Apoplastic transport of ions in the motor organ of *Samanea*

(circadian rhythms/leaf movements/x-ray microanalysis)

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ABSTRACT Turgor-mediated leaf movements of the legume *Samanea saman* are associated with the migration of K^+ and Cl^- between opposing sides of the motor organs (pulvini). We have investigated the pathway of this ion migration by localizing K^+ and Cl^- within the secondary pulvinus at various times during leaf movements. Ion distributions in freeze-dried cryosections of pulvini were determined by scanning electron microscopy/x-ray microanalysis. The results indicate that the apoplast is a major pathway for the migration of K^+ and Cl^- within the pulvinus.

Circadian and light-modulated leaflet movements of *Samanea* and other nyctinastic legumes result from massive rhythmic migrations of K^+ and Cl^- between opposing sides of specialized motor organs, the pulvini (1-4). These ion movements mediate localized changes in the volume of motor cells, causing the pulvinus to alter its shape. In the daytime position, pinnae (leaflets) of the bipinnate leaves of *Samanea* are separated from each other in a horizontal plane (open). At night the pinnae are folded together in a vertical position (closed). Leaflet closure occurs when cells on the flexor side of a pulvinus increase in volume while those on the opposite (extensor) side lose volume (5). These motor cell-volume changes are reversed when leaves reopen in the morning. The leaflet movements and the causal ion migrations are controlled by an endogenous clock and its interaction with light absorbed by the photo-convertible pigment phytochrome (1, 3, 6-9).

The shuttling of K^+ and Cl^- between the extensor and flexor regions of pulvini has been documented by the use of an electron microprobe (1-3). However, the technique lacked the spatial resolution necessary to assess the pathway of this solute redistribution. Because plasmodesmata (intercellular cytoplasmic bridges) were present in all examined regions of the motor tissue (10), the symplast (the living continuum) and the apoplast (the nonliving continuum of cell walls and intercellular spaces) both apparently are available for the lateral movement of ions in the pulvinus. Determining the relative importance of these two possible pathways would further our understanding of the mechanism of leaf movements.

We have investigated the route of ion migration by using scanning electron microscopy/x-ray analysis to measure the K^+ and Cl^- contents of the symplast and apoplast at various stages of leaf movements.

MATERIALS AND METHODS

Plant Materials and Growing Conditions. *Samanea saman* (Jacq.) Merrill plants were entrained to a cycle of 16-hr light/8-hr dark in a growth chamber maintained at 28°C. Illumination was from a mixture of Cool White (Sylvania F20T12.CW) and Gro-Lux (Sylvania F20T12.GRO) fluorescent lamps, with a total fluence of 20 W/m².

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Cryotechniques. Freeze-dried thin sections of pulvini were prepared by using methods designed to minimize redistribution of diffusible ions during sample preparation. Secondary pulvini, which join pinnae to the rachis, were excised from plants, trimmed to a size of about 1 mm³, and mounted on the heads of silver pins (LKB 94-92-0592) with a drop of Tissue-Tek. The pins were then inserted into a spring-loaded gun (LKB) and shot into supercooled Freon-22 (-150°C) prepared according to Somlyo *et al.* (11). Total elapsed time from pulvinus excision to freezing was 30 sec or less. The frozen samples were stored in liquid nitrogen until they could be sectioned.

Thin sections (≈100 nm) were cut with an LKB V ultramicrotome, equipped with a cryokit maintained at -75°C (specimen) and -70°C (dry diamond knife). Cryosections were collected on a polished carbon planchet (E. F. Fullam, Schenectady, NY) and then covered by a Formvar-coated copper slot grid. A second carbon planchet was placed on top of the grid to form a sandwich, and a 200-g weight was added to flatten the sections. These steps were all performed within the chamber of the cryokit; the planchets, grid, and weight were prechilled to -70°C.

We found that drying cryosections under vacuum severely disrupted the structure of the motor tissue, and therefore we dried the sections at atmospheric pressure. The cryosections, still pressed between the carbon planchets, were transferred to a prechilled glass desiccator containing phosphorus pentoxide, a desiccant with an extremely strong affinity for water. To sublimate water from the cryosections, the desiccator was stored for 16 hr at -20°C and then kept for several hours at 5°C. Finally, the desiccator was gradually warmed to room temperature. When the two carbon planchets were separated, the copper grid generally adhered to the upper planchet, with the dried cryosections exposed on the Formvar film of the grid. The planchet could then be mounted in the scanning electron microscope for x-ray microanalysis of the sectioned motor tissue.

When cryosections dried in the manner described above were analyzed, they showed ion distributions very different from the more randomized patterns we observed in control sections that were allowed to dry under conditions such that liquid water could form.

Elemental Analysis. X-ray microanalysis was performed with an AMR-1000 scanning electron microscope equipped with a United Scientific energy dispersive x-ray detector and multi-channel analyzer, interfaced with a Tracor Northern NS-880 minicomputer. The microscope was operated at 20 kV. When the instrument was used as an electron probe, the diameter of the incident electron beam was 100 nm. Potassium and chlorine

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were identified by their characteristic K_{α} peaks in x-ray energy spectra at 3.313 and 2.622 keV, respectively, and the peaks were integrated above background levels prior to analysis of the data.

RESULTS

The secondary pulvinus of *Samanea* has a central vein surrounded by 15–30 layers of motor cells (7, 10) (Fig. 1). The outer portion of the vascular core consists of three to seven layers of parenchymatous cells with unevenly thickened walls. For simplicity, we shall refer to these cells as “vascular collenchyma,” although they are more properly termed “collenchymatous parenchyma” (12). We analyzed the K^{+} and Cl^{-} content of six regions of pulvinar cross sections: the outer cortex, inner cortex, and vascular collenchyma of the extensor side, and comparable regions of the flexor side. The functional extensor–flexor axis of the pulvinus is offset by about 40° from the adaxial (upper)–abaxial (lower) axis (Fig. 1). Consequently, during leaflet closure, paired pinnae move in two planes: toward one another, as well as downward (2). Our cryosections provided complete epidermis-to-epidermis transects along the extensor–flexor axis.

Adequate preservation of plant tissue during freezing, cryosectioning, and sublimation of tissue water presents severe technical difficulties. Our preservation was adequate to allow a clear distinction between cell walls and protoplasts in many cells (Fig. 2), although we were unable to distinguish vacuolar from cytoplasmic compartments because of structural damage. We analyzed only those cells in which the protoplast/wall distinction was obvious. Fig. 3 shows contrasting x-ray spectra from the protoplast and bordering wall of an outer cortical cell.

To assess the relative distribution of ions between the sym-

plast and apoplast, we compared the x-ray emissions from K^{+} and Cl^{-} in protoplasts and in walls of motor cells in different regions of the pulvinus. To ensure that the wall areas we probed did not contain plasmodesmata, we carefully avoided pit fields. Virtually all plasmodesmata in the *Samanea* pulvinus are localized in these thin regions of the wall (10), which were readily detectable in our freeze-dried sections.

The ion distributions in pulvini frozen during the open (hour 3 of the “daytime”) and closed (hour 3 of the “night”) portions of the diurnal cycle are compared in Fig. 4. Both K^{+} and Cl^{-} were concentrated in the extensor region when leaves were open and in the flexor region when leaves were closed. This was true of the vascular collenchyma as well as the motor cells of the inner and the outer cortex. The migration between opposing sides of the pulvinus was more pronounced for K^{+} than for Cl^{-} . Asymmetry of ion content between extensor and flexor sides of the pulvini applied to both the cell walls and protoplasts. The possibility of some ion leakage between protoplasts and walls during tissue preparation must be considered. Nevertheless, extensor and flexor collenchyma cells with nearly equivalent internal K^{+} had very different amounts in their respective cell walls (see Fig. 4, data from closed leaflets).

In order to study the pathway of ion migration from extensor to flexor side, we compared the ion distributions in pulvini from open leaves to those in pulvini that were in the process of closing (Fig. 5). At hour 3 of the light (“daytime”) period, pulvini were excised and frozen. The plant was then placed in the dark, a treatment that induces leaf closure (2, 6). Pulvini were excised and frozen after 20 min, when the rate of dark-induced closure was greatest.

FIG. 1. Light micrograph of a transverse section of the secondary pulvinus of *Samanea*. The extensor–flexor axis is displaced by approximately 40° from the adaxial–abaxial axis. The vein has a cap of thick-walled parenchyma cells. (From ref. 7, reprinted with permission of the publisher; micrograph courtesy of J. Chaudhri.)

FIG. 2. Scanning electron micrograph of cryosectioned pulvinar tissue. The protoplasts (P) can be distinguished from the cell walls (W). These cells are in the vascular collenchyma which surrounds the vein. ($\times 7350$.)

The closing movement was associated with a pronounced increase in the K^+ content of the cell walls in all regions of the pulvinus (Fig. 4). There was a similar, but less striking, change in the Cl^- distribution. Note the large gradient in the K^+ content of the apoplast along the extensor-flexor axis, with K^+ concentration highest in the outer cortex of the extensor region. The increase in the K^+ content of the apoplast during dark-induced closure was concomitant with a loss of K^+ from the protoplasts of extensor cells and an increase in the K^+ content of the protoplasts of flexor cells.

DISCUSSION

Our results corroborate earlier investigations (2, 3) demonstrating that the leaf movements of *Samanea* are associated with the migration of K^+ and Cl^- between extensor and flexor regions of pulvini (Fig. 4). We now propose that the apoplast is a major route for this lateral transport of ions across the pulvinus. There is a large increase in the wall K^+ /protoplast K^+ ratio for cells of the extensor cortex during dark-induced closure of leaves. This change is concurrent with an increase in the flexor-protoplast K^+ /extensor-protoplast K^+ ratio. We interpret these

FIG. 3. X-ray energy spectra from the wall (A) and protoplast (B) of a motor cell in the flexor region of an open leaflet. The probed regions A and B were $2 \mu m$ apart. The K_{α} peaks for chlorine (2.622 keV) and potassium (3.313 keV) are identified. The Cl peak is higher than the K peak for the protoplast; the reverse is true for the wall.

FIG. 4. Ion distributions in pulvini from open and closed leaves. The scale bar for the histograms corresponds to 8000 scintillations during 150 sec of x-ray detection. Each bar in a histogram represents the mean for five cells in a particular region of a single cryosection. Standard deviations were 10–20% of the mean values. Several cryosections were analyzed for both leaf positions, with results similar to those shown in this figure.

results to indicate that leaf closure is accompanied by an efflux of K^+ across the plasma membranes of extensor cells into the apoplast. The K^+ ions then move by diffusion or some other mechanism through the apoplast to the flexor region, where they are taken up by motor cell protoplasts. The results suggest a similar pathway for Cl^- movement, although the extent of Cl^- migration was less than that of K^+ . Along with Cl^- , other anions or cations must provide electrical balance for the K^+ fluxes, as suggested by Satter *et al.* (3). Nitrates, organic anions, and Cl^- all participate in balancing the charge of K^+ in *Phaseolus* pulvini (13).

It is probable that most of the K^+ and Cl^- of a turgid motor cell is compartmentalized within the large, central vacuole (5). Thus, the tonoplast, as well as the plasmalemma, could regulate ion fluxes during leaf movements. If primary control is exercised at the tonoplast, ions could be shuttled between the vacuoles of extensor cells and those of flexor cells via the symplast. Because motor cells have pit fields with plasmodesmata (10), we cannot exclude the symplast from consideration as an accessory pathway for the ion movements; a recent model (14) proposes that some K^+ and Cl^- ions travel through a symplastic shunt in the vascular tissue. However, results presented in this paper support the view that the apoplast is the major pathway—i.e., fluxes across the tonoplast and plasmalemma are coordinated so that a net effect is the movement of ions and water between

FIG. 5. Ion distributions in pulvini when leaves were open and pulvini during dark-induced leaf closure.

the vacuole and the apoplast. Scott *et al.* (4) have demonstrated that the leaf movements of *Trifolium* are associated with K^+ fluxes across the plasma membranes of motor cells.

Both active and passive processes probably participate in dark-induced closure of leaves. The loss of K^+ from extensor cells may result partly from permeability changes and a collapse of electrochemical ion gradients; ion pumps may be involved in the loading of K^+ by the flexor cells (14). The latter postulate is supported by electrophysiological data revealing that flexor cells hyperpolarize when leaflets are transferred from white light to darkness if phytochrome is in the P_r form (15). The Cool White fluorescent light to which our plants were exposed prior to darkness would be expected to establish a high P_r level (16).

Although K^+ is the major osmoticum in the turgor-regulation of motor cells, it is doubtful that this ion is directly pumped by an active transport system. It seems more likely that K^+ fluxes are powered by membrane potentials generated by an electrogenic proton (or hydroxyl) pump. Data supporting this view are summarized in ref. 14. Electrogenic H^+ transport could also energize Cl^- uptake, possibly via a two-proton/anion symport, as proposed for *Chara* (17).

Pulvini of nyctinastic plants are well suited as model systems for the study of such phenomena as circadian rhythms, turgor regulation, control of ion compartmentalization, and lateral transport of ions in plant organs. Earlier investigations have supported the hypothesis that ion migrations within pulvini cause turgor-mediated leaf movements, that these migrations reflect interactions between ion "pumps" and "leaks" of motor cell membranes, and that a biological clock somehow modulates the transport properties of the membranes (7). Demonstration that the apoplast is a major pathway for ion migration in the pulvinus advances our understanding of the mechanism of leaf

movements by pointing to the plasmalemma as an important site of ion fluxes and as a locus for phytochrome-clock interaction.

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