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Distribution and Activity of the Plasma Membrane H^+-ATPase in *Mimosa pudica* L. in Relation to Ionic Fluxes and Leaf Movements[1]

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Plasma membrane H^+-ATPase was immunolocalized in several cell types of the sensitive plant *Mimosa pudica* L., and transmembrane potentials were measured on cortical cells. In comparison with the nonspecialized cortical cells of the petiole or stem, the proton pump was highly expressed in motor cells. These immunological data are in close agreement with electrophysiological data, because the active component of the transmembrane potential was low in the nonspecialized cortical cells and high in motor cells. Therefore, motor cells contain the plasma membrane H^+-ATPase required to mediate the ionic fluxes that are involved in circadian leaf movements and that are necessary to recover the turgor potential that is considerably affected by the large K^+ and Cl^- efflux associated with seismonastic movement. With the exception of sieve tubes, the phloem also had a high density of H^+-ATPase. This suggests that the recovery of the transmembrane ionic gradients (K^+ and Cl^-), which is affected by various stimuli, is more energized by the companion and parenchyma cells than by the sieve elements. In addition, at the phloem/cortex interface collocytes displayed the required properties for lateral transduction of the action potential toward the pulvinal motor cells.

The Leguminoseae plant *Mimosa pudica* has proven to be one of the most attractive plant systems for the study of movement. Circadian as well as stimuli-induced leaf movements are mediated by motor organs called pulvini. As with stomate guard cells, pulvini motor cells are characterized by a high level of K^+, which is the major ion that is implicated in the osmoregulation process (Toriyama, 1955; Mayer, 1977; Kiyosawa, 1979; Satter and Galston, 1981). Osmotic pressures in these pulvinus cells reach 2.5 MPa in *M. pudica* (Aimi, 1963; Fleurat-Lessard, 1988). The mechanism of circadian leaf movements is similar to that in guard cells. It includes a nyctinastic turgor variation that is associated with ionic (K^+ and Cl^-) fluxes between the symplasm and apoplasm. When these ions accumulate in the motor cell, the water potential decreases and subsequent water uptake induces cell swelling, whereas reverse fluxes lead to turgor loss and cell shrinkage (for review, see Satter, 1990).

The seismonastic movement is induced by an AP propagated from the part where the stimuli (cold water drop, electric shock, etc.) are applied. The transmission velocity of the AP (1–5 cm s^{-1}) from the stimulated area to the motor organ is quite slow (Roblin, 1979) when compared with the AP propagated in animal nerves, but is within the range recorded in the epithelium of the Siphonophores and Hydromeduseae. It is thought that in *M. pudica* the cell depolarization results from a Cl^- efflux followed by a K^+ efflux, which initiates the repolarization phase (Samejina and Sibaoka, 1983; Sibaoka, 1991). This AP is propagated in tissues in which a negative resting potential exists, i.e. in the phloem (near −160 mV) and protoxylem parenchyma (near −150 mV) (Sibaoka, 1962; Roblin, 1979). The pathway of AP conduction in the phloem is controversial; it could be through the companion and phloem parenchyma cells (Samejina and Sibaoka, 1983; Sibaoka, 1991) or via the mature sieve elements (Fromm and Eschrich, 1988a). Moreover, the phloem sap composition can be dramatically altered by certain stimuli. For example, a comparison of the ionic content of maize phloem sap before and after electric stimulation shows a loss of Cl^- and K^+ from 170 and 180 mM to 50 and 30 mM (Fromm and Bauer, 1994), respectively.

When the petiole action potential arrives at the main pulvinus, another action potential is elicited in this organ after a latent period of 0.2 s (Sibaoka, 1962). The collapse movement of the leaf, realized in 2 s, takes place 0.02 s after the beginning of this action potential (Oda and Abe, 1972). This response is associated with a decompartmention of intracellular Ca^{2+} and a large efflux of Cl^- ions, K^+ ions, and water toward the wall (Sibaoka, 1991). The upward position is recovered after 20 to 30 min, in association with the progressive turgor increase in the motor cells (Sibaoka, 1991).

Hundreds of motor cells control the regulation of the leaf position. The large apoplasmic space that is associated with the walls of the motor cells functions as an appropriate temporary storage site for solutes and exchange with the symplast (Freudling et al., 1988). The H^+ extrusion in excised *M. pudica* pulvini (Roblin, 1982) is greater than that in the less specialized tissues (Roblin and Fleurat-Lessard, 1983). This extrusion is increased by fusicoccin (Otsiogo-Oyabi and Roblin, 1984). It is also promoted by light (Igle-

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Abbreviation: AP, action potential.

sias and Satter, 1983) and varies during the circadian movements (Lee and Satter, 1987). In this regard, the aim of this study was to compare the distribution and activity of the plasma membrane H^+-ATPase in the motor tissue (Fig. 1, A and C) and nonmotile parts (Fig. 1, B and D) of *M. pudica* L. This intrinsic enzyme, of comparable importance as the plasma membrane Na^+/K^+ ATPase in animal cells, catalyzes a H^+ efflux toward the cell wall and controls many functions directly or indirectly, especially cell nutrition. Indeed, the proton gradient that is generated by this enzyme is the driving force for the active uptake of ions and organic nutrients (Serrano, 1989). Consequently, its distribution was studied in *M. pudica* in relation to (a) the control of turgor variations regulating the day/night rhythmic movements of leaves; (b) the energization of solute absorption, allowing the recovery of motor cell turgor after the realization of the seismonastic movement; (c) the energization of phloem loading (and the recovery of phloem sap traffic by turgor pressure) after the unloading that is induced by electric stimuli; and (d) the maintenance of a high resting potential, an essential condition for the activation of action potentials (Opritov and Pyatygin, 1989).

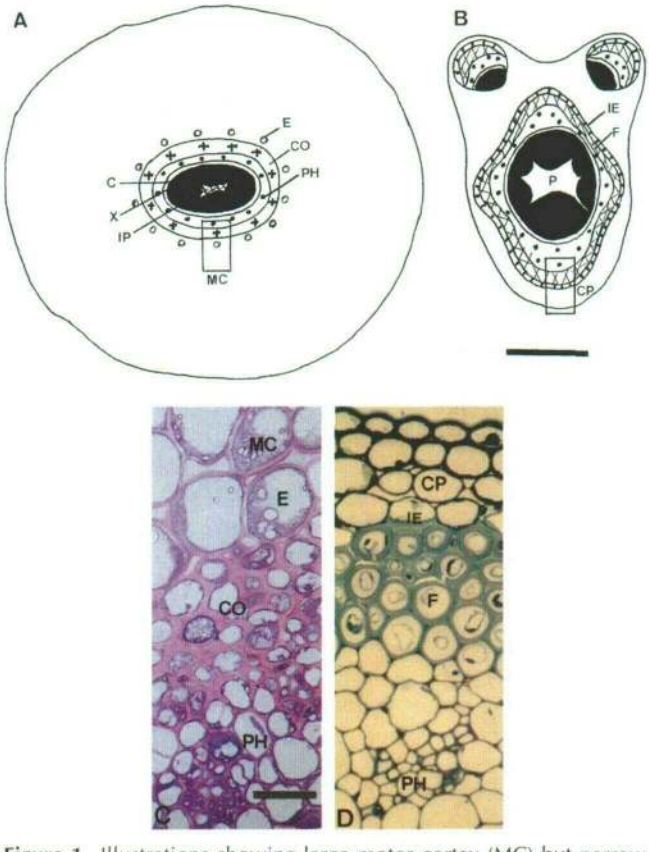

Figure 1. Illustrations showing large motor cortex (MC) but narrow central cylinder in pulvinus (A), and narrow cortical parenchyma (CP) and wide central cylinder in petiole (B). Electron micrograph showing pulvinus phloem (PH) and collenchyma cells (CO) with pectocellulose walls and groups of narrow cells, including sieve tubes, companion, and parenchyma cells (C). In petiole lignified phloem fibers (F) and lignified endodermis layer (IE) (D). C, Cambium; E, endodermis; IP, lignified pith; P, pith; and X, xylem. Bar = 500 μm for A and B; bar = 20 μm for C and D.

MATERIALS AND METHODS

Plant Material and Sampling

Mimosa pudica plants were grown in pots containing a mixture of garden earth, heath-mould, sand, and peat in 50, 20, 15, and 15%, respectively. The following conditions were chosen: the temperature was 28°C ± 5% and illumination was from 7 AM to 9 PM, which was provided daily by a set of white fluorescent tubes ("Phytor", ACEC, Charleroi, Belgium) giving a photon fluence rate (400–700 nm) of 36 μmol m^{-2} s^{-1} at the plant apex (Fleurat-Lessard and Roblin, 1982). The sampling of pulvini and petiole was made on the fourth leaf from the base in plants having six leaves that were reactive to mechanical stimuli and those on the stem of the subtending internode.

Electrophysiology

After excision at its base (the pulvinus), the leaf was secured with terostat in a cuvette containing the standard medium buffered at pH 5.0 (Otsiogo-Oyabi and Roblin, 1985; Roblin et al., 1993). Electrophysiological measurements were made at 28 ± 1°C in a Faraday cage with the equipment previously described (Renault et al., 1989). After 1 h of incubation the reference electrode was dipped into the bathing medium and the glass micropipette was inserted into the cortical cells of the pulvinus or petiole with a mechanical micromanipulator (Prior, UK).

Plasma Membrane Isolation and Western Blots

Membrane isolation, SDS gel electrophoresis, and western blot analysis were performed as previously described (Gallet et al., 1992).

Immunocytochemical Procedures

Antibody

The antibody we used (antibody 758) was raised against the central domain (amino acids 340–650) of isoform 3 of *Arabidopsis thaliana* H^+-ATPase expressed in *Escherichia coli* (Pardo and Serrano, 1989; Roldan et al., 1991). This conserved amino acid stretch has homologies (80%) with the different isoforms of tobacco H^+-ATPase. In *M. pudica* our results probably represent the additive contribution of several isoforms.

Chemical Fixation and Immunoreaction on Semi-Thin and Thin Sections

Mature pulvini, petioles, and stems were cut into 1- or 2-mm-thick pieces and fixed for 15 to 30 min in a mixture of 1.5% (w/v) paraformaldehyde and 0.5% glutaraldehyde in 0.05 mM phosphate buffer, pH 7.2 (Fleurat-Lessard et al., 1995). Abundant washing (at least six baths during 2 h) in the same buffer was followed by a 4-min postfixation in 1% (v/v) OSO_4, dehydration in an ethanol series, and overnight embedding in London Resin White. Polymerization occurred in gelatin capsules at 60°C for 24 h.

The immunofluorescence reaction was carried out in the dark at room temperature on semi-thin sections, collected on glass slides, and spread 24 h at 55°C. These treatments followed previously described protocols (Knox and Singh, 1985; Laskowski and Briggs, 1989). Sections placed for 15 min in PBS-T (PBS, pH 7.2, and 0.1% Triton X-100) and 0.5% Gly were washed in PBS-T. They were dipped for 45 min in a saturation solution (PBS-T, 0.2% Tween 20, 0.1% BSA, and 1/20 goat serum) before overnight incubation with the H$^+$-ATPase antibody or with the preimmune serum (antibody and serum 1/40 diluted in the saturation solution) (control 1) or with the saturation solution (control 2) or with the antibody saturated with the purified enzyme (control 3). A 30-min wash in PBS and 1% BSA was followed by 3 h of incubation in 1/100 diluted fluorescein-coupled goat anti-rabbit antibody (GAR, Biocell, Le Perray en Yvelines, France) and washing in PBS. Autofluorescence was reduced by 1/10,000 Evans blue. Samples were mounted in 50/50 glycerol-PBS and observed under blue light using a light microscope (Zeiss Axioplan). Photographs were taken with 160T film (Ektachrome, Kodak).

The immunogold reaction on thin sections, carefully spread with toluene vapor on parlodion coated gold grids, was performed at 20°C, as previously described (Bouché-Pillon et al., 1994a, 1994b). The procedure accommodated a compromise between the preservation of structure and antigenicity. Solutions were filtered (0.1-μm pores, Millipore MFVCWP,) or centrifuged (500g). The sections, hydrated in deionized water, were then, in the dark, etched by 0.56 M $NaIO_4$ and 0.1 N HCl, and washed for 15 min on PBS, 0.1% (v/v) Triton X-100, and 0.2% (v/v) Gly at pH 7.2. Nonspecific sites were saturated for 45 min by goat serum in PBS, 0.2% (v/v) Triton X-100, 0.2% (v/v) Tween, and 0.1% (w/v) BSA, and sections were incubated overnight with the H$^+$-ATPase antibody at a 1/50 dilution. After washing in PBS, sections were placed for 40 min on TBS, pH 8.2, 0.2% (v/v) Tween, 0.2% (v/v) Triton X-100, 1% (w/v) BSA, and goat serum before the 2-h application of a 15-nm gold particle-labeled goat anti-rabbit IgG (Biocell) at a 1/40 dilution. The sections washed in TBS and deionized water were contrasted in uranyl acetate at saturation in water (8 min) and in lead citrate (3 min). Controls were as for the semi-thin sections. Specimens were observed with a microscope (100C, JEOL) operated at 80 KV.

RESULTS AND DISCUSSION

Distribution of the Plasma Membrane H$^+$-ATPase in Motor and in Nonspecialized Cortical Cells

The plasma membrane H$^+$-ATPase antibody from *A. thaliana* cross-reacted with the plasma membrane vesicles that were isolated from *M. pudica* pulvini, as shown in Figure 2. In light microscopy using fluorescein isothiocyanate labeling a fluorescent line was observed along the plasma membrane of the pulvinar motor cells (Fig. 3A), whereas a very weak response or an absence of response was noted in the nonspecialized cortical cells of the petiole and stem (Fig. 3C). The immunoreaction in the motor cells was blocked when the antibody was saturated by the purified H$^+$-ATPase (Fig. 3B).

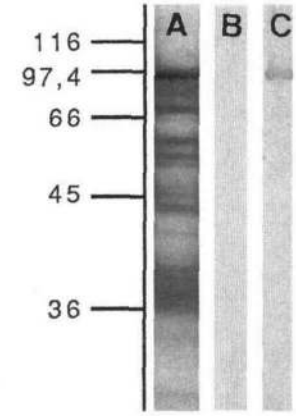

Figure 2. Transfer of polypeptides from plasma membrane- enriched fractions of *M. pudica* pulvini on a nitrocellulose sheet and western blot analysis of H$^+$-ATPase. A, SDS-PAGE electrophoresis of polypeptide profile and position of molecular mass markers (kD). Incubation with preimmune sera (B) or with 1/2000 antibody directed against the central domain (C) of the plasma membrane H$^+$-ATPase.

In electron microscopy the observation of the immunogold reaction in the motor cells showed that the plasma membrane H$^+$-ATPase was located along the meatus (Fig. 4A) as well as at the interface between the adjacent motor cells (compare Fig. 4, B and C, control). At this scale the paucity of the plasma membrane H$^+$-ATPase sites in the nonspecialized cortex of petiole was also confirmed (Fig. 4D). Moreover, the quantification of gold particles per unit length of plasma membrane showed that the density of specifically recognized sites (immunogold minus preimmune labeling) was approximately 50 times higher in the motor cells compared with the cortical cells in the petiole (Table I), and was comparable to that detected in the companion and phloem parenchyma cells (Table I).

Comparison of the Transmembrane Potential in Motor and Nonmotor Cells and Energization of Ion Influx

The immunological data were in agreement with the electrophysiological data. Motor cells had a transmembrane potential (−144 mV; Abe and Oda, 1976; −139 ± 12 mV; mean ± SE, n = 6, our results) almost as negative as the values obtained in the phloem (−160 mV; Fromm and Eschrich, 1988b), but considerably more negative than in cortical parenchyma of the petiole (−52 mV; Sibaoka, 1962) and of the stem (−61 ± 13 mV, mean ± SE, n = 10, our results). Moreover, our results pointed out that the transmembrane potential in the motor cells was hyperpolarized by the addition of fusicoccin in contrast with the nonspecialized cortical cells (Fig. 5). Our electrophysiological and immunological data suggest that, in comparison with the cortical parenchyma of the stem, the motor cortex, a highly specialized tissue, was characterized by a high expression of the plasma membrane H$^+$-ATPase. In addition, the transmembrane potential in cortical cells of the petiole and stem varied considerably (from −40 to −130 mV). The most negative values appear to be in contrast with the

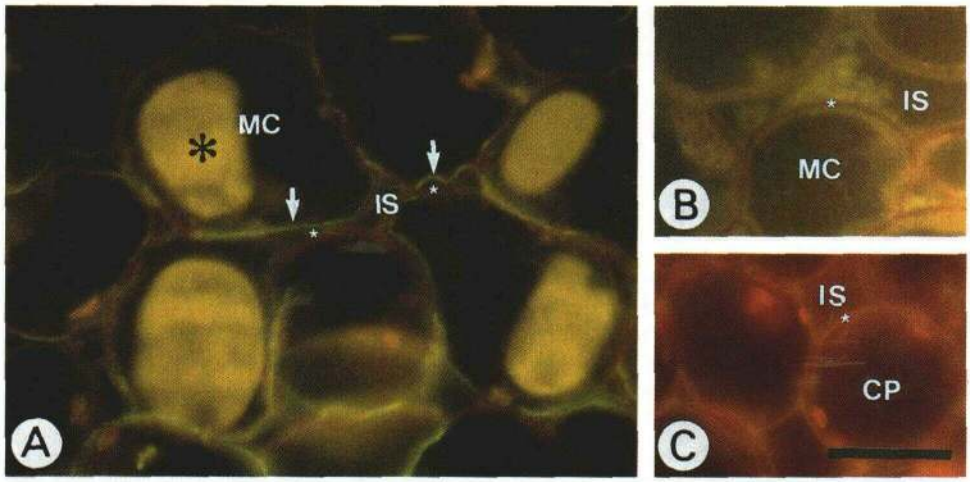

Figure 3. Distribution of the plasma membrane H^+-ATPase in cortex semi-thin sections showing extensive green immunofluorescence (arrow) in the plasma membrane of motor cells (MC) (A), but very low labeling in petiole (C). B, No recognition in control (antibody saturated with the purified protein); yellow autofluorescence of tannin vacuoles (black asterisk) (A and B). White asterisk, Wall; IS, intercellular space; CP, cortical parenchyma. Bar = 20 μm.

paucity of the plasma membrane H^+-ATPase (Figs. 3C and 4D; Table I) and, except the possibility of poorly detected H^+-ATPase isoforms, it may reflect the presence of a H^+ excreting redox system similar to that described for the guard cells (Gautier et al., 1992).

The active absorption of ions such as K^+ and Cl^- (K^+/H^+ cotransport, Cl^-/nH^+ cotransport) being dependent on the proton gradient maintained by the plasma membrane H^+-ATPase, in addition to information on the distribution of vacuolar membrane H^+-ATPase explains the high concentration of solutes in motor cells (Toriyama, 1955). This capacity to accumulate solutes is a required condition to realize the leaflet movements that are linked with a nyctinastic variation of turgor in motor cells (Schrempf et al., 1976; Satter et al., 1977; Schrempf and Mayer, 1980). In this view, the natural behavior of motor cells can be reversed (opening in the dark phase and closing in the light phase) by modifying the H^+-ATPase activity and the K^+ permeability of the plasma membrane. Application of auxin (Kumon and Suda, 1984; Abe, 1985) or fusicoccin, which causes the transmembrane potential to hyperpolarize (Fig. 2), activates inward K^+ currents, leading to leaflet opening in *M. pudica* in the dark period (Bonnemain et al., 1978; Roblin and Fleurat-Lessard, 1983). In reverse, ABA, which indirectly prevents influx and/or causes efflux of K^+ from cells, induced the closing of

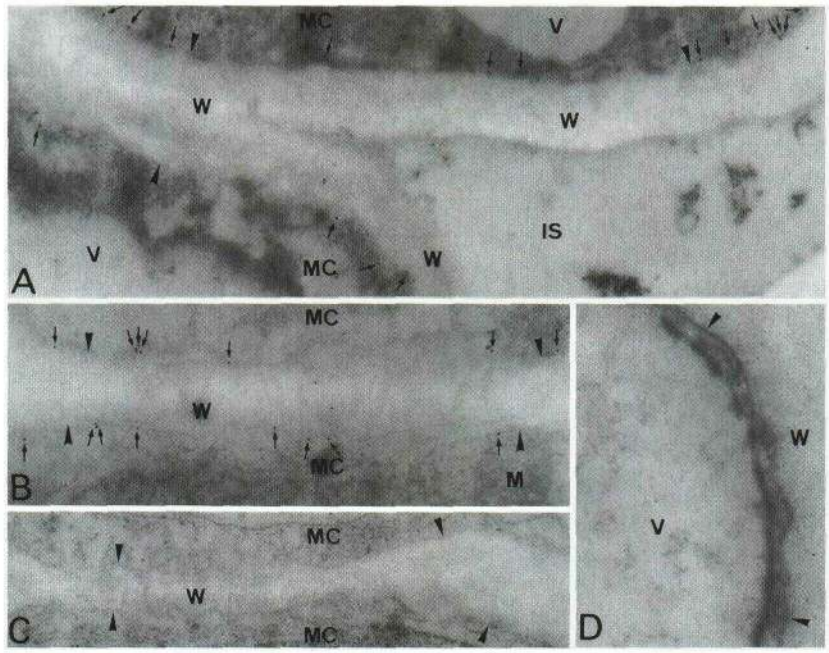

Figure 4. Distribution of the plasma membrane H^+-ATPase in cortex thin sections. In motor cells (MC) high immunogold labeling (arrow) on the interfaces with an intercellular space (IS) (A) and with another motor cell (B). D, Scarce labeling in petiole. Note the absence of labeling in motor cells treated with the antibody saturated with the purified protein (C). W, Wall; V, vacuole; arrowhead, plasma membrane; M, Mitochondria. Bar = 1 μm.

Table I. *Comparative density of the H^+-ATPase*

We made 3 fixative-embedding procedures and 4 immunoreactions in view of this quantitative study performed on 30 to 40 cells, or cell regions, in each type. The total measured length of the plasmalemma was a mean of 100 μm in 6 pulvini. The mean and SE were calculated and a comparison of the means in a series of values superior to 30 were done.

Cell Types	Gold Particles[a]	
	Immune labeling	Preimmune labeling
	15 nm 10 μm^{-1} plasma membrane	
Petiole		
Cortical cell	0.5 ± 0.1	0.3 ± 0.1
Pulvinus		
Motor cell	10.5 ± 2.3	0.4 ± 0.1
Endodermis	3.2 ± 0.3	0.5 ± 0.1
Collenchyma	6.0 ± 0.6	0.7 ± 0.1
Phloem parenchyma	8.3 ± 0.7	0.3 ± 0.1
Companion cell: area		
Opposite to sieve tube	11.5 ± 1.2	0.6 ± 0.2
Next to sieve tube	6.6 ± 1.2	0.3 ± 0.3
Sieve tube	1.3 ± 0.4	0.6 ± 0.2

[a] Mean ± SE.

M. pudica leaflets (Bonnemain et al., 1978), similar to what happens with stomata in the light phase of the photoperiodic cycle (Mansfield and Jones, 1971).

Among the numerous types of induced movement, those resulting from light or mechanical stimuli have been widely studied. The capability of motor cells to accumulate solutes in response to the proton gradient maintained by the plasma membrane H^+-ATPase is a required condition to realize the seismonastic movement, which is characterized by a decompartmentation of intracellular Ca^{2+} (Toriyama and Jaffé, 1972; Campbell et al., 1979) and by a rapid efflux of Cl^-, K^+, and water into the wall (Campbell et al., 1981). In parallel with about 1 MPa of turgor loss (Allen, 1967; Fleurat-Lessard, 1988, 1990), an increase of solutes occurs in the apoplastic compartment. In the lower half of the main pulvinus, the extracellular Cl^- and K^+ concentrations are increased from 10 to 80 mM and from 27 to 200 mM, respectively, during the bending movement (Samejina and Sibaoka, 1983; Kumon and Suda, 1984). During the leaf recovery toward the upward position, the motor cell has to restore its ATP level, which has fallen by 50% during the seismonastic response (Roblin, 1979), to reestablish the transmembrane proton gradient after the long depolarization of wide amplitude following the action potential (Roblin et al., 1993), and to reimport the released solutes. Although, the wall is a K^+ reservoir playing its role during rhythmic movements in some species (Mayer, 1977; Freudling et al., 1988; Antkowiak and Engelmann, 1995), a large portion of the ions released into the apoplasm of motor cells, during the seismonastic response, leave the lower part of the pulvinus (Sibaoka, 1991). Thus, recovery of the initial solute content in the motor cell involves a competition with the other plant parts, and, in this context, by its density in plasma membrane H^+-ATPase the motor tissue functions as a dominant sink.

Distribution of the H^+ Pump in Relation to the Propagation of an AP

Our results also pointed out that the *M. pudica* phloem contained a large amount of plasma membrane H^+-ATPase. This thinking is consistent with the negative transmembrane potential (about −160 mV) previously reported for this tissue (Sibaoka, 1962; Fromm, 1991). However, the number of recognized sites of the antibody varied with the cell type, being high in phloem parenchyma and companion cells and low in the sieve element (Table I; Fig. 6A). A low and nonspecific distribution was noted in the control (Table I; Fig. 6B). If this difference in labeling density, obtained using an antibody directed against a conserved region of the plasma membrane H^+-ATPase, which does not discriminate between several isoforms of this enzyme (Palmgren and Christensen, 1994), reflects the true distribution in situ of the plasma membrane H^+-ATPase in the phloem of *M. pudica*, it can be concluded that the negative transmembrane potential, noted in the sieve element (−160 mV) (Fromm and Eschrich, 1988b, 1988c), could be maintained by the companion cell. The symplasmic links, i.e. branched plasmodesma, between the companion cell and the sieve element would allow such a control, taking into account the electric conductivity of these cell-to-cell connections (Robards and Lucas, 1990). Our results therefore suggest that the companion cells and the parenchyma cells of the phloem play a major role in maintaining the resting potential in sieve element system, a condition required for the transmission of AP in response to stimuli (Opritov and Pyatygin, 1989). The companion cell may also play a lead role in the recovery of K^+, Cl^-, and Suc in stimulated sieve elements. In this condition, the K^+ content in the phloem sap decreases 6 times and that of Cl^- 3.5 times after an electric stimulation (Fromm and Bauer, 1994). A large amount of the plasma membrane H^+-ATPase was already observed in the companion cells of *Vicia faba* (Bouché-Pillon et al., 1994). Furthermore, the AHA_3 isoform is specifically expressed in the companion cells of Arabidopsis (Dewitt and Sussman, 1995).

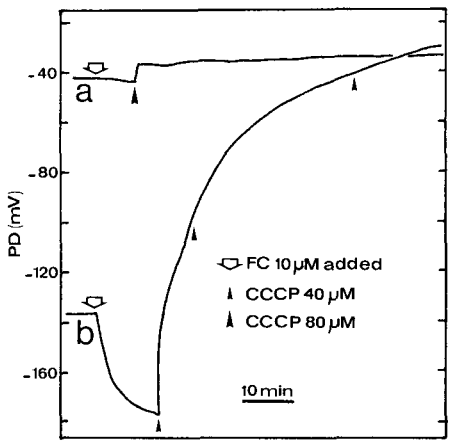

Figure 5. Effect of fusicoccin (FC) and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) on the transmembrane potential difference (PD) in cortical cells of mature internodes (a) and motor cells of pulvini (b).

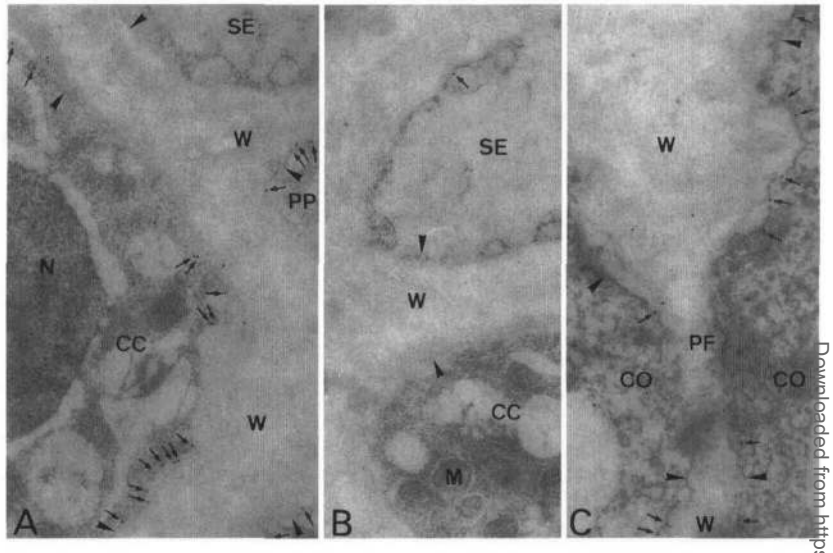

Figure 6. Distribution of the plasma membrane H^+-ATPase in thin sections of pulvinar phloem. Many gold particles (arrows) distributed along the plasma membrane in companion (CC) and parenchyma cells (PP), whereas they were scarce in the sieve element (SE) (A). Stronger labeling (arrowheads) appeared at the border than at the bottom part of pit-fields (PF) between collenchyma cells (CO) (C). B, Very low labeling in control. N, Nucleus; other symbols are as in Figure 4.

It should also be stressed that in the companion cell the density of H^+-ATPase sites was twice as high along the cytoplasm, opposite from that facing the sieve element (Table I). This polarized distribution of the plasma membrane H^+-ATPase, previously noted in phloem and epidermal transfer cells (Bouché-Pillon et al., 1994a, 1994b), can be compared with that of the plasma membrane Na^+/K^+ ATPase observed in several cell types in the animal kingdom (Zurgolo and Rodiguez, 1993).

At the interface phloem/motor cortex collenchyma cells have a density of the H^+-ATPase sites close to that noted in the phloem parenchyma cells (Table I; Fig. 6C). These cells therefore are different from other supporting cells, either dead (fibers between phloem and cortical parenchyma in *M. pudica* petiole or stem) or living, but with a low plasma membrane H^+-ATPase density, as found in the xylem parenchyma cells (Fromard et al., 1995). These results suggest that collenchyma cells present in the pulvini are able to maintain a transmembrane potential close to that noted in the phloem. Such a property of the plasma membrane and their structural features (large pit-fields with many plasmodesmata [Fleurat-Lessard and Bonnemain, 1978; Fleurat-Lessard et al., 1995]) are among the required conditions to propagate the AP from the phloem to the motor cortex. The only discontinuity concerning the distribution of the plasma membrane H^+-ATPase in the pulvini is located in the endodermis, in which the density is twice as low as in collenchyma cells but 6 times higher than in the nonspecialized cortical parenchyma of the petiole (Table I).

In conclusion, parallel studies utilizing immunological and electrophysiological techniques establish a correlation between the high expression of H^+-ATPase in the plasma membrane of the motor cortex and the presence of a hyperpolarized membrane potential. These studies underscore the difference between the motor cortex and the nonspecialized cortical cells. This finding helps to explain the functioning of motor cells widely linked with ionic and water fluxes. Our results suggest that the negative transmembrane potential in the phloem, a condition required for the longitudinal propagation of AP, is mainly due to the high expression of the plasma membrane H^+-ATPase in companion and parenchyma cells, but not in the sieve cells. This work shows that collenchyma cells in pulvini are not only supporting cells but also possess structural and biochemical properties that are necessary for the lateral propagation of AP between the stele and the cortex.

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