Plant Physiol. (1995) 108: 913–918

Control of Vascular Sap pH by the Vessel-Associated Cells in Woody Species[1]

Physiological and Immunological Studies

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In *Robinia* wood, the vessel-associated cells form a continuous sleeve around the vessels. Variations in pH of the solution perfused through the vessels during the annual cycle and the opposing effects of carbonyl cyanide-*m*-chlorophenylhydrazone and fusicoccin on this pH value indicate that some living cells of the wood are involved in the control of vascular sap pH and that this control fluctuates with the seasons. The immunolocalization of the plasma membrane H^+-ATPase in *Robinia* wood was studied by the immunogold-silver-staining technique using an antibody raised against a conserved stretch of the cytoplasmic domain of the H^+-ATPase. The immunostaining is much stronger in vessel-associated cells than in other living cell types (ray and axial parenchyma elements) of the secondary xylem. Our data show an efficient involvement of this cell type in the control of vascular sap pH.

VAC belong to the group of "contact cells" that join conducting elements (vessel element or sieve element) with other cell types (donor or receiver parenchyma cells) (Czaninski, 1987). The term VAC was used for the first time in the 1960s to refer to the specialized cells surrounding the vessels in *Robinia* that are derived from both radial and axial xylem mother cells (Czaninski, 1964, 1968). This cellular type has since been described in several herbaceous and woody species (Foster, 1967; Czaninski, 1972, 1977; Fujita et al., 1975; Catesson et al., 1982).

VAC are small cells with a high nucleoplasmic ratio. Their dense cytoplasm contains many ribosomes and mitochondria with numerous cristae, starch granules in plastids are absent or of reduced size, the ER is well developed, and the vacuoles are small. VAC thus show many structural analogies to the companion cells of the phloem.

Nevertheless, VAC differ from companion cells in some features. The VAC wall is lignified, except in the pits that are common to the vessels. Furthermore, at the latest stage of differentiation, VAC produce a new pecto-cellulosic parietal layer, called a "protective layer," along the wall common to the vessels. But the type of relation between the contact cell and the conducting element is the main character differentiating the VAC from the companion cells. Whereas the companion cell and the sieve element are connected by branched plasmodesmata, no plasmodesmata are seen in the pits at the interface between the VAC and the vessel element (Bonnemain and Fromard, 1987; Czaninski, 1987). Consequently, ions and organic molecules moving from the VAC to the vessel, or in the opposite direction, must cross the plasmalemma of this cellular type (or must be exchanged by a pinocytosis process). From their position, their structural features, and their enzymatic activities (Sauter et al., 1973; Czaninski, 1977; Sauter, 1982; Braun, 1984), it was assumed that VAC could control nutrient exchange between the storage parenchyma and the vessels. However, for a long time the mechanisms of these exchanges have been questioned, in particular those involved in sugar efflux toward the vessels during the winter: exosmosis (Münch, 1930), active transport (Sauter et al., 1973; Ziegler, 1975), and facilitated diffusion (Sauter, 1982). The results seem to depend in part on the species chosen, and the matter remains to be explored as underlined by Sauter (1988).

As a general rule, transport of ions and organic nutrients across the plasma membrane of plant cells depends largely on the electrical and pH gradients established on each side of the membrane (Larsson and Moller, 1990). There is much evidence that the pH of the vascular sap varies with the annual cycle. The pH values are close to neutrality in winter, whereas they are clearly acidic (pH 5.5) at the beginning of spring (Essiamah, 1980; Ferguson et al., 1983; Sauter and Ambrosius, 1986; Sauter, 1988; Fromard, 1990).

[1] This work was supported by the Centre National de la Recherche Scientifique (URA CNRS 574) and the Ministère de l'Enseignement Supérieur et de la Recherche Eureka-Eurosilva Programme (No. 1416A).

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FC, fusicoccin; VAC, vessel associated cell(s).

The acidification mechanism is not understood, but it appears that the cell type best localized to play a major role in control of the vascular sap pH is VAC. Nevertheless, this role has not been mentioned among the possible functions of VAC (Braun, 1984).

In recent years, some plasma membrane proteins directly (sugar and amino acid transporters) or indirectly (H^+-ATPase) involved in membrane exchanges have been characterized (Serrano, 1989; Sauer et al., 1990; Li et al., 1992; Frommer et al., 1993; Riesmeier et al., 1993). Identifying the location of these intrinsic proteins in the various living cell types of the secondary xylem would improve our understanding of nutrient compartmentation occurring in this tissue and its seasonal changes, especially the variations of vascular sap components during the annual cycle. The present work is a contribution to this subject, the target chosen being the H^+-ATPase of the plasma membrane.

MATERIALS AND METHODS

Plant Material

Robinia pseudoacacia is suitable for studying the properties of VAC because they form a continuous or almost continuous sleeve around the vessels (Czaninski, 1968) (Fig. 1). Furthermore, in this ring-porous species, the wide xylem vessels allow easy perfusion through short stem segments. The 2-year-old branches used in these experiments were cut from *Robinia* trees growing on the campus of Poitiers University.

Perfusion Technique

This technique (initially used by Van Bel [1974] to study the uptake of amino acids by tomato xylem tissue) was chosen to investigate the properties of the cells bordering the vessels, the VAC in the present case. *Robinia* branches were cut into 5-cm-long segments. Segments with a wood diameter of 10 mm were selected. The two ends of each segment were stripped of bark over a length of 7 mm and covered with paraffin film to isolate the phloem tissue (Fig. 2). The segment was turned upside down, and its morphologically lower part was inserted into a plastic tube containing a standard solution. The pH of this solution (0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.1 mM KCl) was adjusted to 6.0 by addition of 0.01 N HCl or NaOH.

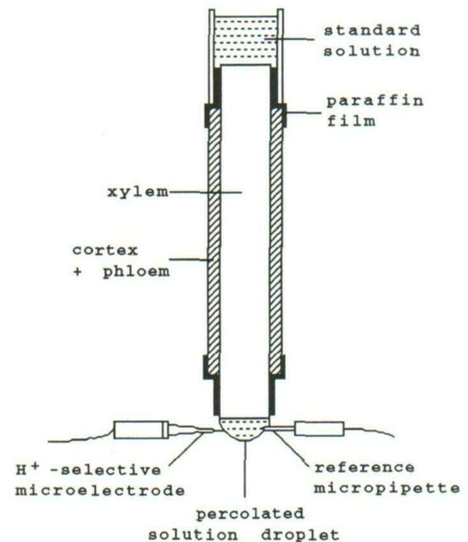

Figure 2. Scheme showing the xylem perfusion device, with measurement of the pH of the percolated solution droplet (the stem segment being placed upside down, the perfusing solution flowed down by gravity through the vessels in the circulation mode of the vascular sap). The measurements were made at room temperature (22 ± 2°C).

After the vascular sap was eliminated from these segments and they were rinsed with standard solution, this setup was used to measure the pH of the perfused solution droplets and to study the effects of CCCP and FC.

pH Measurement

The pH of the perfused solution was measured continuously with H^+-selective liquid membrane microelectrodes prepared according to a method already described (Amman et al., 1981; Renault et al., 1989). Briefly, the H^+-selective microelectrode and the reference micropipette (filled with 3 M KCl in 1% agar) were connected to an electrometer amplifier (model FD 233; WP Instruments, Inc., New Haven, CT) and to a chart recorder (Kipp and

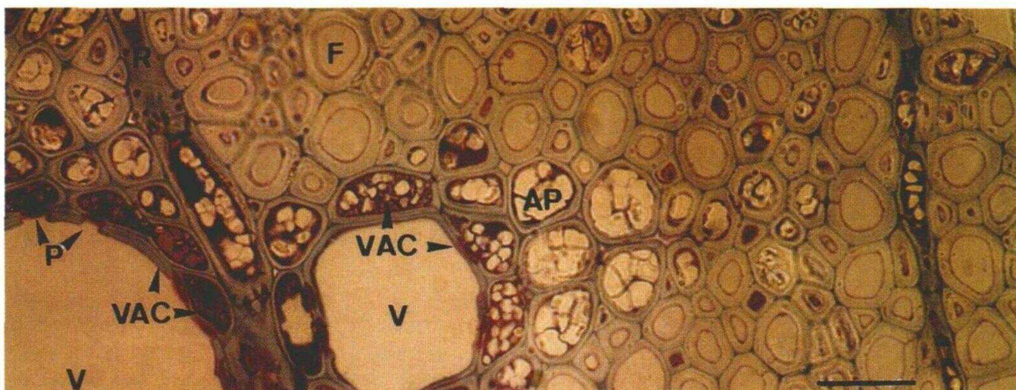

Figure 1. The cell types in *Robinia* wood. F, Fiber; P, pit of the vessel/VAC interface; R, ray; V, vessel; AP, axial parenchyma with large starch granules; VAC, without starch (large vessel) or with small starch granules (small vessel). Bar = 20 μm.

Zonen, Delft, The Netherlands). The pH measurements were made in a Faraday cage. After the H^+-selective microelectrode was calibrated in a series of buffer solutions (pH 5.0–7.0), its tip and the reference micropipette tip were both inserted into the droplet of the perfused solution, as close as possible to the wood section but without touching it (Fig. 2). Thus, it was possible to measure the pH of the percolated solution from droplet to droplet and even during an increase in size of the droplet.

Immunogold-Silver-Staining Procedures

The highly lignified walls of the xylem and its extreme hardness in *Robinia* have so far prevented the use of the immunolocalization technique already used to study the distribution of the plasma membrane H^+-ATPase at the subcellular level (Bouché-Pillon et al., 1994a, 1994b). Therefore, the immunolocalization of this pump was studied at the light microscope level.

Two-millimeter-thick transverse sections were taken from 2-year-old branches and immediately fixed (1.5% paraformaldehyde plus 0.5% glutaraldehyde plus 0.05 M phosphate buffer, pH 7.4, 30 min at 4°C). After the sections (1.5 μm thick) of the fixed samples were washed in 0.1 M phosphate buffer, pH 7.4, plus 7.5% Suc and then in PBS, pH 7.2, they were cut on a freezing microtome (Decimu, Paris, France) at 2 ± 2°C, collected in PBS, and then put in methanol at the same temperature for 12 min. Sections were incubated at room temperature first in PBS plus 0.1% (v/v) Triton plus 0.5% Gly for 15 min and then in PBS plus 0.1% (v/v) Triton for 10 min to enhance the permeability of tissues.

The immunoreaction was carried out according to the method of Bouché-Pillon et al. (1994a, 1994b). Briefly, nonspecific sites were saturated for 45 min by normal goat serum in PBS plus 0.1% (v/v) Triton plus 0.2% (v/v) Tween plus 0.1% (w/v) BSA and then incubated overnight at room temperature with 1/40 diluted primary antibody against H^+-ATPase. After the samples were washed rapidly in Tris-buffered saline, pH 8.2, and in Tris-buffered saline plus 1% BSA for 30 min, the secondary antibody (goat anti-rabbit IgG, 1/100 dilution; Biocell, Cardiff, UK), labeled with 1-nm gold particles, was applied for 3 h in darkness.

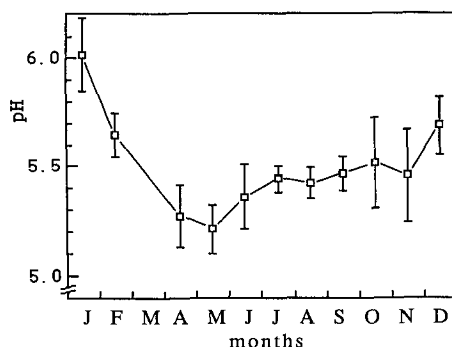

Figure 3. Seasonal variations (first letter of months on abscissa) of the pH of the perfused droplets. The pH of the standard solution was 6.0. The mean values ± SE are reported monthly (n = 6).

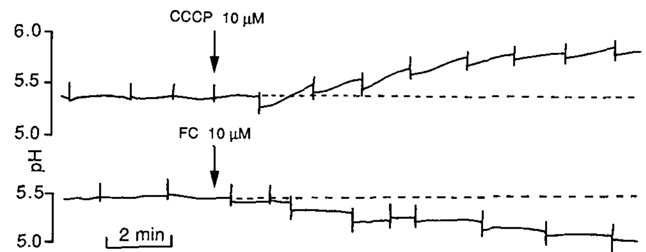

Figure 4. Effects of CCCP or FC added to the standard perfusion solution on the pH of the perfused droplets. The measurements were made during the LD period (June) and repeated three times with similar results. The vertical lines of the plot are artifacts induced by the fall of each droplet of the perfused solution. These substances had no (CCCP) or only a slight (FC) effect during the autumn/winter transition (see Table I).

Sections were then washed for 30 min in PBS and 5 min in filtered deionized water before a controlled immersion (at least 15 min in the dark) in the silver enhancement reagent (Silver Enhancing Kit for light microscopy, Biocell). Finally, sections to be observed by light microscopy were carefully washed in water and mounted in PBS plus 20% glycerol.

The antibody used was raised against a conserved stretch of 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). The antibody does not discriminate between the different isoforms of this enzyme (Palmgren and Christensen, 1994). Control sections were subjected to the preimmune serum.

RESULTS AND DISCUSSION

Figure 3 shows that the pH of the perfusing bath was modified after the flow of this solution in the vessels, but the magnitude of the acidification was variable according to the season. Indeed, a clear oscillation of the pH was observed over the annual cycle, with an unchanged pH value in January and a maximal acidification from April until June.

These data are similar to the pH variations occurring in the vascular sap throughout the year in various woody species and are characterized by a pronounced acidification at the beginning of spring (see introduction). The origin of this acidification is unknown. According to Es-

Table I. *Effects of FC and CCCP on the pH of the solution perfused through stem segments of R. pseudoacacia during various periods of the annual cycle (mean ± SE, n ≥ 6)*

Addition	ΔpH		
	April to June	July and August	Mid-November to mid-February
FC (10 μM)	−0.33 ± 0.07	−0.20 ± 0.05	−0.06 ± 0.03
CCCP (10 μM)	+0.41 ± 0.10	+0.25 ± 0.04	0

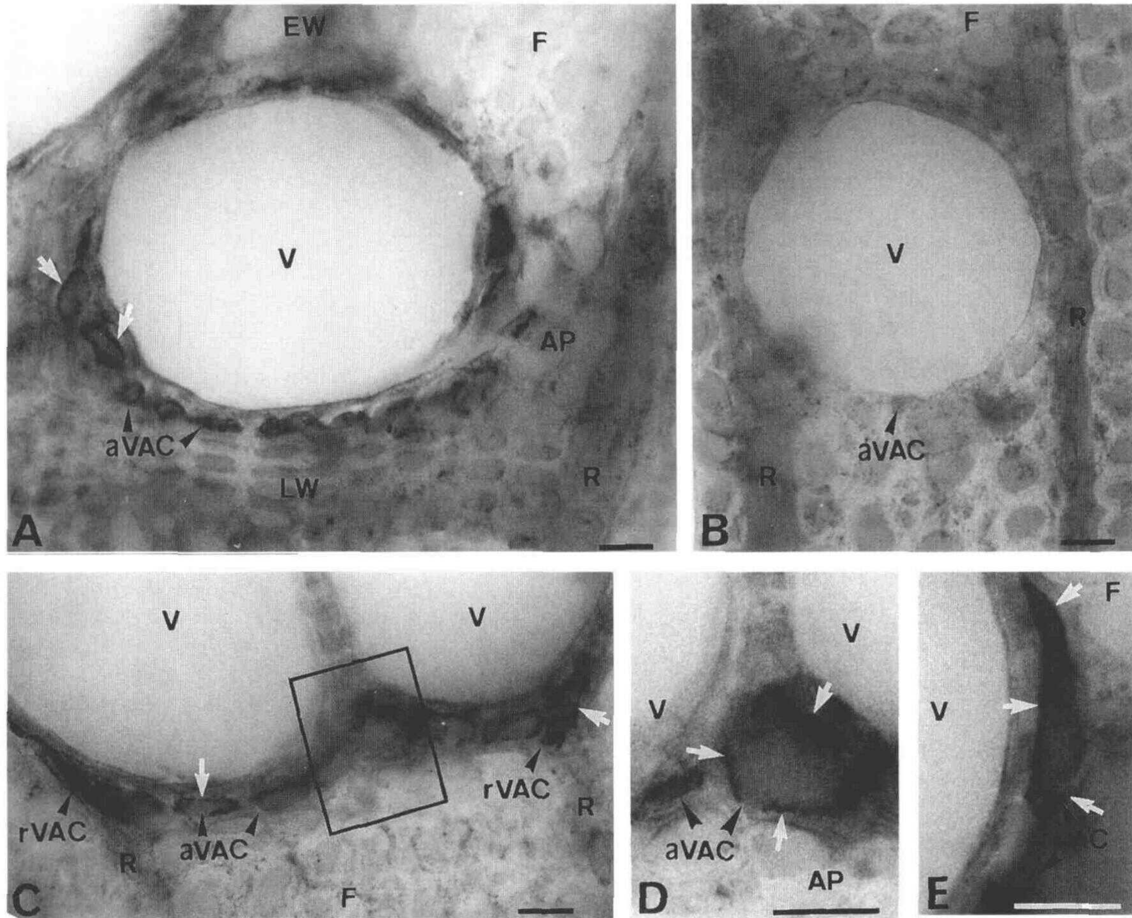

Figure 5. Immunogold-silver staining of H^+-ATPase sites in cross-sections of secondary xylem tissue of *R. pseudoacacia* sampled in June. A and C to E, Immunotreated sections. B, Control section subjected to a preimmune serum. Note in A and C to E a black deposit lining the wall (white arrow) in VAC. D is a detail (frame in C) showing axial VAC (aVAC). E is a detail of ray VAC (rVAC). Note the absence of significant labeling in rays (R) and in axial xylem parenchyma (AP). EW, Early wood; F, fiber; LW, late wood; V, vessel. Bar = 10 μm.

siamah (1980), the pH decrease of vacular sap noted in young branches of *Acer pseudoplatanus* is due to the arrival of a more acidic sap coming from the base of the tree. According to Sauter (1988), this decrease could be explained, at least in part, by proton-coupled Suc efflux from parenchyma cells into the vessels.

It could also be suggested that the spring acidification of the vascular sap is closely related to the activity of the plasma membrane H^+-ATPase in certain living cells of the xylem. Figure 4 shows that the protonophore CCCP induced an alkalinization of the perfusing liquid, whereas the reverse effect was obtained using FC, a specific activating agent of the plasma membrane H^+-ATPase. However, the amplitude of alkalinization caused by CCCP and that of acidification by FC showed seasonal variations, with the responses being highest in spring and lowest during the autumn/winter transition (Table I). These results indicate that some living cells of the xylem have a plasma membrane H^+-ATPase population large enough to control the pH of the vascular sap, this control varying according to the season. The results may also be compared with data from perfusion experiments in which young roots of herbaceous species were used. Here the opposite effects of anoxia and FC on the pH of the perfusion liquid flowing through the vessels of primary xylem indicated that the vascular sap pH depends on the activity of the plasma membrane H^+-ATPase of cells probably located in the stele or surrounding the stele (De Boer et al., 1983; Clarkson and Hanson, 1986). Immunocytolocalization of this enzyme or in situ expression in sections of young roots indicated that the H^+-ATPase was highly concentrated in the endodermis (Parets-Soler et al., 1990) or pericycle (Samuels et al., 1992), which is adjacent to the primary vessels. On the other hand, in the secondary wood of many stems, VAC line or even completely surround the vessels as in *R. pseudoacacia;* they are therefore located in the ideal place to fulfill this control function of controlling vascular sap pH.

In spring, a clear immunolabeling was apparent in VAC (Fig. 5), whereas such labeling was extremely low in rays and axial lignified parenchyma. No reaction occurred in controls (Fig. 5B). Furthermore, the strongest labeling was observed in the VAC of both axial and ray parenchyma

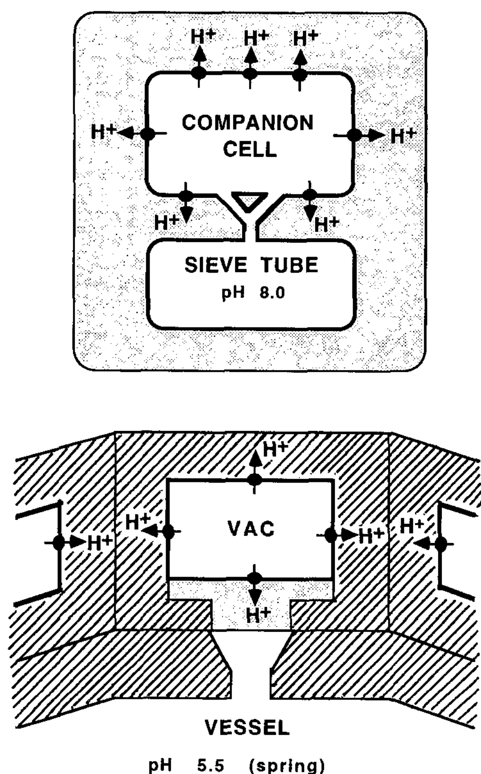

Figure 6. Comparison of the companion cell-sieve tube complex and the VAC-vessel complex: relations between cells and main characteristics of the plasma membrane H^+-ATPase distribution (from Bouché-Pillon, 1994; Bouché-Pillon et al., 1994a; and this work). In the species studied (*V. faba*, *Mimosa pudica*, and *Prunus domestica*), the density of the H^+-ATPase was very low in the sieve tube (Bouché-Pillon, 1994). Only a part of the VAC-vessel complex was drawn. The lignified wall is hatched, and the plasma membrane is indicated by a thick line.

(Fig. 5, C–E). More precisely, this labeling lined the wall like the plasma membrane (Fig. 5, A and C–E). These results demonstrate that the plasma membrane H^+-ATPase is mainly located in VAC.

In recent years, localization of the plasma membrane H^+-ATPase has been achieved using immunocytochemical procedures instead of cytochemical techniques, which have been proven to be nonspecific (Katz et al., 1988). These immunotechniques and in situ expression procedures have demonstrated the abundance of this enzyme in certain cell types of primary tissues: rhizodermis (Parets-Soler et al., 1990; Samuels et al., 1992), endodermis (Parets-Soler et al., 1990), pericycle (Samuels et al., 1992), root cap (Stenz et al., 1993), pollen tube (Obermeyer et al., 1992), guard cells (Villalba et al., 1991), epidermal transfer cells (Bouché-Pillon et al., 1994b), and phloem (Parets-Soler et al., 1990; De Witt et al., 1991; Villalba et al., 1991; Samuels et al., 1992). In *Vicia faba* minor veins, the distribution of this H^+-ATPase is not homogeneous in the phloem, its density being particularly high in transfer cells (modified companion cells) and very low in the sieve tubes (Bouché-Pillon et al., 1994a). Our present immunological data reveal a new case of nonhomogeneous distribution of the plasma membrane H^+-ATPase. It concerns a secondary tissue, the highly lignified wood of *Robinia*.

The present study points out the physiological importance of the VAC, a cell type poorly studied until now with the exception of ultrastructural examination and the localization of some enzyme activities (Czaninski, 1987, and refs. therein).

Our data indicate that the analogies noted between companion cell and VAC are not restricted to the anatomical features already described (Bonnemain and Fromard, 1987; Czaninski, 1987) but may also involve the location of a major intrinsic protein. However, the plasma membrane H^+-ATPase enrichment observed in the companion cell (Bouché-Pillon, 1994; Bouché-Pillon et al., 1994a) and the VAC (this study) generates opposite physiological effects on the sap pH due to the fact that the companion cell communicates with the sieve element (living cell) by branched plasmodesmata, whereas the VAC have no symplastic link (Bonnemain and Fromard, 1987; Czaninski, 1987) with the adjacent vessel element (a nonliving cell belonging to the apoplasmic compartment) (Fig. 6). Consequently, the proton excretion catalyzed by the plasma membrane of the companion cell contributes to the alkalinization of the phloem sap (which flows in the symplastic compartment), whereas the activity of the VAC induces an acidification of the vascular sap (which flows in the apoplastic compartment).

Several factors (variation of the density of the plasma membrane H^+-ATPase, regulation, variation of energetic charge) may be implicated in the seasonal variations of control of the vascular sap pH by the VAC. We are at present examining these parameters.

ACKNOWLEDGMENTS

We are grateful to J.M. Perault and C. Besse of the EM service (Service Universitaire de Microscopie électronique appliqué à la Biologie, Poitiers, France) for their technical assistance. We thank Dr. J. Dainty, Dr. S. Delrot, and Dr. E. Martinoia for critical reading of the manuscript.

Received December 16, 1994; accepted March 16, 1995.
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