Phloem Loading by the PmSUC2 Sucrose Carrier from *Plantago major* Occurs into Companion Cells

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High levels of mRNA for the sucrose-H^{+} symporter PmSUC2 have been found in the vascular bundles of petioles from *Plantago major*. The possible role of PmSUC2 in phloem loading was studied with antiserum raised against the recombinant PmSUC2 protein. This antiserum labeled a single 35-kD protein band in detergent extracts of *P. major* vascular bundles. It showed no cross-reaction with the *P. major* sucrose carrier PmSUC1, which was tested with detergent extracts from plasma membranes of transgenic yeast strains containing either the *P. major* sucrose transporter PmSUC1 or PmSUC2. The antiserum was used to determine the site of *PmSUC2* expression in leaves, petioles, and roots of *P. major*. In cross-sections and longitudinal sections, the PmSUC2 protein was found in only one single cell type. These cells were identified as companion cells because they are nucleated, contain a dense cytoplasm, and are always adjacent to a sieve element. The labeled cells had the same longitudinal extension as did their sister sieve elements and always ended next to the sieve plates, which were characterized by specific staining. *PmSUC2* mRNA and PmSUC2 protein were also detected in *P. major* roots. The function of PmSUC2 in the different organs and its role in phloem loading are discussed.

INTRODUCTION

In many plants, sucrose is the long-distance transport form for carbohydrates. During recent years, cDNAs for several sucrose transporters have been cloned (Riesmeier et al., 1992, 1993; Gahrtz et al., 1994; Sauer and Stolz, 1994). They were all expressed in baker's yeast and shown to catalyze the uptake of sucrose across yeast plasma membranes. Invertase-deficient yeast cells expressing the *Plantago major* PmSUC2 sucrose transporter accumulate sucrose >200-fold, and in vitro studies with the recombinant PmSUC2 protein from yeast have shown that the necessary energy can be supplied by an artificial proton motive force (PMF) generator (Gahrtz et al., 1994). This is consistent with earlier studies of sucrose-H^{+} symporters performed with whole plant cells (for review, see Delrot, 1989) or with studies of plasma membrane vesicles purified from plant tissue (Buckhout, 1989; Bush, 1989). First evidence for phloem localization of one of the cloned sucrose transporters was obtained for the potato StSUT1 transporter by using in situ hybridization experiments (Riesmeier et al., 1993). The abundance of *PmSUC2* mRNA in vascular tissue (Gahrtz et al., 1994), investigations with *StSUT1* antisense potato plants (Riesmeier et al., 1994), and the phloem specificity of an *AtSUC2* promoter–β-glucuronidase (*GUS*) reporter gene

construct in Arabidopsis (Truernit and Sauer, 1995) have confirmed these results.

The phloem of angiosperms is composed of phloem parenchyma cells, sieve elements, and companion cells, and a large number of structural differences suggest a clear functional separation of these cell types. Mature, fully developed sieve tubes lose their nuclei, and most of their other cell organelles, such as vacuoles, dictyosomes, and ribosomes, are degraded during the final steps of phloem differentiation. However, other organelles, such as the endoplasmic reticulum, mitochondria, and plastids, are found in varying amounts during all stages of sieve element ontogeny (for review, see Behnke, 1989). Companion cells, in contrast, contain large nuclei and, in addition to all other organelles, numerous mitochondria, plastids, and ribosomes. The extraordinary density of their protoplasts distinguishes them not only from the sieve elements but also from the phloem parenchyma cells (Behnke, 1989). For many plants, plasmodesmal frequencies between the so-called sieve element–companion cell (se-cc) complex and the surrounding parenchymatic cells have been studied, and three groups with practically no connections, with few plasmodesmata, or with many plasmodesmata have been identified (Gamalei, 1989). A large number of plasmodesmal connections between companion cells and sieve elements on one hand and a symplastic isolation of the se-cc complex on the other have also been described (Hayes et al., 1985; van Bel and Kempers, 1990; van der Schoot and van Bel, 1990). The work of Gamalei (1989) shows that the members of the Plantaginaceae display virtually complete symplastic isolation of the se-cc complex. As

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early as 1929, sucrose was shown to be the main sugar within the vascular bundles from *P. major*, and it became clear that sucrose accumulates within the vascular tissue (Ritschl, 1929).

These results can be explained only by the existence of an energy-dependent sucrose transporter that catalyzes phloem loading from the apoplast. This concept was developed by Giaquinta (1977) and confirmed by Komor et al. (1977) and others. A more general correlation between symplastic isolation of the se-cc complex and apoplastic phloem loading was described recently (van Bel et al., 1994). In light of this work, there was a need to identify the cells in which the actual loading step occurs. Morphological adaptations, such as the increased surface of the companion cells in certain plants (so-called transfer cells) and the immunolocalization of a H⁺-ATPase within such transfer cells (Bouché-Pillon et al., 1994), suggest that these cells might represent the actual site of loading.

Phloem loading does not seem, however, to be the sole function of sucrose transporters. Analyses of the tissue-specific expression of sucrose transporters by RNA gel blotting and in transgenic Arabidopsis plants (Sauer and Stolz, 1994; Truernit and Sauer, 1995) have clearly pointed to an expression of Arabidopsis sucrose transporters outside the classical source tissue, such as fully developed leaves. In this study, we addressed how sucrose enters the phloem and into which cells the actual loading step occurs by using a combination of different histochemical techniques. The use of highly specific anti-PmSUC2 antibodies permitted the localization of the PmSUC2 protein in companion cells.

RESULTS

Specificity of the Anti-PmSUC2 Antiserum

The PmSUC2 protein has a calculated molecular mass of 54 kD and an apparent molecular mass of ~35 kD when separated on 10% SDS–polyacrylamide gels (Gahrtz et al., 1994). The recombinant PmSUC2 protein from transgenic baker's yeast was isolated from preparative SDS gels and used to raise antibodies in rabbits. Because PmSUC2 is not the only sucrose-H⁺ symporter from *P. major* and because the second *P. major* protein, PmSUC1, shows high sequence homology (59.9% identical amino acids), the specificity of the resulting anti-PmSUC2 antiserum had to be tested. SDS extracts from plasma membranes of transgenic yeast cells expressing either the *P. major* PmSUC1 or the PmSUC2 sucrose-H⁺ symporter, and extracts from yeast cells expressing the respective proteins from Arabidopsis (AtSUC1 and AtSUC2; Sauer and Stolz, 1994), were separated by SDS-PAGE, gel blotted, and treated with affinity-purified anti-PmSUC2 antiserum. A 35-kD band and a much weaker 70-kD band (the dimeric form), which correspond to the PmSUC2 protein, were recognized only in lane 3 of Figure 1A. A faint cross-reaction can be seen only with the 43-kD band of the AtSUC1 sucrose carrier (lane 4). All other

bands in lanes 2, 4, and 5 of Figure 1A can also be seen in extracts of a control yeast strain expressing no sucrose transport protein (lane 1). This shows that the anti-PmSUC2 antiserum is specific for the PmSUC2 protein and does not cross-react with the protein of the PmSUC1 sucrose carrier.

Does the anti-PmSUC2 antiserum recognize proteins other than PmSUC2 in *P. major*? To test this possibility, proteins were solubilized from total membranes prepared from *P. major* leaves or from vascular bundles extracted from petioles. Protein gel blot analysis of these fractions gave no signals in extracts from sink or source leaves but gave a clear, single band in the extract from vascular bundles (Figure 1B). This not only shows that the anti-PmSUC2 antiserum is monospecific in *P. major* but also proves that the PmSUC2 protein is strongly enriched in vascular tissue.

Because the anti-PmSUC2 antiserum recognized the recombinant PmSUC2 protein with high specificity and because
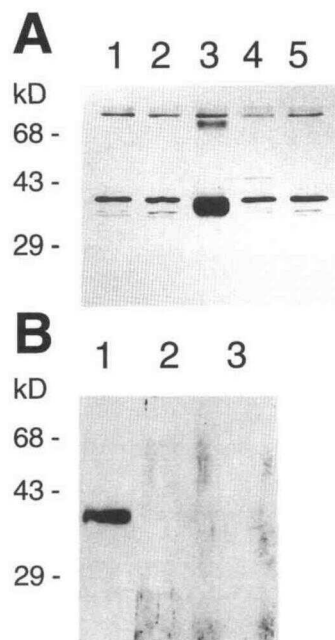

Figure 1. Protein Gel Blot Analyses of the Specificity of the Anti-PmSUC2 Antiserum in Transgenic Yeast Cells and in Leaves of *P. major*.

Plasma membranes from transgenic yeast strains expressing four different plant sucrose transporters, as shown in **(A)**, or total membranes from *P. major* sink leaves, source leaves, or extracted vascular bundles, as shown in **(B)**, were tested for their reaction with anti-PmSUC2 antiserum.

(A) Lane 1 contains the control (strain MGY1); lane 2, PmSUC1 (strain MGY6); lane 3, PmSUC2 (strain MGY2); lane 4, AtSUC1 (strain NSY611); and lane 5, AtSUC2 (strain NSY612).

(B) Ten micrograms of SDS-solubilized proteins from vascular bundles (lane 1), sink leaves (lane 2), or source leaves (lane 3) of *P. major* was separated on SDS gels, gel blotted, and treated with anti-PmSUC2 antiserum (diluted 1:16). The apparent molecular masses of the size markers are given at left in kilodaltons.
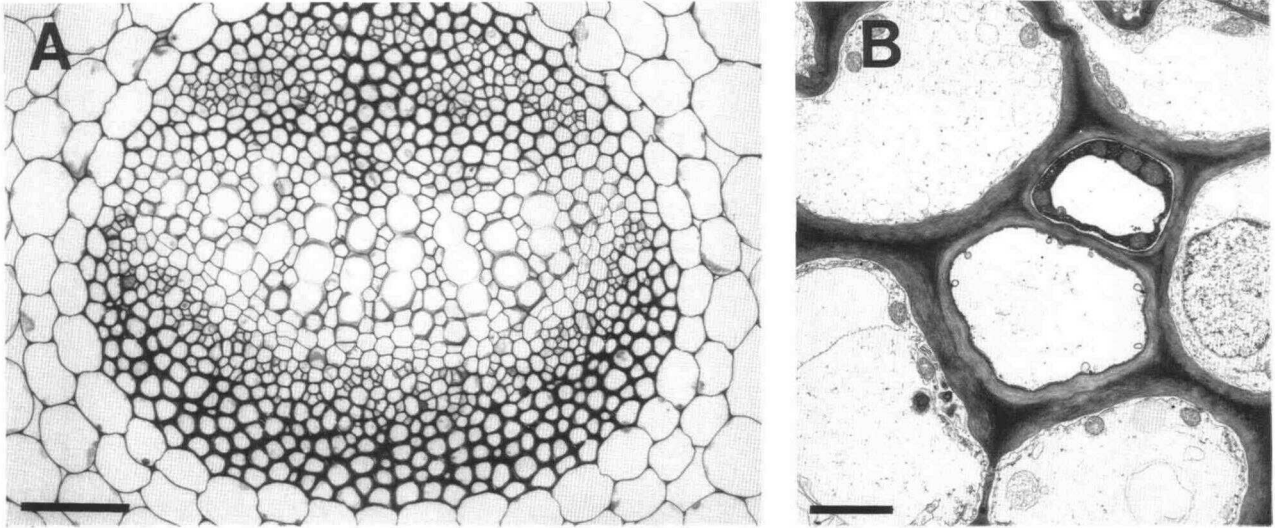
Figure 2. A Vascular Bundle and a se-cc Complex from *P. major* Petioles.

Petiole sections were photographed under the light microscope, as shown in **(A)**, or the electron microscope, as shown in **(B)**.
(A) An overview of a vascular bundle from a petiole. Scale bar = 50 μm.
(B) A typical se-cc complex surrounded by five phloem parenchyma cells; the dense cytoplasm of the smaller companion cell is rich in mitochondria. Scale bar = 2 μm.

cross-reactions could be detected with neither the second *P. major* sucrose transporter nor any other membrane protein of *P. major*, the serum was used for immunohistochemical analysis of PmSUC2 localization in *P. major*.

Localization of the PmSUC2 Protein

The cross-section of a typical vascular bundle from petioles of *P. major* is presented in Figure 2A. The large xylem vessels in the center are sandwiched between two layers of phloem (bicollateral bundle) that can be identified as groups of smaller cells above and below the xylem. Typical pairs of sieve tubes and smaller companion cells are found only in these parts of the vascular bundle. The darker cells at the top and the bottom of the bundle represent sclerenchymatic fibers that are partly lignified but still alive. At higher magnification (Figure 2B), the dense cytoplasm of the companion cells and the comparatively empty sieve tubes can be distinguished easily. Typically, the companion cells are smaller in diameter than are the sieve elements. We wanted to determine in which cell type(s) of the phloem the *PmSUC2* gene was expressed — the phloem parenchyma, or one or both cell types of the se-cc complex?

Sections from petioles of *P. major* were incubated with the anti-PmSUC2 antiserum (Figure 3B) or with the anti-STP1 (sugar transport protein 1; Stolz et al., 1994) antiserum (control; Figure 3A) and decorated with a fluorescein isothiocyanate (FITC) isomer 1–conjugated secondary antibody. Fluorescence microscopy of labeled sections shows a green, anti-PmSUC2

antibody–dependent fluorescence in defined regions on both sides of the xylem (Figure 3B). This localization of the fluorescence label is in perfect agreement with the bicollateral organization of the *P. major* vascular bundles in the petioles, indicating that the cells labeled with the anti-PmSUC2 antiserum are located within the phloem. A second anti-PmSUC2 antiserum, which was raised independently in a second rabbit, gave identical results. Controls in which the anti-PmSUC2 antibody was either omitted or replaced by a control antiserum raised against the Arabidopsis STP1 monosaccharide-H^+ symporter showed no FITC labeling (Figure 3A). The yellow staining of the xylem vessels is due to phenolic compounds in the cell walls of the respective cells.

In contrast to the large vascular bundles of the petioles, the minor and medium-sized veins of *P. major* leaves revealed a collateral organization with only one layer of phloem below a small number of xylem elements. Figures 3C to 3E show sections of minor and medium-sized veins stained with anti-PmSUC2 antiserum and labeled with FITC. Again, the PmSUC2 protein is seen only in the phloem portion. Figure 3F shows the venation pattern of a *P. major* leaf with the same types of vein shown in cross-section in Figures 3C (minor vein) and 3E (medium-sized vein).

Characterization of the *PmSUC2*-Expressing Cells

For a more precise characterization of the cell type, it was necessary to look at additional properties of the labeled cells. One of the characteristics typical of sieve elements is
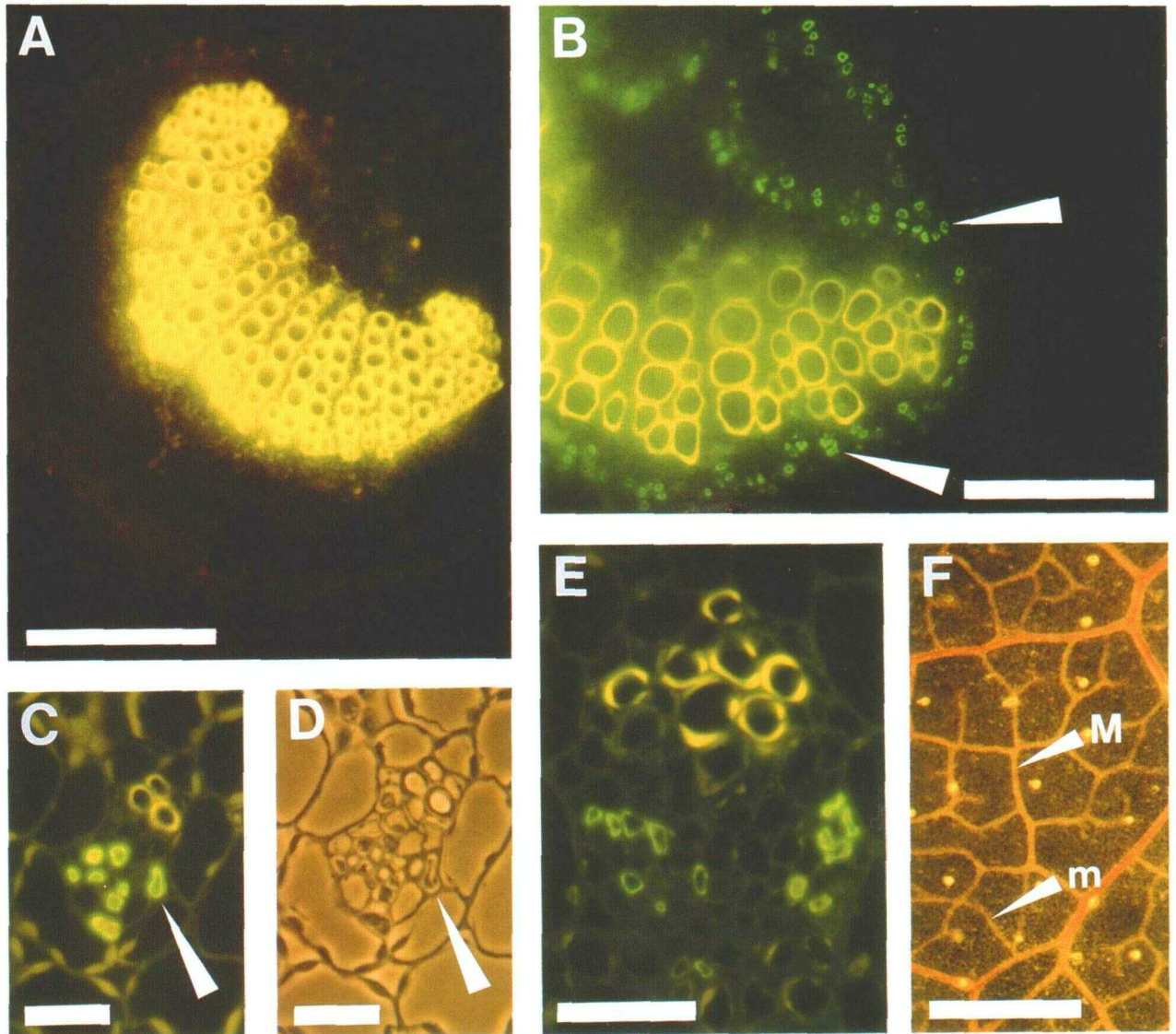
Figure 3. Immunocytochemical Identification of the PmSUC2 Protein in Vascular Bundles from *P. major* Leaves and Petioles.

Hand-cut sections of petioles are shown in **(A)** and **(B)**, and thin sections of methacrylate-embedded leaves are shown in **(C)** to **(E)**. Sections were treated with anti-PmSUC2 antiserum or with control serum; antibody binding was detected with an anti-rabbit IgG–FITC conjugate.
(A) Control section of a vascular bundle from petioles (stained with anti-STP1 antiserum and labeled with FITC). Scale bar = 100 μm.
(B) Cross-section of a vascular bundle from petioles (stained with anti-PmSUC2 antiserum and labeled with FITC). The large xylem vessels (yellow fluorescence) are sandwiched between two layers of phloem (arrowheads), which contain small cells labeled with anti-PmSUC2 antiserum. Scale bar = 50 μm.
(C) Minor vein from a leaf (labeled with anti-PmSUC2 antiserum and stained with FITC). Scale bar = 10 μm.
(D) Same minor vein as that shown in **(C)**. The arrowheads in **(C)** and **(D)** mark one of the fluorescence-labeled cells. Scale bar = 10 μm.
(E) Medium-sized vein from leaves labeled with anti-PmSUC2 antiserum and stained with FITC. Scale bar = 10 μm.
(F) Venation pattern of a *P. major* leaf after clearing with 70% ethanol and staining of the xylem with phloroglucinol-HCl. Arrowheads indicate minor (m) and medium-sized (M) veins of the types shown in **(C)** and **(E)**, respectively. Scale bar = 1 mm.

denucleation during development. Double staining of thin sections with the anti-PmSUC2 antiserum/FITC conjugate and with 4′,6-diamidino-2-phenylindole (DAPI), which binds to DNA and results in a blue fluorescence staining of the nucleus, revealed

that the PmSUC2-containing cells have a well-developed nucleus (Figure 4). Because these cells are very long (a longitudinal section is shown in Figure 4B), only some of the antiserum/FITC conjugate–labeled cells showed stained nuclei

in the cross-sections (Figure 4A). This result provides strong evidence that the *PmSUC2* gene is not expressed in the sieve tubes. The shape of the double-labeled cells and the size of their nuclei suggest that these cells may be companion cells. Typically, companion cells are narrow and contain large nuclei that often occupy comparatively great portions of the cell (Behnke, 1989).

These results were confirmed by double staining with the anti-PmSUC2 antiserum/FITC conjugate and aniline blue. This dye binds to callose, which is deposited in the pores of the sieve plates, and gives a bright blue fluorescence under excitation light. Figure 5A shows a sieve plate stained with aniline blue in a cross-section from a *P. major* petiole. Double staining of sections with aniline blue and the antiserum/FITC conjugate (Figures 5B to 5H) resulted in a reduction of brightness for both signals; nevertheless, it is clear that the sieve tubes (which are characterized by the blue staining of their sieve plates) were not labeled with the anti-PmSUC2 antibody. The greenish color of the antiserum/FITC conjugate reaction is restricted to the smaller cells that are always in the immediate vicinity of these sieve elements. As can be seen in the double-stained longitudinal sections in Figures 5F to 5H, the ends of two companion cells meet next to the sieve plate connecting their sister sieve elements.

Expression of the PmSUC2 Protein in *P. major* Roots

Histochemical analysis of Arabidopsis plants that had been transformed with a chimeric construct of promoter and coding sequences from the *AtSUC2* gene and *GUS* (*AtSUC2–GUS*) revealed that the *AtSUC2* promoter directed GUS activity not only to the vascular bundles of green, photosynthetically active tissue, but also to the phloem of Arabidopsis roots (Truernit and Sauer, 1995). RNase protection studies of tissue-specific expression of the *PmSUC2* gene confirmed these results and showed similar expression levels in different organs of *P. major* plants (Figure 6). The lower levels of *PmSUC2* mRNA in younger sink leaves may reflect their physiological situation and low export capacity (for review, see Turgeon, 1989).

We used thin sections of *P. major* roots to identify the precise localization of the PmSUC2 protein. The organization of the vascular systems differs between roots and shoots: the individual xylem strands are arranged in the central cylinder of the root like the spokes of a wheel, with clusters of phloem tissue in between. Figures 7A and 7B show cross-sections of a root with four xylem strands. Staining of this root with the anti-PmSUC2 antiserum/FITC conjugate resulted in fluorescence labeling in the phloem. A comparison of Figures 7C and 7D shows that only small cells with denser cytoplasm reacted. The intensity of this fluorescence was weaker than that in the leaves, but clearly the PmSUC2 protein is also found in the companion cells of these roots. Cross-sections of the youngest roots (Figures 7E and 7F), however, showed no detectable FITC staining.

DISCUSSION

To study the cell-specific expression of the *P. major PmSUC2* gene, antibodies were raised against recombinant PmSUC2 protein. This protein was isolated from SDS–polyacrylamide gels of plasma membrane extracts from transgenic yeast cells. The protein migrated as a separate, prominent band with an apparent molecular mass of ~35 kD (Gahrtz et al., 1994). We used an anti-PmSUC2 antiserum that is highly specific for PmSUC2 and that showed no cross-reactivity with either the second *P. major* sucrose transporter, PmSUC1 (Figure 1A), or with any other membrane protein from *P. major* leaves (Figure 1B). This serum was used to study the distribution of the PmSUC2 protein in longitudinal sections and cross-sections of *P. major* leaves, petioles, and roots. Incubation of these
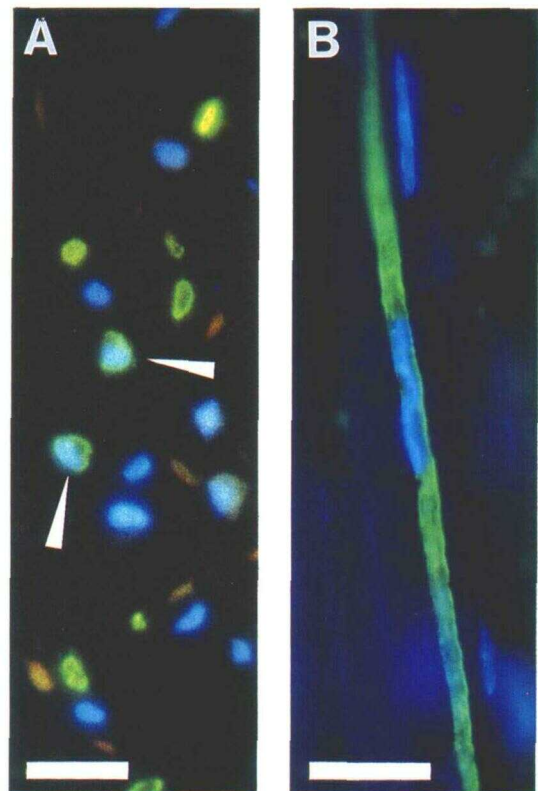

Figure 4. Histochemical Staining of Nuclei with DAPI in Cells Treated with the Anti-PmSUC2 Antiserum/FITC Conjugate.

Petiole sections of 3 to 6 μm were double stained with anti-PmSUC2 antiserum/FITC conjugate and DAPI.

(A) Cross-section, through the phloem portion, of a vascular bundle. Two double-stained cells with a ring of green FITC staining and blue DAPI staining of the nucleus are indicated by arrowheads. Scale bar = 5 μm.

(B) Longitudinal section, to the phloem portion, of a vascular bundle. One single companion cell is shown. Scale bar = 10 μm.
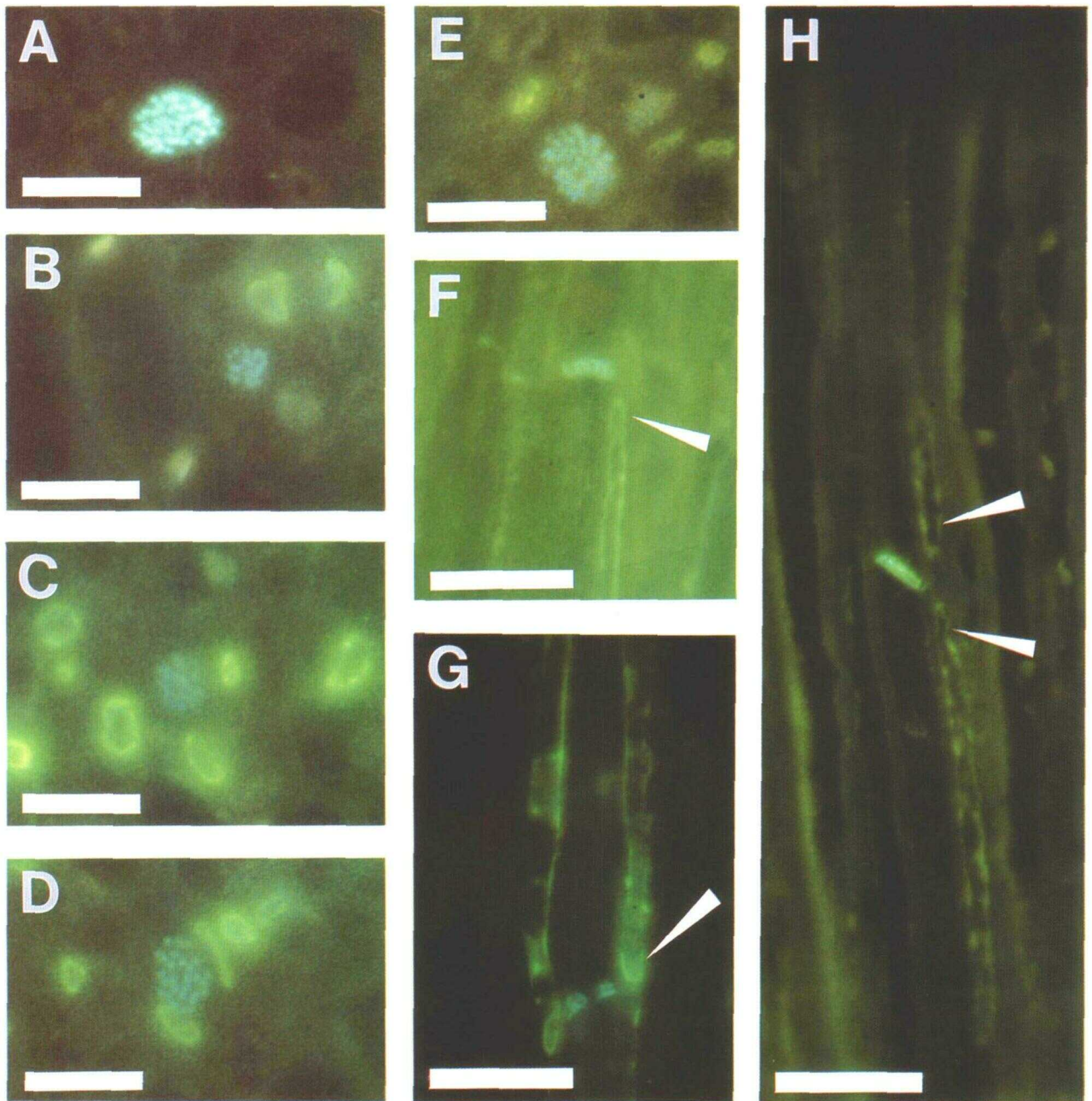
Figure 5. Double Staining of Sieve Plates with Aniline Blue and of Companion Cells with the Anti-PmSUC2 Antiserum/FITC Conjugate.

(A) Sieve plate from a petiole cross-section stained with aniline blue only.

(B) to **(E)** Sieve plates (blue dots) and companion cells (green rings) from petiole cross-sections double stained with aniline blue and the anti-PmSUC2 antiserum/FITC conjugate. Scale bars in **(A)** to **(E)** = 5 μm.

(F) to **(H)** Sieve plates and companion cells from petiole longitudinal sections double stained with aniline blue and the anti-PmSUC2 antiserum/FITC conjugate, respectively. Arrowheads indicate the ends of companion cells next to the respective sieve plate. Scale bars = 10 μm.

sections with the anti-PmSUC2 antiserum and with the FITC-labeled second antibody revealed a specific localization of the PmSUC2 protein in one single cell type within the phloem. The additional use of classic histochemical techniques for light microscopy showed that the antiserum/FITC conjugate–labeled

cells are longitudinally extended and relatively narrow, with a well-developed nucleus. In petioles and major veins, the diameter of these cells is smaller than the diameter of the adjacent sieve tubes. However, in minor veins, these cells have at least the same diameter as the adjacent sieve elements.

The sieve tubes themselves, whose sieve plates were stained with aniline blue, did not have detectable amounts of the PmSUC2 protein.

The se-cc complex originates from one cambial or procambial derivative; typically, the last, usually unequal division of this cell gives rise to a larger sieve element and a narrow companion cell. The companion cell may then undergo a number of anticlinal divisions or perform the same longitudinal extension as its sister sieve element. In any case, the outermost ends of a companion cell will be just at the ends of the respective sieve tube. This can be seen in Figure 5E, in which the fluorescence of the antiserum/FITC conjugate–labeled companion cells ends at the sieve plate connecting their sieve elements.

The gene for the PmSUC2 sucrose-H$^+$ symporter is expressed in the companion cells of the minor and medium-sized veins of the leaves that collect the assimilates and that are the primary sites of sucrose loading. PmSUC2 is also expressed in the companion cells of the largest vascular bundles of leaves and petioles that are primarily responsible for the export of sucrose and other assimilates from the leaves. To a lesser extent, these cells are responsible for sucrose loading. A direct comparison of antibody/FITC conjugate–labeled thin sections from veins of different sizes revealed that the highest intensity of FITC fluorescence is seen reproducibly in the minor veins. This suggests that more PmSUC2 protein is synthesized in the companion cells of these minor veins, which are the sites of maximal phloem loading, than in the companion cells of the larger exporting veins.

The identification of PmSUC2 mRNA in root tissue and the localization of PmSUC2 protein in companion cells of the root phloem confirm results obtained earlier from Arabidopsis plants transformed with an AtSUC2–GUS fusion (Truernit and Sauer, 1995). Therefore, the function of PmSUC2 cannot be restricted to the loading of newly synthesized sucrose into the phloem of source leaf minor veins. PmSUC2 also may be involved in one of the following two reactions: First, the high concentration of sucrose within the sieve tubes causes a permanent passive leak of sucrose out of the sieve tubes. During the long-distance transport from the source leaf to the different sinks, a dramatic loss of assimilate could occur and cause reduced flux rates within the sieve tubes. To avoid this loss of sucrose

and to maintain an optimal mass flow within the sieve tubes, it may be necessary to reimport the lost sucrose into the se-cc complex. Such retrieval mechanisms have been discussed previously as the primary or even exclusive function of plant sugar transporters (Maynard and Lucas, 1982). Second, the PmSUC2 protein may catalyze not only energy-dependent phloem loading but, under appropriate conditions in the sinks, also the export of sucrose from the se-cc complex.

In this study, we addressed how sucrose enters the sieve tubes of P. major. Sucrose is loaded from the apoplast into the companion cells of the phloem; from there, sucrose diffuses symplastically through the plasmodesmata of the se-cc complex into the sieve elements. Companion cells, which are rich in mitochondria, do not supply the sieve tubes with energy for sucrose uptake; rather, they use this energy themselves. The energy could be provided from sucrose hydrolysis by a companion cell–specific sucrose synthase (Nolte and Koch, 1993). A plasma membrane, H$^+$-ATPase, which is expressed predominantly in transfer cells and which generates the PMF necessary for sucrose loading, has previously been described in broad bean (Bouché-Pillon et al., 1994).

The PmSUC2 protein studied by heterologous expression in yeast is extremely pH sensitive and shows increased transport rates with decreasing extracellular pH values (Gahrtz et al., 1994). The respective H$^+$-ATPase in P. major may therefore not only supply the PMF needed for sucrose transport but also regulate the transport rates of the PmSUC2 protein by influencing the extracellular pH. It has been shown recently (Buckhout, 1994) that changes in the extracellular pH strongly affect the K_m value of a sucrose carrier from sugar beet, suggesting that such changes may in fact represent a regulatory mechanism. However, in contrast with these results, Bush (1990) proposed a model in which, according to the proposed pH optimum, the proton binding site of the sucrose transporter is always occupied under physiological conditions. According to this model, changes in the extracellular pH would not influence the activity of the transporter, and only changes in the extracellular sucrose concentration or in the membrane potential could affect the rates of phloem loading.

METHODS

Strains

Plants (Plantago major) were grown in the greenhouse. The specificity of the anti-PmSUC2 antiserum was tested with different strains of baker's yeast. All were transformants of strain DBY2617 (MATa, his4-539, lys2-801, ura3-52, suc2-438; Kaiser and Botstein, 1986). The strains included MGY2 (Gahrtz et al., 1994) and MGY6, which express the P. major sucrose-H$^+$ symporters PmSUC2 and PmSUC1, respectively, and strains NSY611 and NSY612, which express the Arabidopsis sucrose-H$^+$ symporters AtSUC1 and AtSUC2, respectively. Strain MGY1, which harbors the PmSUC2 construct of strain MGY2 in an antisense orientation (Gahrtz et al., 1994), was used as a control strain. For construction of strains MGY1, MGY2, NSY611, and NSY612, the yeast–
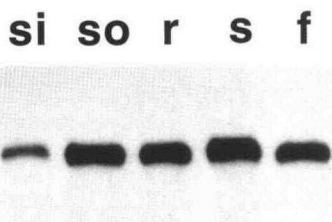
si   so   r   s   f

Figure 6. RNase Protection Analysis of the Organ-Specific Expression of the PmSUC2 Gene.

Twenty micrograms of total RNA from each organ was used in each lane. si, sink leaves; so, source leaves; r, roots; s, stems; f, flowers.
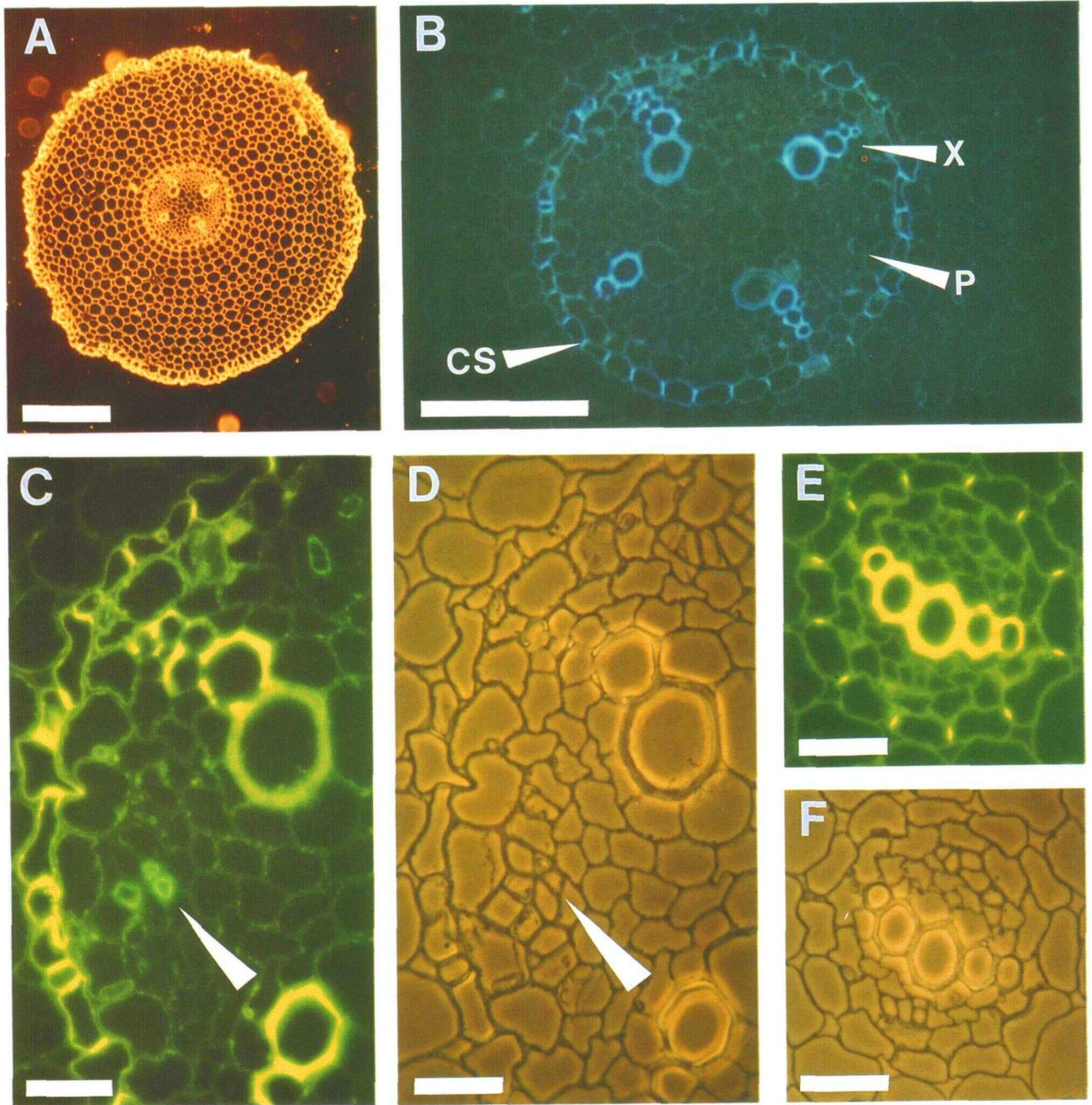
Figure 7. Immunocytochemical Identification of the PmSUC2 Protein in *P. major* Roots.

(A) Cross-section of a root.

(B) Central cylinder stained with aniline blue to demonstrate its organization. CS, Casparian stripe; P, phloem; X, xylem.

(C) Section of a central cylinder (anti-PmSUC2 antiserum/FITC conjugate treated) with FITC staining of companion cells (arrowhead) in the root phloem.

(D) Control of the central cylinder shown in **(C)**. The arrowhead indicates the cells that were antibody labeled in **(C)**.

(E) Central cylinder of a very young root (anti-PmSUC2 antiserum/FITC conjugate treated) with no detectable FITC staining.

(F) Control of the central cylinder shown in **(E)**.

Scale bar in **(A)** = 100 μm; scale bar in **(B)** = 50 μm; scale bars in **(C)** to **(F)** = 10 μm.

Escherichia coli shuttle vector NEV-E was used (Sauer and Stolz, 1994). For construction of strain MGY6, a full-length cDNA clone of the *P. major* PmSUC1 sucrose-H$^+$ symporter (EMBL accession number X84379) was cloned into the unique NotI site of the yeast–*E. coli* shuttle vector NEV-N (Sauer and Stolz, 1994).

Preparation of Yeast Plasma Membranes and of Total Membranes from *P. major* Tissue

Plasma membranes were isolated from yeast cells as described previously by Stolz et al. (1994). Total membranes from *P. major* leaves or isolated vascular bundles were isolated as described by Stolz et al. (1994).

Preparation of the Anti-PmSUC2 Antiserum, SDS-PAGE, and Protein Gel Blotting

The recombinant PmSUC2 protein was isolated from the yeast strain MGY2 (Gahrtz et al., 1994). Plasma membranes of MGY2 cells were isolated (Stolz et al., 1994), plasma membrane proteins were solubilized with SDS, and the proteins were separated by preparative SDS-PAGE (Laemmli, 1970). The recombinant PmSUC2 protein was visualized, excised, and eluted from the gel as described by Sauer and Stadler (1993). From 1 mg of SDS–solubilized yeast plasma membrane proteins, 30 to 40 µg of purified PmSUC2 protein was obtained. Three to four portions of ∼10 µg were injected into rabbits at intervals of ∼3 weeks.

Crude anti-PmSUC2 antisera were affinity purified by adsorption to the recombinant PmSUC2 protein, which had been immobilized on nitrocellulose filters (Sauer and Stadler, 1993). Residual unspecific antibodies were removed (Sauer and Stadler, 1993) by adsorption to immobilized plasma membrane proteins from yeast strain MGY1 (Gahrtz et al., 1994). The quality of the purified anti-PmSUC2 antiserum was tested on protein gel blots (Dunn, 1986). Antibody binding was detected with the chemiluminescence Western blot detection kit from Amersham, according to the manufacturer's protocol.

Preparation and Fixation of *P. major* Sections for Light Microscopy

Fragments from leaves, petioles, and roots of *P. major* plants were briefly degased in 1 mL of Mops buffer, pH 6.9 (50 mM 2-[*N*-morpholino]-propanesulfonic acid/NaOH, pH 6.9, 5 mM EGTA, 2 mM MgCl$_2$). The Mops buffer was replaced by 1 mL of fixation solution (ethanol–formaldehyde [37%]–acidic acid, 60:40:2 [v/v]; Schlüter, 1976), and the tissue was fixed at room temperature for 1 hr. After washes with 70 and 100% ethanol, the tissue was embedded into methacrylate (Baskin et al., 1992) as described previously (Stadler et al., 1995). Semithin sections (3 to 6 µm) were made with an ultramicrotome (Reichert, Vienna, Austria), and sections were put on poly-L-lysine–coated coverslips. Hand-cut sections (shown in Figures 3A and 3B) were made with a razor blade, fixed as described above, and washed with Mops buffer, pH 6.9.

Staining of Sections with Fluorescent Dyes

For removal of methacrylate from the thin sections, coverslips were incubated for 30 sec in 100% acetone, washed three times with blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% skim milk powder), and incubated in blocking buffer for 1 hr; hand-cut sections were not treated with acetone. After overnight incubation with affinity-purified anti-PmSUC2 antiserum (diluted 1:2 in blocking buffer), the coverslips were washed three times with blocking buffer containing 0.1% Triton X-100 and incubated for 1 hr with the anti-rabbit IgG–fluorescein isothiocyanate (FITC) isomer 1 conjugate (Sigma; diluted 1:300 in blocking buffer with 0.1% Triton X-100). After three final washes with blocking buffer containing 0.1% Triton X-100, the coverslips were rinsed with water and mounted in FITC-Guard (Testoc Inc., Chicago, IL). Photographs were taken with a fluorescence phase microscope (Standard 16; Carl Zeiss, Oberkochen, Germany) with an excitation light of 450 to 490 nm.

For double staining of the PmSUC2 protein with the antiserum/FITC conjugate and of nuclei with 4',6-diamidino-2-phenylindole (DAPI; Serva, Heidelberg, Germany), sections were treated as described above. After the three final washing steps, slides were incubated for 1 hr at room temperature in DAPI (0.2 µg/mL). DAPI fluorescence was detected with an excitation light of 365 nm.

For double staining of the PmSUC2 protein with the antiserum/FITC conjugate and of sieve plates with aniline blue (Water Blue; Fluka, Buchs, Switzerland), the slides were incubated for 5 min in aniline blue (0.5% in 50 mM Na/K-PO$_4$ buffer, pH 7.2). Aniline blue fluorescence was detected with an excitation light of 365 nm.

RNA Isolation and RNase Protection Analysis

P. major tissue was collected, and RNA was isolated as described previously (Sauer et al., 1990). RNase protection assays were performed with a radiolabeled 214-bp *PmSUC2* antisense RNA. RNA was separated on 10% polyacrylamide gels.

Electron Microscopy

For fixation, longitudinal hand-cut sections (10 × 3 × 0.5 mm) from petioles of *P. major* were immediately immersed in a formaldehyde-glutaraldehyde mixture (Karnovsky, 1965) in 100 mM sodium cacodylate buffer, pH 7.3. After rinsing with the same buffer, postfixation with 1% OsO$_4$, dehydration through an acetone series, and embedding with Spurr's resin, the ends of the sections (2 mm) were discarded, and middle portions were oriented for transverse sectioning and polymerized in molds at 60°C for 36 hr. Semithin sections (1 µm thick) were stained with 0.5% crystal violet for light microscopy (Figure 2A). Ultrathin sections (50 nm thick) were treated with uranyl acetate and lead citrate and observed with an electron microscope (CM 10; Philips, Eindhoven, The Netherlands).

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