1992). In the light period, however, the situation is more complex (Kim et al., 1993). Premature darkness alone is sufficient to close the open channels of extensor protoplasts, but both darkness and a preceding pulse of red light are required to open the closed channels in flexor protoplasts. The effects of red light on protoplasts, like the effects of red light on the intact plant, are prevented by far-red light, which implicates phytochrome as the photoreceptor.

The web of signal transduction in light control of leaf movement is thus of great complexity. Considering the regulation of inward-directed K^+ channels alone, light of two different colors, as well as darkness, have different effects, which vary with time and between two different cell types. Preliminary progress has recently been made in puzzling out the intricacies of this information flow.

PHOSPHOINOSITIDE SIGNALING IN LEAF MOVEMENT

Ca^{2+} has long been implicated in leaf movement in legumes, although the evidence has been indirect (reviewed by Coté and Crain, 1994; Coté et al., 1995). Regulation by Ca^{2+} suggests a possible role for the second messenger $Ins(1,4,5)P_3$, a key regulator of cytosolic Ca^{2+} levels in animal cells. $Ins(1,4,5)P_3$ is produced in animal cells by the receptor-triggered hydrolysis of the lipid $PI(4,5)P_2$ by the enzyme phospholipase C. $Ins(1,4,5)P_3$ thus produced binds to a specific receptor channel, which releases Ca^{2+} from intracellular stores into the cytoplasm, and the Ca^{2+} thus released mediates at least some of the cellular responses to receptor activation.

$PI(4,5)P_2$ has been conclusively demonstrated in plant tissues, including *S. saman* pulvini (reviewed in Coté and Crain, 1993). Plant phospholipases C have been demonstrated (reviewed by Coté and Crain, 1993; see also Huang et al., 1995) and recently cloned (Hirayama et al., 1995; Shi et al., 1995; Yamamoto et al., 1995). $Ins(1,4,5)P_3$ has been shown to release Ca^{2+} from plant vacuoles (Allen et al., 1995). Given this evidence, the involvement of $Ins(1,4,5)P_3$ signaling in leaf movement is plausible.

Kim et al. (1995) presented evidence that $Ins(1,4,5)P_3$ might trigger release of Ca^{2+}, which then closes K^+ channels in pulvinar cells. They measured $Ins(1,4,5)P_3$ production in both flexor and extensor protoplasts stimulated with various light signals using a radioreceptor assay in which unlabeled $Ins(1,4,5)P_3$ in the cell sample competes with added tritiated $Ins(1,4,5)P_3$ for binding to a specific $Ins(1,4,5)P_3$ receptor isolated from bovine adrenal glands. They demonstrated that *S. saman* pulvini appear not to contain any metabolites that compete with $Ins(1,4,5)P_3$ in binding to the bovine receptor, allaying concerns that plant tissues might contain such metabolites.

In flexor protoplasts, blue light during the dark period not only closed the inward-directed K^+ channels but also increased $Ins(1,4,5)P_3$ levels 2-fold during a time course of seconds. The effect of blue light on flexor $Ins(1,4,5)P_3$ levels was specific, since $Ins(1,4,5)P_3$ levels did not increase in extensor protoplasts exposed to blue light; on the contrary, basal $Ins(1,4,5)P_3$ levels decreased. Thus, $Ins(1,4,5)P_3$ production might mediate blue-light-induced channel closure in flexor cells, whereas some other signal transduction pathway must mediate blue-light-induced channel opening in extensor cells. It is tempting to speculate that some basal $Ins(1,4,5)P_3$ level might maintain inward-directed K^+ channels in a closed state so that a blue-light-induced reduction in basal $Ins(1,4,5)P_3$ levels might facilitate channel opening in extensor cells, but there is as yet no evidence to support this.

Closure of K^+ channels in extensor cells in the light by red light and darkness may also involve $Ins(1,4,5)P_3$, but the signaling pathway appears to be more complex than that used by blue light. A red-light pulse followed by darkness, a red-light pulse followed by dim red light, or direct transfer to darkness all lead to closure of K^+ channels in extensor cells in the light period (Kim et al., 1993). $Ins(1,4,5)P_3$ levels did increase upon transfer to darkness or dim red light, suggesting that $Ins(1,4,5)P_3$ might close K^+ channels during these treatments (Kim et al., 1995). However, the absolute level of $Ins(1,4,5)P_3$ cannot be the sole determinant of channel closure, because $Ins(1,4,5)P_3$ levels decreased during the preceding red-light pulse, and the levels following the final transfer to darkness or dim red light were not greater than the levels preceding the red-light treatment. This may reflect compartmentalization of $Ins(1,4,5)P_3$ so that some of the $Ins(1,4,5)P_3$ detected may not be active in signal transduction. Compartmentalization

of some portion of cellular Ins(1,4,5)P_3 has been suggested previously (reviewed by Balla and Catt, 1994). In any event, the changes in Ins(1,4,5)P_3 levels were specific since none of the treatments affected Ins(1,4,5)P_3 levels in flexor cells, in which they did not trigger channel closure.

The antibiotic neomycin is an inhibitor of PI(4,5)P_2 hydrolysis by phospholipase C; it is presumed to bind the substrate and sequester it from the enzyme. Not surprisingly, 10 μM neomycin blocked blue-light-induced Ins(1,4,5)P_3 production in flexor protoplasts and darkness-induced Ins(1,4,5)P3 production in extensor protoplasts. At the same concentration it prevented closure of inward-directed K^+ channels both in flexor protoplasts stimulated with blue light and in extensor protoplasts stimulated by transfer to darkness (Kim et al., 1995). It had no effect on K^+ channel opening induced by any light treatment of either type of protoplast. This strengthens the correlation between Ins(1,4,5)P_3 production and channel closure and suggests that the Ins(1,4,5)P_3 is produced by phospholipase C action on PI(4,5)P_2.

In animal cells, G proteins carry information from the signal-activated receptor to activate phospholipase C. The effects of mastoparan on protoplasts suggest that G proteins might similarly mediate blue-light- and darkness-induced changes in Ins(1,4,5)P_3 levels in protoplasts (Kim et al., 1995). Mastoparan, a tetradecapeptide toxin from wasp venom, is a potent nonspecific activator of G proteins. In the dark period, 10 μM mastoparan mimicked blue-light illumination in its effects on flexor cells; it increased Ins(1,4,5)P_3 levels 2.7-fold and closed the open K^+ channels. Similarly, in the light period mastoparan mimicked darkness in its effects on extensor protoplasts; it increased Ins(1,4,5)P_3 levels about 2-fold and closed the open K^+ channels. In each case the effect of mastoparan on Ins(1,4,5)P_3 levels was blocked by neomycin, suggesting that mastoparan was activating a G protein-dependent phospholipase C.

Of course, mastoparan may have effects other than activation of G proteins (see refs. in Kim et al., 1995); it is known to antagonize calmodulin at micromolar levels, and, at higher concentrations, it can directly activate phospholipase C and disrupt cell membranes. In fact, it now appears that membrane damage rather than G protein activation is the explanation for inhibition of Golgi transport by micromolar levels of mastoparan (Weidman and Winter, 1994). It is thus not possible to say with certainty that mastoparan activates G protein(s) in pulvinar protoplasts. Nonetheless, the effects of mastoparan again link Ins(1,4,5)P_3 production and channel closure, strengthening the case for a causal relation between these phenomena.

It is interesting that mastoparan also opened K^+ channels, mimicking the effects of blue light on extensor protoplasts in darkness and the effects of darkness on flexor protoplasts in the light (Kim et al., 1995). In extensor protoplasts at least, Ins(1,4,5)P_3 levels were not increased when mastoparan triggered channel opening. This suggests that G proteins not linked to phospholipase C might regulate inward-directed K^+ channel opening, either directly or through another signaling pathway. Evidence for direct regulation of K^+ channels by G proteins in guard cells has been reported (Li and Assmann, 1993).

THE ACTION OF CALCIUM

All of these results are consistent with Ins(1,4,5)P_3 production, triggered by appropriate signals, mediating K^+ channel closure in pulvinar cells. Presumably Ca^{2+} released by the Ins(1,4,5)P_3 signal regulates the K^+ channels. How might Ca^{2+} regulate the channels? This remains mysterious, but some possibilities are suggested by studies of stomatal guard cells, in which Ins(1,4,5)P_3 production also appears to trigger closure of inward-directed K^+ channels and cause cell shrinking (Coté et al., 1995). Patch-clamp studies have shown that micromolar levels of cytosolic Ca^{2+} close the inward-directed K^+ channels in guard cells of *Vicia faba* (Schroeder and Hagiwara, 1989; Luan et al., 1993), and *Zea mays* (Fairley-Grenot and Assmann, 1992). This effect of Ca^{2+} can be reversed with *V. faba* guard cells by adding the immunosuppressant drug FK506 along with its specific binding protein, FK506-binding protein, to the cytoplasmic side of the channels through the patch pipet (Luan et al., 1993). Another immunosuppressant drug, cyclosporin A, is also effective, although the addition of its specific binding protein (cyclophilin) is not required (Luan et al., 1993). In animal cells these immunosuppressant/binding protein complexes are potent inhibitors of calcineurin (also called protein phosphatase 2B), a Ca^{2+}/calmodulin-activated protein phosphatase (Liu et al., 1991; Clipstone and Crabtree, 1993).

The effects of immunosuppressants on guard cells suggest that Ca^{2+} might activate a Ca^{2+} or Ca^{2+}/calmodulin-dependent protein phosphatase, homologous to calcineurin, which dephosphorylates and inactivates inward-directed K^+ channels. Guard cells contain a Ca^{2+}-stimulated protein phosphatase activity against a synthetic peptide substrate, and this activity is inhibited by FK506/FK506-binding protein and cyclosporin A/cyclophilin complexes (Luan et al., 1993). An activated tryptic fragment of bovine brain calcineurin can substitute for Ca^{2+} in closing guard cell K^+ channels in patch-clamp experiments (Luan et al., 1993), demonstrating that the inward-directed K^+ channel is potentially a substrate for a calcineurin homolog.

The *Arabidopsis thaliana* ABI1 locus encodes a product essential for several responses to ABA, including ABA regulation of stomata (Koornneef et al., 1984). Expression of the wild-type gene in transgenic tobacco leads to loss of ABA regulation of both inward and outward K^+ channels (Blatt et al., 1995). The ABI1 gene has been cloned and shown to encode a protein with high homology to protein phosphatase 2C at the carboxyl terminus and a putative Ca^{2+}-binding domain at the amino terminus (Leung et al., 1994; Meyer et al., 1994). The ABI1 gene product has thus been proposed as a good candidate for mediating K^+ channel closure through Ca^{2+}-induced protein dephosphorylation (Coté et al., 1995), although Ca^{2+} regulation of this enzyme has yet to be demonstrated biochemically.

A MODEL FOR SIGNAL TRANSDUCTION DURING CELL SHRINKING

Current evidence thus supports the following model for one signal transduction pathway contributing to the loss of turgor pressure and consequent cell shrinking during pulvinar movements (Fig. 3). Blue light acts on flexor cells in darkness and darkness acts on extensor cells in the daylight to trigger phosphoinositide turnover, possibly through G protein-linked receptors. The resulting increase in Ins(1,4,5)P_3 levels triggers Ca^{2+} release from internal stores and high levels of Ca^{2+} trigger closure of the inward-directed K^+ channels. We can speculate that Ca^{2+} might act, as it appears to do in guard cells, by activating a Ca^{2+}-dependent protein phosphatase that dephosphorylates and inactivates the channels.

It is not enough, however, for the cell to close its inward-directed K^+ channels; outward-directed K^+ channels must also be opened. How this occurs remains to be elucidated. In *V. faba* guard cells, micromolar cytosolic Ca^{2+} enhances outward currents through anion channels in the plasma membranes. Since Cl^- levels are much higher inside the cell, these channels would permit Cl^- efflux, depolarizing the cell, even to positive potentials (Schroeder and Hagiwara, 1990), and this depolarization would activate the outward-directed K^+ channels. The net result of inactivating the inward K^+ channels and activating anion channels, thereby activating outward-directed K^+ channels, is that K^+ and Cl^- now exit the cell instead of entering, and water follows; the cell shrinks.

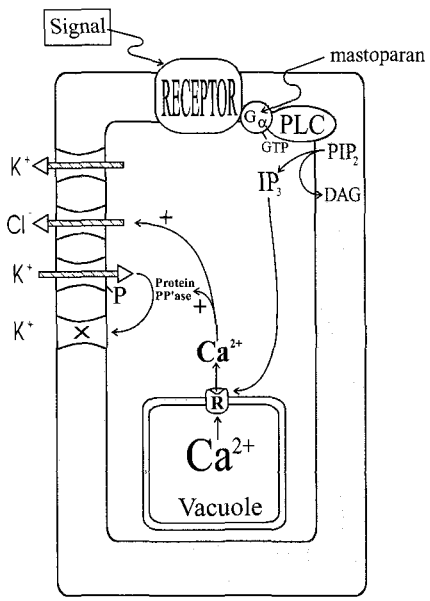

Figure 3. A model for signal transduction leading to K^+ channel closure and cell shrinking during leaf movement. Environmental signals activate a receptor that activates a phospholipase C (PLC), possibly through a mastoparan-sensitive G protein (G_α). The phospholipase C hydrolyzes PI(4,5)P_2, (PIP_2), producing Ins(1,4,5)P_3 (IP_3) and diacylglycerol (DAG). Ca^{2+} released by Ins(1,4,5)P_3 activates an anion channel that permits Cl^- efflux and inactivates the inward-directed K^+ channels, possibly through protein phosphatase (Protein PP'ase) action. Cl^- efflux activates the outward-directed K^+ channels and drives K^+ efflux. There is now evidence for each of the steps leading to Ins(1,4,5)P_3 production. Subsequent steps are speculative in pulvinar cells, although there is evidence for them during similar cell shrinking of stomatal guard cells. R, The Ins(1,4,5)P_3 receptor.

WHAT WE DON'T KNOW

How is cell shrinking regulated? Despite the recent insights described here, we are far from a complete understanding. As noted above, regulation of the outward-directed channels, which are critical to cell shrinking, has not yet been explored in pulvinar cells. Regulation of the plasma membrane ATPase, which polarizes the cell and provides the driving force for K^+ uptake and must, therefore, be inhibited during shrinking, also remains largely unexplored in these cells.

How is cell swelling regulated? We know even less about the answer to this question. The signal transduction pathways by which environmental signals trigger opening of the inward-directed K^+ channels, activation of the proton ATPase, and the other changes in ion fluxes that lead to swelling are still unknown. The effects of mastoparan suggest that G proteins are part of the signal transduction pathway, but the downstream mediators that might be activated by these G proteins are unknown. We also do not know what endogenous signals might be produced by the internal circadian clock to control the channels and lead to spontaneous swelling and shrinking and ultimately to rhythmic leaf movement. Nor do we know how environmental signals control the clock, which can be reset by red light or blue light if it drifts out of synchrony with local sunrise and sunset. Dissecting these multiple signal pathways in the two kinds of pulvinar cells, which appear identical except for their signaling pathways, will continue to challenge plant physiologists for some time to come.

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