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Immunolocalization of the Plasma Membrane H^+-ATPase in Minor Veins of *Vicia faba* in Relation to Phloem Loading[1]

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The immunolocalization of the plasma membrane H^+-ATPase, which generates a proton motive force energizing the uptake of inorganic and organic solutes, was studied by electron microscopy. The cells studied were in minor veins of *Vicia faba* L. exporting leaves, where photosynthates are supposed to be absorbed from the apoplast by phloem transfer cells. Immunologically detectable H^+-ATPase varied among the different cell types and was considerably denser in the transfer cells than in the other cell types, particularly in the sieve tube. Moreover, the distribution of the H^+-ATPase was not homogeneous in transfer cells, that pump being more concentrated in the region adjacent to the bundle sheath, phloem parenchyma, and xylem vessels than along the smooth part of the wall bordering the sieve tube. These results show that the plasma membrane infoldings of transfer cells possess the proton-pumping machinery required to energize an efficient uptake of photosynthates from the phloem apoplast and an efficient retrieval of nitrogenous compounds from the vascular sap.

The plant cell membrane H^+-ATPase that catalyzes a H^+ efflux toward the cell wall belongs to the P-type family of cation pumps (Sussman and Surowy, 1987; Serrano, 1989; Palmgren, 1991). This intrinsic 100-kD polypeptide is considered a "master enzyme" that controls, directly or indirectly, many important functions such as cytoplasmic pH regulation, cell elongation and division, stomata movements, dormancy break, and nutrient uptake (Serrano, 1989).

The plasma membrane H^+-ATPase plays an important function in long-distance nutrient transport in plants where phloem loading is thought to be apoplastic, i.e. sugar beet (*Beta vulgaris*) (Giaquinta, 1976) and broad bean (*Vicia faba*) (Delrot, 1981; Delrot and Bonnemain, 1981; Bourquin et al., 1990). In these plants, sugars and amino acids enter the apoplastic compartment at some point en route to the conducting cells to be further accumulated in the sieve tube-companion cell or sieve tube-transfer cell complex. Suc and amino acids are accumulated in the phloem via a H^+-cotransport process energized by the proton motive force resulting from the activity of the H^+-ATPase of vascular tissues (Despeghel and Delrot, 1983; Giaquinta, 1983; Delrot and Bonnemain, 1984; Bush, 1993).

The model of apoplastic loading is supported by physiological data such as the inhibition of phloem loading of endogenous sugars by *p*-chloromercuribenzenesulfonic acid (Giaquinta, 1976; Bourquin et al., 1990), a nonpermeant thiol reagent that blocks the Suc carrier without affecting the proton motive force (Delrot et al., 1980). These results are strengthened by anatomical data, particularly the modification, in some species, of the companion cells into transfer cells. Those cells are characterized by the presence of wall ingrowths increasing the surface area of the plasma membrane from 3 to 10 times, so that they are considered to be highly specialized in nutrient exchanges between the apoplast and the symplast (Pate and Gunning, 1972; Gunning, 1977; Wimmers and Turgeon, 1991). These data also show a scarcity of plasmodesmata between the conducting complex and the surrounding cells such as those of phloem parenchyma and bundle sheath cells (Pate and Gunning, 1972; Evert and Mierzwa, 1986; Bonnemain et al., 1991).

On the other hand, in numerous species there is considerably more symplastic continuity between photosynthetic and conducting cells, suggesting a symplastic loading of the conducting complex. Although the physiological evidence for symplastic phloem loading is growing, the mechanism by which sugars are selectively accumulated in the conducting complex remains a source of controversy (Delrot, 1987; Van Bel, 1987, 1993; Turgeon and Beebe, 1991).

For a better understanding of assimilate transport toward the conducting complex and to identify strategic steps, information about the location of proteins involved in the H^+ cotransport of nutrients is needed. For this reason, the immunolocalization of the H^+-ATPase polypeptide has been studied with the light microscope in several tissues (Parets-Soler et al., 1990; Villalba et al., 1991; Samuels et al., 1992). These studies showed that most plant cells contain a low amount of this enzyme and that it is highly concentrated in tissues specialized in nutrient transport such as root epidermis and phloem. However, these studies lacked the necessary resolution to identify the phloem cell types with accuracy. Thus, a more detailed knowledge of the H^+-ATPase protein could be gained with immunocytological procedures coupled with EM.

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The ultrastructural observations made in the present work were aimed at the comparative quantification of the distribution of the H^+-ATPase sites on the plasma membrane in the phloem. Our study dealt with the exporting leaves of broad bean, whose minor veins differentiate phloem transfer cells as soon as nutrient transport occurs (Pate and Gunning, 1972; Bonnemain et al., 1991).

MATERIALS AND METHODS

Plant Material

Broad bean (*Vicia faba* L. cv Aguadulce) plants were grown under conditions previously described (Delrot et al., 1980).

Immunocytochemical Procedures

We improved the procedures for immunochemistry already used to localize hormones in cell compartments (Sossountzov, 1986) and proteins in protoplasts (Fleurat-Lessard et al., 1993). This necessitates a compromise between the preservation of infrastructure and antigenicity.

Fixation, Embedding, and Sectioning

Sampling was from the fourth and fifth bifoliate leaves counted from the stem base in 3-week-old plants having six mature leaves. Pieces of blade (1 mm^2) were fixed for 45 min at 4°C in a mixture of 3% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde, and phosphate buffer, pH 7.2. Abundant washing in phosphate buffer, pH 7.4, was followed by a postfixation for 7 min in 1% (v/v) OsO_4, rapid dehydration in an ethanol series, and embedment overnight in hydrophilic resins. We tried several embedding media (glycolmethacrylate, butylmethacrylate, Spurr); finally the London White Resin (medium, TAAB Laboratories, J. Delville St. Germain en Laye, France) was chosen. It was polymerized in gelatin capsules at 55°C for 24 h.

Semithin (1 μm) sections were used to select veins 50 to 100 μm wide (see Fig. 1A), and thin (60 nm) sections, carefully spread with xylene vapors, were laid on parlodion-coated gold grids (200 mesh).

Immunogold Staining

The immunoreaction was performed at 18°C on thin sections collected on grids. Solutions were filtered (Millipore MF VCWP, 0.1-μm pores) or centrifuged (500*g*). The sections, hydrated in deionized water and etched by 0.56 M $NaIO_4$ and 0.1 N HCl, were laid for 15 min on PBS, 0.1% (v/v) Triton X-100, 0.2% (v/v) Gly at pH 7.2 (Sossountzov et al., 1986). After washing in the PBS-Triton medium, nonspecific sites were saturated for 45 min by normal goat serum in PBS, 0.2% (v/v) Triton X-100, 0.2% (v/v) Tween, 0.1% (w/v) BSA and incubated overnight, with 1/50 diluted antibody against H^+-ATPase. After washing in PBS, the sections were laid for 40 min on Tris-buffered saline, pH 8.2, 0.2% (v/v) Tween, 0.2% (v/v) Triton X-100, 1% (w/v) BSA, and normal goat serum. The secondary antibody (goat anti-rabbit IgG, Biocell, TEBU, le Perray en Yuelines, France), diluted 1/40 and labeled with 15-nm gold particles, was applied for 2 h in darkness. The specimens were then washed for 1 h in Tris-buffered saline and deionized water, and they were treated for contrast in uranyl acetate (10 min) and in lead citrate (5 min).

Antibody

The polyclonal antibody used 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 (around 80%) with the different isoforms of tobacco H^+-ATPase (Perez et al., 1992). Thus, it is unlikely that this antibody would discriminate between different isoforms of *V. faba* H^+-ATPase, and the results of our immunogold localizations probably represent the additive contribution of all isoforms (Palmgren and Christensen, 1994). On western blots of crude membranes the antibody specifically recognized a band of about 100 kD (Roldan et al., 1991).

Observation and Quantification of the Results

Observations were made under 80 kV with a 100 C Jeol microscope. Samples for quantification include tissue from three independent fixation-embedding procedures and four immunoreactions. Quantification of gold particles was made per 10 μm of plasma membrane, the total measured length being 200 μm in a total of six minor veins. Two sections in each vein were observed, so that countings were made on 12 sections (see Table I).

Controls

Controls were as follows: (a) omission of the first antibody, (b) sections submitted to preimmune serum, and (c) sections submitted to the specific antibody saturated with purified H^+-ATPase (Palmgren and Christensen, 1993).

RESULTS

The typical structure of minor veins of *V. faba* is shown in Figure 1A. In these veins, the transfer cells are modified companion cells, and some of them are adjacent to both a sieve tube and a xylem vessel (Fig. 1A). Wall ingrowths are located on the interfaces with all surrounding cells, with the exception of the associated sieve tube, so that a marked structural polarity occurs in those transfer cells (Fig. 1B). The polarity is reinforced by the presence of branched plasmodesmata in the smooth part of the wall, which allow symplastic continuity with the sieve tube (Fig. 1B).

We noticed increased immunolabeling on the plasma membrane in the ingrowth area of the transfer cells (Figs. 2 and 3). This suggests that a large number of H^+-ATPase sites were present in the transfer cell plasma membrane at the interface with bundle sheath (Fig. 2A), phloem parenchyma (Fig. 2B), and other transfer cells (Fig. 3A). When transfer cells joined xylem and phloem members simultaneously, the plasma membrane infoldings facing the vessel also had a strong immunoreaction (Fig. 3B). The transfer cell interface with the sieve tube did not develop ingrowths. The immu-

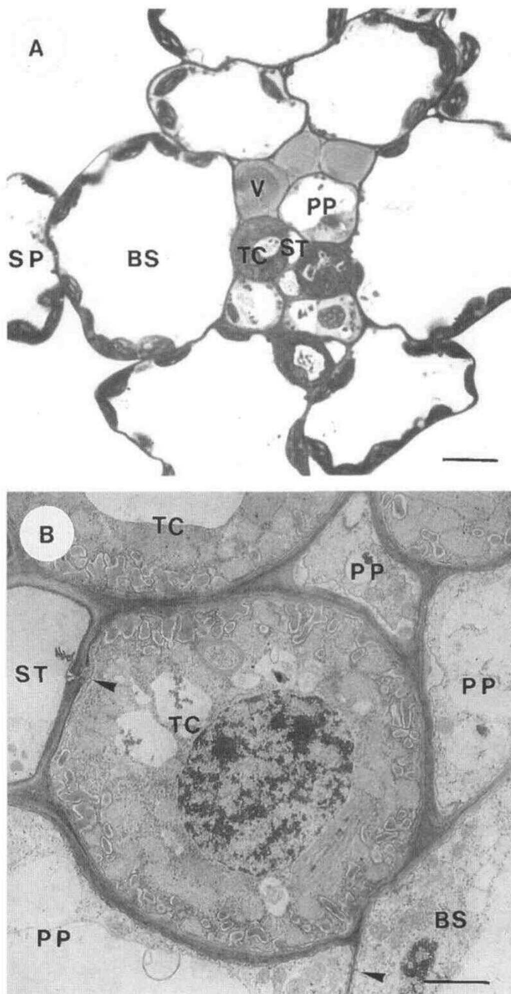

Figure 1. Location of the vascular cells in a cross-section of a *V. faba* minor vein. A, Transfer cells (TC), which have a dense cytoplasm, are adjacent to bundle sheath cells (BS), to phloem parenchyma (PP), to a sieve tube (ST) (smallest structure), and, in the central part of the vein, to xylem vessels (V). SP, Spongious parenchyma cell. Scale bar = 10 μm. B, Detail of the interfaces between a transfer cell (TC) and adjacent cells in a minor vein: another transfer cell with many wall ingrowths and a dense cytoplasm, a bundle sheath cell (BS), phloem parenchyma cells (PP), and a sieve tube (ST). Arrowheads, Plasmodesmata. Scale bar = 2 μm.

nolabeling of plasma membrane on this area was less intense (Fig. 2C) than that observed on the plasma membrane lining the wall ingrowths (Fig. 2, A and B).

By contrast with the labeling associated with transfer cells, the plasma membrane in the adjacent bundle sheath and phloem parenchyma cells had comparatively little labeling (Fig. 2, A and B). Similarly, the plasma membrane in the sieve tube was poorly immunolabeled (Fig. 2C). In all the experiments, some gold particles were present in the wall, the mitochondria, and the plastids, but this labeling was nonspecific, since a similar pattern was observed in preimmune controls (Fig. 4, A and B). In control treatments (Fig. 4C) consisting of the antibody saturated with the purified H^+-ATPase, the immunoreaction was similar to that observed with preimmune sera. In controls in which the first antibody was omitted no labeling occurred (data not shown).

The density of gold particles on a given length of plasma membrane (because labeling occurred only on the surface of a section) provided an additional argument concerning the distribution of H^+-ATPase at the various interfaces between cells. In transfer cells, gold particles were 1.5 to 2.5 times as abundant in the area of plasma membrane infoldings as on the plasma membrane lining the smooth wall facing a sieve tube (Table I). Because the plasma membrane surface was increased at least 5 times in the wall ingrowth area, the total number of H^+-ATPase sites was considerably enlarged in that cell region by comparison with the wall area facing the sieve tube. The density of gold particles on the plasma membrane in bundle sheath cells and phloem parenchyma cells confirms their relatively poor labeling compared with that in transfer cells (Table I). No polarity in the distribution of the H^+-ATPase was found in the bundle sheath and phloem parenchyma cells (Table I).

DISCUSSION

The proton electrochemical potential difference generated by the plasma membrane H^+-ATPase is the driving force for active transport of nutrients, especially sugars and amino acids. Thus, knowledge about the distribution of this enzyme in source (leaf) and sink organs is essential in understanding how the compartmentation of photosynthates is controlled and in identifying the main transport steps.

The first method used to localize this enzyme at the subcellular level consisted of cytochemical staining for ATP hydrolysis by lead-induced precipitation of released Pi (see refs. in Katz et al., 1988). However, it was practically impossible to identify the H^+-ATPase among the cytochemical activities observed. Some of the reasons were the mobility of the liberated phosphate, the affinity of the plasma membrane for the reacted product, and the large number of enzymes that hydrolyze ATP, some of which have no known specific inhibitors (Katz et al., 1988). Therefore, a more specific approach to the H^+-ATPase localization was needed.

In the present work the dramatic reduction of immunolabeling when the antibody was titrated with purified H^+-ATPase (compare Figs. 2B and 4C) argues for the validity and specificity of the methodological approach. Compared with the immunolabeling of the ATPase obtained in light microscopy (Parets-Soler et al., 1990; Villalba et al., 1991), EM provides reliable identification of cell types and makes possible the quantification of gold particles on the plasma membrane. In this way, a comparative study of enzyme distribution was made between different cell types and in different areas of the same cell (Table I).

First, our work confirms the abundance of H^+-ATPase in the phloem tissue, which had already been described with light microscopy (Parets-Soler et al., 1990; Villalba et al., 1991). In contrast with this observation, the number of gold particles is less than 1 per 10 μm in nonspecialized tissues (Bouché-Pillon et al., 1994). It has been suggested that multiple H^+-ATPase genes provide expression of specific isozymes in specialized cell types. With regard to this point the plasma membrane H^+-ATPase isoform 3 is expressed pre-

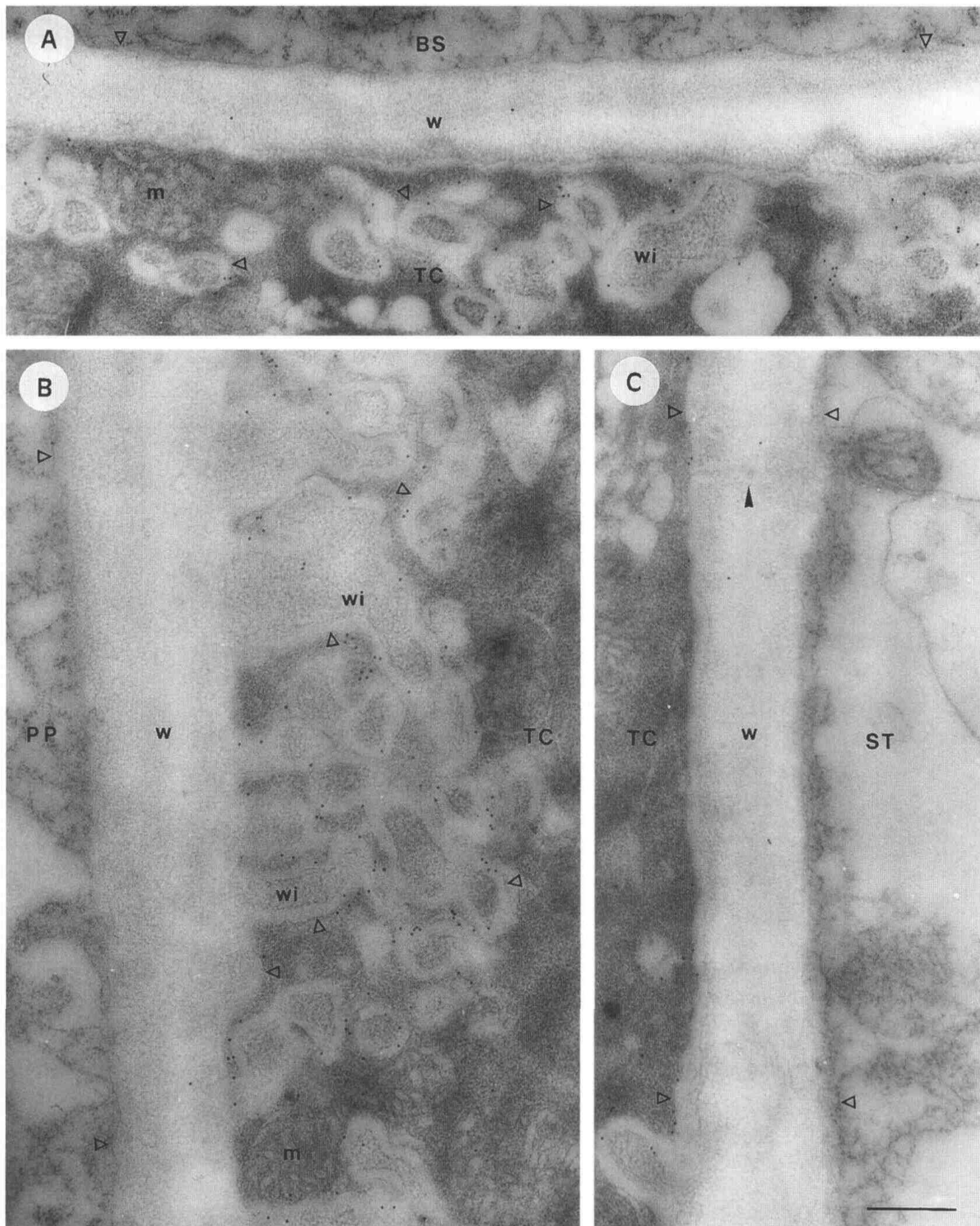

Figure 2. Immunolocalization of H^+-ATPase sites in cross-sections of phloem transfer cells (TC) and adjacent cells. A, At the interface with a bundle sheath cell (BS). B, At the interface with a phloem parenchyma cell (PP). C, At the interface with a sieve tube member (ST). The highest density of 15-nm gold particles is observed along the plasma membrane lining the wall ingrowths (wi). Note the poor labeling of the plasma membrane in the bundle sheath cell (A), the phloem parenchyma cell (B), and in the sieve tube (C). m, Mitochondria; w, wall; Δ, plasma membranes; arrowhead, plasmodesmata. Scale bar = 0.5 μm.

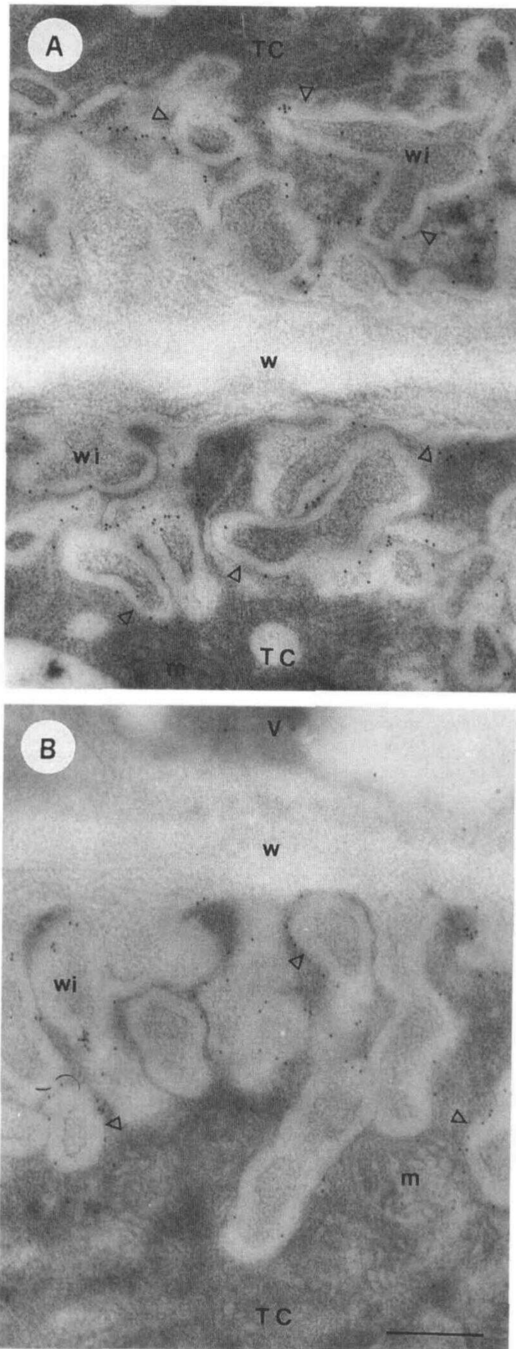

Figure 3. Immunolocalization of the H^+-ATPase sites at the interface between two transfer cells (A) and at the transfer cell/xylem vessel interface (B). Note that the immunogold labeling is abundantly distributed on the plasma membrane infoldings. Abbreviations as in Figures 1 and 2. Scale bar = 0.5 μm.

dominantly in the phloem tissue of *Arabidopsis thaliana* (De Witt et al., 1991). However, it is likely that the immunoreaction in *V. faba* phloem represents the additive contribution of all the isoforms, as stated in "Materials and Methods." Indeed, antibodies directed against the central domain (amino acids 340–650) do not differentiate between the H^+-ATPase isoforms (Palmgren and Christensen, 1994). Furthermore, in the *Vicia* embryo, the highest number of gold particles occurred in the cell type (epidermal transfer cells) (Bouché-Pillon et al., 1994) having the more negative transmembrane potential difference (Bonnemain et al., 1991).

Second, the data demonstrate the heterogeneity, according

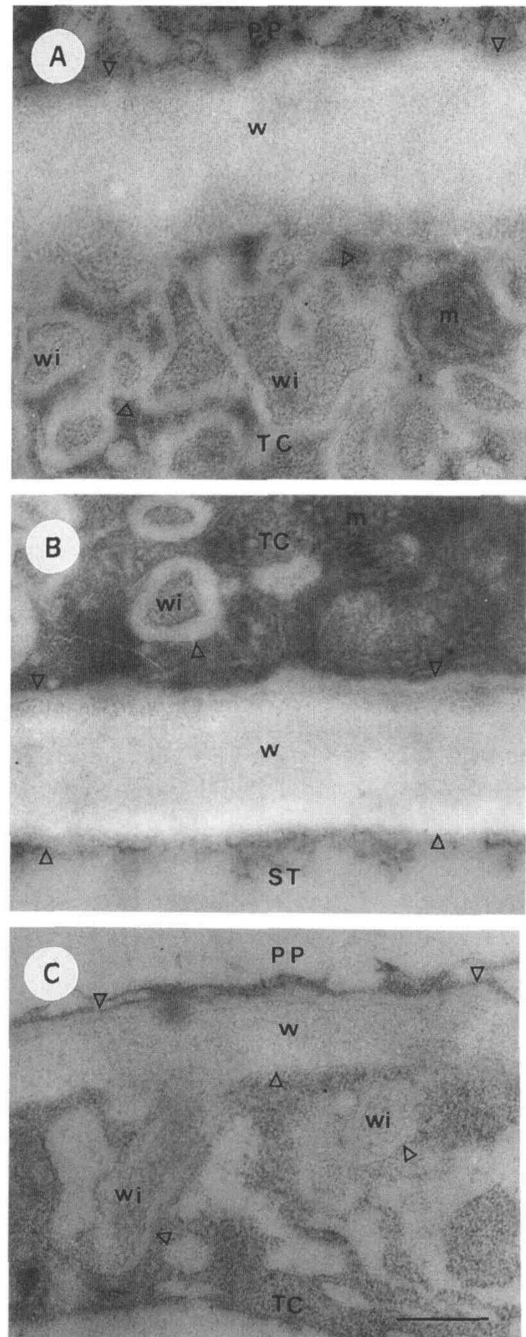

Figure 4. Controls. With a preimmune serum (A and B) or an antibody blocked by a purified H^+-ATPase (C), very weak and nonspecific labeling is observed on the transfer cell/phloem parenchyma and transfer cell/sieve tube interfaces. Abbreviations as in Figure 2. Scale bar = 0.5 μm.

Table I. *Comparative distribution of H^+-ATPase in the cells of V. faba minor veins*

Counts were made on the two sides of the interface between adjacent cells. The distribution of 15-nm gold particles is reported per 10 μm of plasma membrane (mean ± SE, n = 20). pi, Plasma membrane infoldings.

Interface between	Immune Serum	Preimmune Serum
Transfer cell (pi) and	19.3 ± 1.4	0.8 ± 0.2
Bundle sheath	4.1 ± 0.7	0.5 ± 0.2
Transfer cell (pi) and	24.4 ± 1.8	0.7 ± 0.2
Phloem parenchyma	6.1 ± 1.1	0.5 ± 0.2
Transfer cell (pi) and	33.1 ± 1.6	0.4 ± 0.2
Transfer cell (pi)	32.8 ± 1.6	0.4 ± 0.3
Transfer cell and	14.5 ± 1.7	0.4 ± 0.1
Sieve tube	3.0 ± 0.6	0.5 ± 0.2
Phloem parenchyma and	5.9 ± 0.9	0.3 ± 0.2
Bundle sheath	4.3 ± 1.1	0.5 ± 0.2
Phloem parenchyma and	4.0 ± 0.6	0.4 ± 0.2
Phloem parenchyma	4.0 ± 0.8	0.4 ± 0.3

to cell types, of the distribution of the H^+-ATPase in the phloem of minor veins, since the density of label is 6 to 11 times higher in transfer cells (plasma membrane infoldings area) than in sieve tubes. Transfer cells, which are widespread in the plant kingdom, generally occur at strategic locations: the interface between the plant and the environment, the interface between generations, and, in veins, the interface between vascular and parenchyma cells. From the location of this cell type, its anatomical features (the increase of the plasma membrane surface area and the abundance of mitochondria), and its physiological activity (a strongly negative transmembrane potential difference, at least in epidermal transfer cells [Renault et al., 1989; Bonnemain et al., 1991]), we may infer that this cell type plays an important role in apoplast-symplast exchanges (Pate and Gunning, 1972; Gunning, 1977; Kursanov, 1984; Bonnemain et al., 1991) and in the increased uptake of Suc from the phloem apoplast (Wimmers and Turgeon, 1991). The present work supports this hypothesis based on the differentiation of the plasma membrane of the phloem transfer cell, which is particularly rich in H^+-ATPase.

In the veins, as in other tissues, the location of the Suc and amino acid carriers is still unknown. However, the asymmetry of H^+-ATPase distribution in transfer cells, caused by a higher density in the region of wall ingrowths (Figs. 2 and 3), makes possible the efficient collection of photosynthetic products from the apoplast not only at the interface lining the bundle sheath but also at the interface lining the phloem parenchyma. Then, the photosynthates absorbed by each transfer cell can enter the associated sieve tube through branched plasmodesmata (Fig. 1B) (Delrot, 1987; Van Bel, 1987, 1993). A similar asymmetry both in the wall ingrowth and in the H^+-ATPase distribution was found in the epidermal transfer cells of *V. faba* embryo (Bouché-Pillon et al., 1994). These cells collect apoplastic Suc supplied by the maternal organism by a H^+ cotransport process (El Ayadi, 1987; Bonnemain et al., 1991). In this context, a 62-kD extrinsic protein involved in Suc uptake has been immunolocalized on plasma membrane infoldings of transfer cells of *V. faba* cotyledons (Franceschi et al., 1992).

The other function attributed to vein transfer cells is transfer to the sieve tubes of inorganic and organic solutes, delivered via the xylem to the mature leaves (Pate and Gunning, 1972; Gunning, 1977; Madore and Lucas, 1989). This function is of special interest in the Leguminoseae, the vascular sap of which is particularly rich in nitrogenous compounds (Pate, 1991). In that respect, our data show that the plasma membrane of the transfer cells adjacent to a xylem vessel and a sieve tube has abundant H^+-ATPase sites facing the vessel (Fig. 3B). Because no plasmodesmata occurred between vessels and transfer cells, the uptake of nitrogenous compounds by the latter has to be a case of apoplastic retrieval. It should be stressed that H^+-ATPase distribution is the same on plasma membrane infoldings next to a vessel (which are involved in apoplastic retrieval of nitrogenous compounds) or next to a cell of the phloem parenchyma or bundle sheath (which are involved in the uptake of photosynthates from the apoplastic compartment) (Figs. 2, A and B, 3B).

In conclusion, our results (a) show the heterogenous distribution of the H^+-ATPase pump in the phloem cell types, among which transfer cells have the highest density of the proton pump, especially in areas of plasma membrane infoldings; (b) support the occurrence of an apoplastic loading of photosynthates in *V. faba*; (c) suggest that the strategic steps of this apoplastic loading of photosynthates are localized at the bundle sheath/transfer cell and at the phloem parenchyma/transfer cell interfaces; (d) point out that the H^+-ATPase density in the transfer cell region adjacent to vessels makes possible an efficient retrieval of nitrogenous compounds toward the sieve tubes. The immunolocalization of Suc and amino acid carriers needs to be performed in order to examine in detail the two latter points.

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