Differential Expression of Sucrose Transporter and Polyol Transporter Genes during Maturation of Common Plantain Companion Cells

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The cDNAs of two sorbitol transporters, common plantain (*Plantago major*) polyol transporter (PLT) 1 and 2 (PmPLT1 and PmPLT2), were isolated from a vascular bundle-specific cDNA library from common plantain, a dicot plant transporting Suc plus sorbitol in its phloem. Here, we describe the kinetic characterization of these sorbitol transporters by functional expression in Brewer's yeast (*Saccharomyces cerevisiae*) and in *Xenopus* sp. oocytes and for the first time the localization of plant PLTs in specific cell types of the vascular tissue. In the yeast system, both proteins were shown to be uncoupler sensitive and could be characterized as low-affinity and low-specificity polyol symporters. The K_m value for the physiological substrate sorbitol is 12 mM for PmPLT1 and even higher for PmPLT2, which showed an almost linear increase in sorbitol transport rates up to 20 mM. These data were confirmed in the *Xenopus* sp. system, where PmPLT1 was analyzed in detail and characterized as a H^+ symporter. Using peptide-specific polyclonal antisera against PmPLT1 or PmPLT2 and simultaneous labeling with the monoclonal antiserum 1A2 raised against the companion cell-specific PmSUC2 Suc transporter, both PLTs were localized to companion cells of the phloem in common plantain source leaves. These analyses revealed two different types of companion cells in the common plantain phloem: younger cells expressing PmSUC2 at higher levels and older cells expressing lower levels of PmSUC2 plus both PLT genes. The putative role of these low-affinity transporters in phloem loading is discussed.

The export of photoassimilates from higher plant source leaves occurs via the sieve element/companion cell complex (SE/CCC) of the phloem. In many plant species, such as in Arabidopsis, maize (*Zea mays*), sugar beet (*Beta vulgaris*), or tobacco (*Nicotiana tabacum*), assimilated CO_2 is exported exclusively in the form of Suc. In numerous other plants, however, additional carbohydrates are used for this long distance transport. Examples are raffinose or stachyose in Cucurbitaceae (Kandler and Hopf, 1982; Keller and Pharr, 1996) or reduced monosaccharides, such as mannitol or sorbitol in Rosaceae, Plantaginaceae, or several other families (Barker, 1955; Webb and Burley, 1962; Zimmermann and Ziegler, 1975). The common properties of Suc and these additional compounds are that they are highly soluble, chemically inert, and not readily accessible for primary cellular metabolism. They can thus be stored and transported in high concentrations with no damage to the cells and without being degraded or modified.

cDNAs encoding Suc transporters involved in phloem loading have been cloned from several plants (Riesmeier et al., 1992; Riesmeier et al., 1993; Gahrtz et al., 1994; Sauer and Stolz, 1994; Bürkle et al., 1998; Aoki et al., 1999; Noiraud et al., 2000; Williams et al., 2000). In contrast, only a single cDNA of a mannitol transporter, *AgMAT1* from celery (*Apium graveolens*; Noiraud et al., 2001) and two cDNAs for sorbitol transporters, *PcSOT1* and *PcSOT2* from sour cherry (Gao et al., 2003), has been cloned. So far, no transporters for raffinose or stachyose have been identified. This does not at all reflect the relative importance of Suc versus these other substances in phloem transport, because in many plants, phloem concentrations of oligosaccharides from the raffinose family or of polyols are comparable with or even higher than the concentrations of Suc. For example, Suc, raffinose, and stachyose concentrations in pumpkin (*Cucurbita maxima*) were found to be 180, 120, and 180 mM, and in peach (*Prunus persica*), the concentrations of Suc and sorbitol were shown to be 140 and 550 mM in the phloem sap. In common plantain, Suc and sorbitol concentrations are 800 and 300 mM, respectively (Lohaus and Fischer, 2002; G. Lohaus, unpublished data).

It is not really understood, why different plants use different compounds for long distance carbon allocation. For raffinose-transporting symplastic phloem loaders, such as cucurbits, it has been postulated that the difference in the Stoke's radii between Suc and raffinose (or stachyose) represents the actual "driv-

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ing force" for long distance transport (Turgeon, 1996). The so-called "polymer trap model" is based on the assumption that Suc, the first precursor in raffinose biosynthesis, can traffic symplastically from mesophyll cells into the SE/CCC. The model is also based on the observation that galactinol, the second precursor in raffinose formation, is synthesized inside the companion cells (also called intermediary cells) of these plants (Bebee and Turgeon, 1992; Haritatos et al., 2000). The unproven conclusion is that Suc and monosaccharides can traffic into the companion cells, but raffinose cannot traffic back due to its higher Stoke's radius (trapping mechanism). According to this model, phloem transport of oligosaccharides from the raffinose family might be essential to drive phloem transport in symplastic loaders. The major drawback of this model is, however, that it has never been possible to show that plasmodesmata can discriminate between molecules, such as Suc or raffinose.

In contrast, there are several physiological reasons that might explain the long distance transport of polyols. Obviously, phloem transport of more reduced sugar alcohols may be advantageous for $NADPH^+$-dependent reactions in sinks, such as the reduction of NO_3^-, which in many plants is performed in roots (Hansch et al., 2001). Mannitol has also been shown to act as antioxidant (Shen et al., 1997) and may have important functions in plant-pathogen interactions (Jennings et al., 1998). Moreover, it is known that polyols can serve as compatible solutes and the role of mannitol as an osmoprotectant in celery is well documented (Tarczynski et al., 1993; Everard et al., 1994; Stoop and Pharr, 1994a). Interestingly, mannitol synthesis is not up-regulated under stress (Everard et al., 1994), and increased mannitol concentrations in roots of stressed plants result primarily from reduced degradation of mannitol (Stoop and Pharr, 1994a, 1994b), which is constantly supplied by the phloem. Finally, phloem polyols were also shown to influence the phloem mobility of boron (B) by the formation of soluble mannitol-B-mannitol complexes in celery or of sorbitol-B-sorbitol complexes in peach (Penn et al., 1997). These complexes were identified in the phloem sap of these plants and are assumed to be the basis for the increased B efficiency of these plants (Hu et al., 1997). Genetically modified tobacco plants with enhanced sorbitol synthesis were shown to transport B in their phloem, whereas control plants did not (Bellaloui et al., 1999; Brown et al., 1999).

Despite these important functions of mannitol or sorbitol in higher plant physiology, little is known about the proteins involved in phloem loading of polyols—only the mannitol transporter AgMAT1 is thought to be involve in phloem loading (Noiraud et al., 2001)—and nothing is known about the identity of the cells catalyzing this step. Common plantain, a sorbitol-translocating plant (Wallart, 1981; Lohaus and Fischer, 2002), is highly resistant to drought, trampling, and other environmental stresses. Moreover, vascular tissue is easily purified from common plantain leaves and has been used to clone and characterize the Suc transporters PmSUC2 (Gahrtz et al., 1994; Stadler et al., 1995a) and PmSUC3 (Barth et al., 2003). Here, we describe the identification, characterization, and cellular localization of two phloem-localized sorbitol transporters from plantain, an important step toward the understanding of potential physiological roles of sorbitol in higher plants.

RESULTS

Cloning of Two Polyol Transporter (PLT) cDNAs

The only characterized plant PLT putatively involved in phloem loading is the product of the *AgMAT1* cDNA from celery (Noiraud et al., 2001), a Suc and mannitol translocating plant. Homologous cDNAs encoding sorbitol transporters were identified in fruits of sour cherry (Gao et al., 2003), and related genes have also been found in plants that neither transport polyols inside their phloem nor store polyols, such as in Arabidopsis (Munich Information Center for Protein Sequences nos. At2g16120, At2g20780, At2g16130, At3g18830, At2g18480, and At4g36670) and sugar beet (accession nos. U64902 and U64903). None of the encoded gene products has been characterized so far, but the presence of these genes suggests that they may have functions different from phloem loading and that mannitol or similar substrates may be transported in these plants under specific physiological conditions.

AgMAT1, PcSOT1, PcSOT2, and the uncharacterized proteins from Arabidopsis and sugar beet share a high degree of similarity (about 70% similarity on the protein level). Therefore, we hoped that a low-stringency screening (Sauer et al., 1990) of a vascular tissue-specific cDNA library (Gahrtz et al., 1994) from sorbitol-translocating common plantain (Wallart, 1981; Lohaus and Fischer, 2002) with an *AgMAT1*-derived probe might identify cDNAs potentially encoding transporters involved in the phloem loading of sorbitol.

After the first screening, 10 to 15 positive signals were obtained per filter (7,000 plaque forming units), suggesting that between 0.05% and 0.1% of the clones in this library may encode PLT-like sequences. This was confirmed by sequencing the inserts of numerous randomly chosen positive λ-clones. All sequences could be assigned to two different, highly homologous cDNAs. The genes were named *P. major* PLT 1 and 2 (*PmPLT1* and *PmPLT2*). The encoded proteins (Fig. 1A) are 529 amino acids (PmPLT1) and 530 amino acids (PmPLT2) long and share 83.0% identical amino acids. Moreover, they share 67.5% (PmPLT1) and 65.8% (PmPLT2) identity with AgMAT1. The number of identical amino acids shared with the cherry sorbitol transporters are similar.

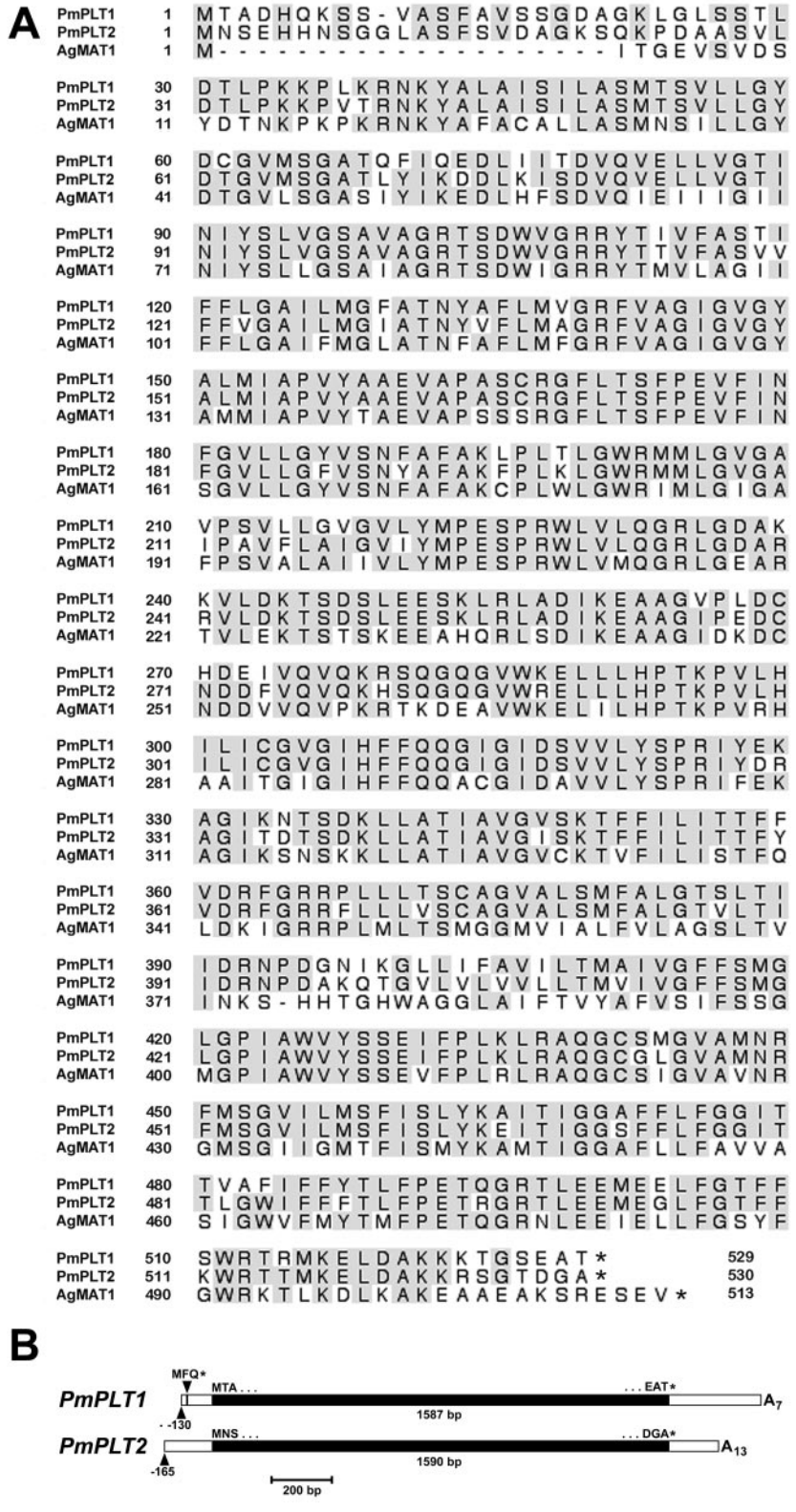

Figure 1. Comparison of PmPLT1, PmPLT2 (sorbitol transporters), and AgMAT1 (mannitol transporter) protein sequences and of the cDNA structures of *PmPLT1* and *PmPLT2*. A, Amino acid sequences of PmPLT1, PmPLT2, and AgMAT1 were aligned with the program SeqVu (James Gardner, Garvan Institute of Medical Research, Sydney), and residues identical in all three sequences were highlighted. B, The structure of the longest *PmPLT1* and *PmPLT2* cDNAs are presented including the information from the 5′-RACE reactions. Arrowheads indicate the transcriptional start sites and the start (−111 bp) of the short ORF in the 5′-untranslated region of PmPLT1. The complete cDNA sequences were deposited in the EMBL data library. Accession numbers are AJ532589 (*PmPLT1*) and AJ532590 (*PmPLT2*).

Interestingly, the longest cDNA of *PmPLT1* (1,987 bp) with 115-bp 5′-flanking sequence and 285-bp 3′-flanking sequence contained a short open reading frame (ORF) for the tripeptide Met-Phe-Gln starting 111 bp upstream from the predicted start-ATG of the cDNA. Such a 5′-ORF was absent from the 5′-flanking sequence of the longest *PmPLT2* cDNA (1,891 bp with 145-bp 5′-flanking sequence and

156-bp 3′-flanking sequence). To test, whether this short 5′-ORF in *PmPLT1* corresponds to the C terminus of an even longer ORF and whether a similar 5′-ORF is present in the complete 5′-untranslated sequence of *PmPLT2*, 5′-RACEs were performed with total RNA from common plantain vascular tissue. The *PmPLT1*-specific RACE-reactions showed that translation of the *PmPLT1* gene starts at position −130 bp and that the 5′-ORF encodes only the tripeptide Met-Phe-Gln (Fig. 1B). The *PmPLT2*-specific 5′-RACE reactions showed that translation in the *PmPLT2* gene starts at −165 and that the corresponding mRNA does not have a 5′-ORF (Fig. 1B).

Functional Expression in Yeast Depends on the 5′-Flanking Sequences

It had been claimed that acyclic polyols cannot be metabolized by Brewer's yeast (*Saccharomyces cerevisiae*; Canh et al., 1975). This was disproven later by the observation that bakers' yeast can induce expression of the sorbitol dehydrogenase gene, SDH1, when grown on sorbitol as the sole carbon source for at least 2 weeks (Sarthy et al., 1994). Such a delayed induction of sorbitol catabolism does not interfere with sorbitol transport tests performed with Glc-grown yeast cells, where endogenous genes for polyol uptake and metabolism stay repressed. Therefore, transport properties of PmPLT1 and PmPLT2 were analyzed by expressing of the longest cDNAs in the bakers' yeast strain SEY2102 (Emr et al., 1983) in sense and antisense orientation in the unique *Eco*RI-site of the NEV-E expression vector (Sauer and Stolz, 1994).

However, uptake analyses with ^{14}C-sorbitol showed no detectable transport activity for any of the sense transformants (data not shown). For PmPLT1, this might be explained by the presence of the 5′-ORF described above. Therefore, truncated clones were generated by PCR lacking this 5′-ORF. But again, no transport activities could be observed after NEV-E-based expression of these cDNAs in yeast (data not shown). In a final attempt, cDNA constructs were generated for *PmPLT1* and *PmPLT2* with modified 5′-flanking sequences. This approach had been applied successfully before (Stadler et al., 1995b) and replaces the 5′-flanking sequence of a given cDNA by the sequence AAGCTTGTAAAAGAA*ATG*. This sequence was taken from the 5′-flanking region of *AtSTP1*, the first higher plant transporter successfully expressed in yeast (Sauer et al., 1990). This sequence seems to be ideal for the bakers' yeast translation machinery and fits well to the consensus sequence (A/Y) A(A/Y) A(A/Y) A*ATG* published for bakers' yeast (Hinnebusch and Liebman, 1991). With the modified *PmPLT1* and *PmPLT2* cDNAs, a third set of sense and antisense yeast lines was generated for and transport of ^{14}C-sorbitol was analyzed.

Obviously, the native 5′-flanking sequences of both cDNAs were the reason for the observed lack in expression with the first two sets of constructs. Sense yeast strains harboring *PmPLT1* (strain MRYs1) or *PmPLT2* (strain MRYs2) cDNA constructs with modified 5′-flanking sequences expressed the cDNAs and were able to incorporate ^{14}C-sorbitol (Fig. 2). No transport activity was observed in control strains harboring the cDNAs in antisense direction (strains MRYas1 and MRYas2).

Transport Properties and Kinetic Parameters

All previously analyzed plant sugar transporters as well as the celery mannitol transporter were described as energy-dependent H^+ symporters driven by the proton motive force (*pmf*) across the plasma membrane (Boorer et al., 1994, 1996; Williams et al., 2000; Noiraud et al., 2001). It was, therefore, expected that sorbitol transport by PmPLT1 and PmPLT2 might also be energy dependent. A first clue came from the observation that ^{14}C-sorbitol transport in transgenic yeast was enhanced in the presence of Glc (Fig. 2). This has also been described for Suc transporters expressed in yeast cells (Riesmeier et al., 1992; Gahrtz et al., 1994; Sauer and Stolz, 1994), and the idea is that Glc metabolism provides energy for active transport of non-metabolizable substrates or activates the plasma membrane H^+-ATPase.

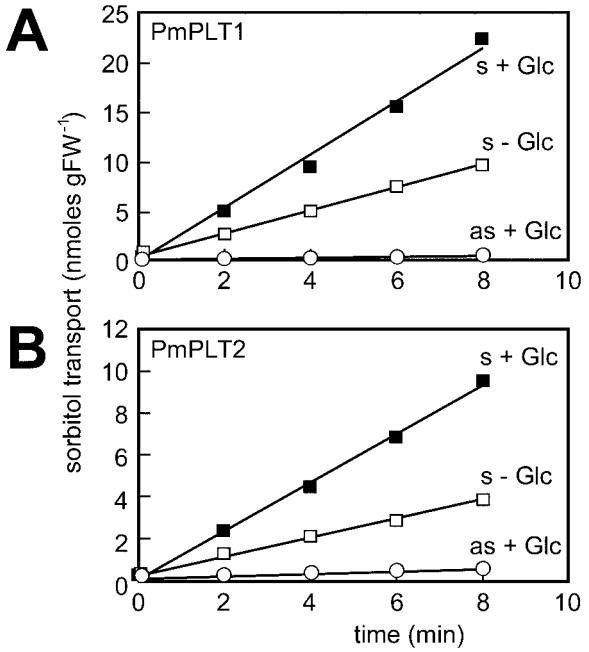

Figure 2. *PmPLT1* and *PmPLT2* can be expressed in yeast cells and catalyze the uptake of ^{14}C-sorbitol. Transport rates for ^{14}C-sorbitol were determined with the transgenic yeast cells MRYs1 and MRYas1 (expressing *PmPLT1* in sense or antisense orientation) or with MRYs2 and MRYas2 (expressing *PmPLT2* in sense or antisense orientation). The concentration of ^{14}C-labeled sorbitol was 0.1 mM in all experiments. Where indicated, D-Glc was added to a final concentration of 10 mM.

In addition, active sorbitol transport by PmPLT1 was shown more directly by comparing intracellular and extracellular sorbitol concentrations in MRYs1 cells after a 60-min incubation in 0.1 mM ^{14}C-sorbitol. During this time, the extracellular concentration of ^{14}C-sorbitol was reduced to 0.075 mM, and from the incorporated amount of label, it was calculated that the cells should have an intracellular sorbitol concentration of 1.6 mM sorbitol, if sorbitol is not metabolized. This was confirmed by thin-layer chromatography of cellular extracts showing that the entire incorporated label was still ^{14}C-sorbitol (Fig. 3). Thus, accumulation of sorbitol inside the yeast cells was more than 20-fold, which strongly supports an active transport mechanism by PmPLT1.

Finally, analyses of the sensitivities of PmPLT1 and PmPLT2 to uncouplers of proton gradients, such as carbonyl cyanide-*m*-chlorophenylhydrazone and dinitrophenol, confirmed that this active transport is driven by the proton motive force (Fig. 4).

PmPLT1 is also sensitive to the SH-group inhibitor *p*-(chloromercuri) benzene sulfonic acid (PCMBS; Fig. 4A), whereas PmPLT2 is not (Fig. 4B). So far, PCMBS was known to inhibit the activity of plant Suc transporters with high specificity (Riesmeier et al., 1992; Sauer and Stolz, 1994) but not the activity of plant monosaccharide transporters (Ludwig et al., 2000). For mannitol transport in celery, it has been reported that the transporter is not (Salmon et al., 1995) or is only slightly (Noiraud et al., 2001) inhibited by PCMBS; the sorbitol transporters from cherry

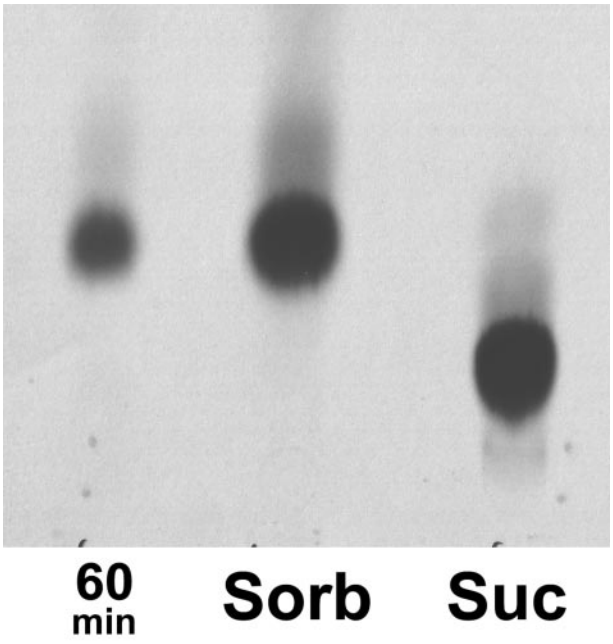

Figure 3. Thin-layer chromatography of ^{14}C-sorbitol accumulated in *PmPLT1*-expressing yeast cells. Cell extracts prepared with 80% (v/v) ethanol from yeast strain MRYs1 after a 60-min incubation in ^{14}C-labeled sorbitol (60 min). ^{14}C-Sorbitol (Sorb) and ^{14}C-Suc (Suc) were used as standards.

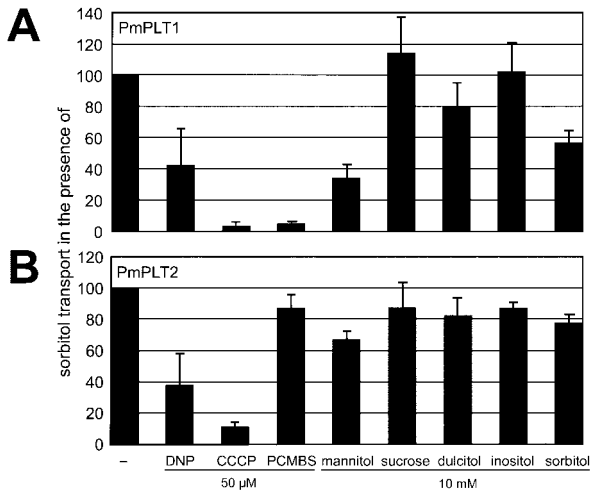

Figure 4. Transport properties of PmPLT1 and PmPLT2 in yeast cells. Transport of ^{14}C-sorbitol (0.1 mM initial outside concentration) was analyzed in the presence of uncouplers (dinitrophenol or carbonyl cyanide-*m*-chlorophenylhydrazone) or in the presence of the SH-group inhibitor PCMBS. Inhibitors were added to a final concentration of 50 µM. Transport of 0.1 mM ^{14}C-sorbitol was also analyzed in the presence of potential substrates added at a 100-fold excess (final concentrations 10 mM). Each bar results from at least three independent analyses (mean ± SD).

were not sensitive to PCMBS (Gao et al., 2003). The observed PCMBS sensitivity of PmPLT1 can be explained by comparing the Cys residues in PmPLT1 (six Cys residues) and PmPLT2 (five Cys residues). All five Cys residues found in PmPLT2 are conserved in PmPLT1. The sixth Cys is specific for PmPLT1 (Cys_{61} in Fig. 1) and is located in the predicted first extracellular loop between transmembrane helices 1 and 2. At this position, it seems to be accessible for the SH-group inhibitor. This Cys is not conserved in AgMAT1 (six Cys residues), PcSOT1 (five Cys residues), or PcSOT2 (six Cys residues) explaining the poor inhibition of these transporters by PCMBS (Noiraud et al., 2001).

Competition of ^{14}C-sorbitol uptake with other compounds, such as unlabeled sorbitol, mannitol, dulcitol, inositol, or Suc (Fig. 4) revealed little effect of any of these compounds on ^{14}C-sorbitol transport by PmPLT2, whereas a significant inhibition was observed for mannitol and unlabeled sorbitol on PmPLT1-driven ^{14}C-sorbitol transport. On one hand, the observed lack of inhibition with PmPLT2 was in agreement with the high specificity described for the sorbitol transporters in cherry (Gao et al., 2003) and with earlier results from Salmon et al. (1995). These latter authors analyzed mannitol transport in plasma membrane vesicles prepared from celery phloem tissue and showed that a 20-fold excess of sorbitol had no inhibitory effect on ^{3}H-mannitol transport. On the other hand, more recent analyses of recombinant AgMAT1 protein showed strong inhibition of mannitol transport by other sugar alcohols, such as sorbitol, xylitol, or dulcitol (Noiraud et al., 2001). Therefore,

the mannitol transport capacity of *PmPLT1*- and *PmPLT2*-expressing yeast cells was tested directly using radiolabeled ^{14}C-mannitol. Interestingly, both common plantain transporters catalyzed the transport of ^{14}C-mannitol despite a poor inhibition of ^{14}C-sorbitol transport by unlabeled mannitol in MRYs1. But again, the transport of ^{14}C-mannitol (initial concentration 0.1 mM) was hardly inhibited by a 10-fold excess of unlabeled sorbitol (data not shown).

This result can only be explained with K_m values that are significantly higher than the K_m values published for AgMAT1 (K_m = 0.3 mM) or PcSOT1 (K_m = 0.6 mM) and PcSOT2 (K_m = 0.3 mM). In this case, unlabeled polyol could be transported in addition to but not in competition with a second ^{14}C-labeled polyol.

Analysis of the K_m values confirmed this interpretation of the competition data. Figure 5 shows that the K_m value for sorbitol of PmPLT1 is 12.3 $\pm$ 0.9 mM and even higher for PmPLT2 (the K_m values for mannitol are about 5 and 30 mM for PmPLT1 and PmPLT2 in the yeast system; average of two analyses; data not shown). Thus, the K_m values for both tested substrates are 1 to 2 orders of magnitude higher than the K_m values of AgMAT1 (Noiraud et al., 2001) or the PcSOTs (Gao et al., 2003). These results characterize both common plantain PLTs as low-specificity and low-affinity sorbitol transporters.

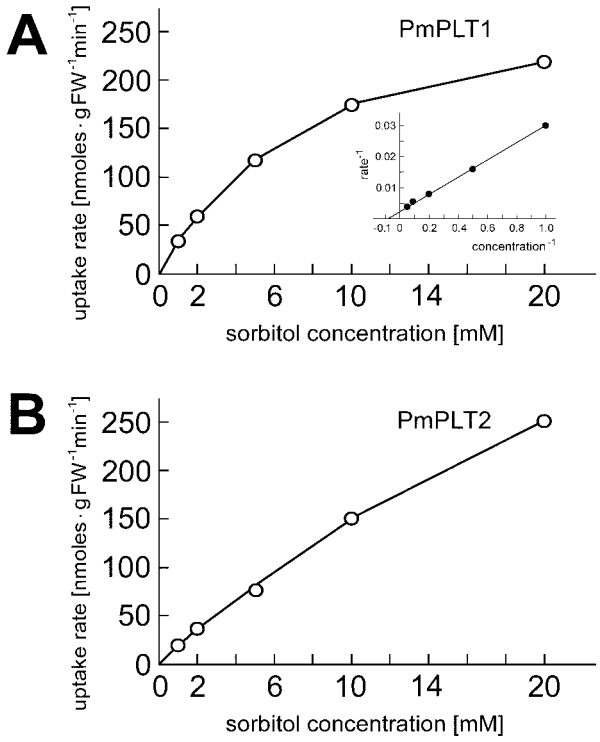

Figure 5. Analysis of substrate affinities of PmPLT1 and PmPLT2 for sorbitol in transgenic yeast cells. A, Michaelis-Menten plot for the sorbitol uptake by PmPLT1. Inset, Lineweaver-Burk plot of the same data set. B, Michaelis-Menten plot for the sorbitol uptake by PmPLT2. Uptake rates could not be saturated under the conditions analyzed. Each curve represents one out of three independent transport tests. From these analyses, the K_m value for sorbitol of PmPLT1 was calculated to be 12.3 $\pm$ 0.9 mM. No K_m value could be calculated for PmPLT2.

Functional Expression in *Xenopus* sp. Oocytes

The accumulation of sorbiol in *PmPLT*-expressing yeast cells, the sensitivity of polyol transport to uncouplers, and the increased transport rates in the presence of D-Glc provide indirect evidence for H^+ polyol transport. For a direct analysis of the potential driving force, PmPLT1 transport was also analyzed in *Xenopus* sp. oocytes expressing injected *PmPLT1* cRNA.

Inward currents were obtained in the presence of 30 mM sorbitol or mannitol, but little or no currents were observed with myoinositol (Fig. 6A) or Suc (data not shown) confirming the specificity of PmPLT1 for linear polyols. Due to the higher currents obtained with mannitol, this polyol was used for all further analyses. The mannitol-derived currents increased over a wide range of concentrations (Fig. 6B). K_m values for mannitol (18.03 $\pm$ 2.38 mM at 0 mV, 16.94 $\pm$ 2.03 mM at -60 mV, and 15.15 $\pm$ 1.97 mM at -120 mV; mean $\pm$ SD; Fig. 6C) as well as analyses at different membrane potentials (Fig. 6D) revealed the dependence of this low-affinity transporter of the membrane potential. The slightly lower K_m value (5 mM) that was determined for mannitol in the yeast system may reflect a higher membrane potential and other differences between the two expression systems.

Figure 6, D and E, show that mannitol import by PmPLT1 is also proton dependent, and that PmPLT1 has a pH optimum at about pH 6.5. Addition of 10 mM Na^+ did not alter the polyol-induced currents (data not shown).

Immunolocalization of the Sorbitol Transporters in Planta

The large number of *PmPLT1* and *PmPLT2* cDNAs identified within the vascular tissue-specific library (0.05%–0.1% of all clones) suggests that both encoded proteins are strongly expressed in the phloem and involved in phloem loading of sorbitol. However, for a detailed understanding of their physiological function it is necessary to identify the precise cell type(s) where each of these genes is expressed. Therefore, antibodies were raised against peptides corresponding to the very N termini and the very C termini of the two transporters (amino acids 1–11 and 522–529 of PmPLT1 and amino acids 1–12 and 523–530 of PmPLT2). The specificity of the obtained antisera was tested on cross sections of the yeast strains MRYs1 (expresses *PmPLT1*) and MRYs2 (expresses *PmPLT2*). Figure 7 shows that the anti-PmPLT1 antiserum binds only to sections of yeast strain MRYs1 but not

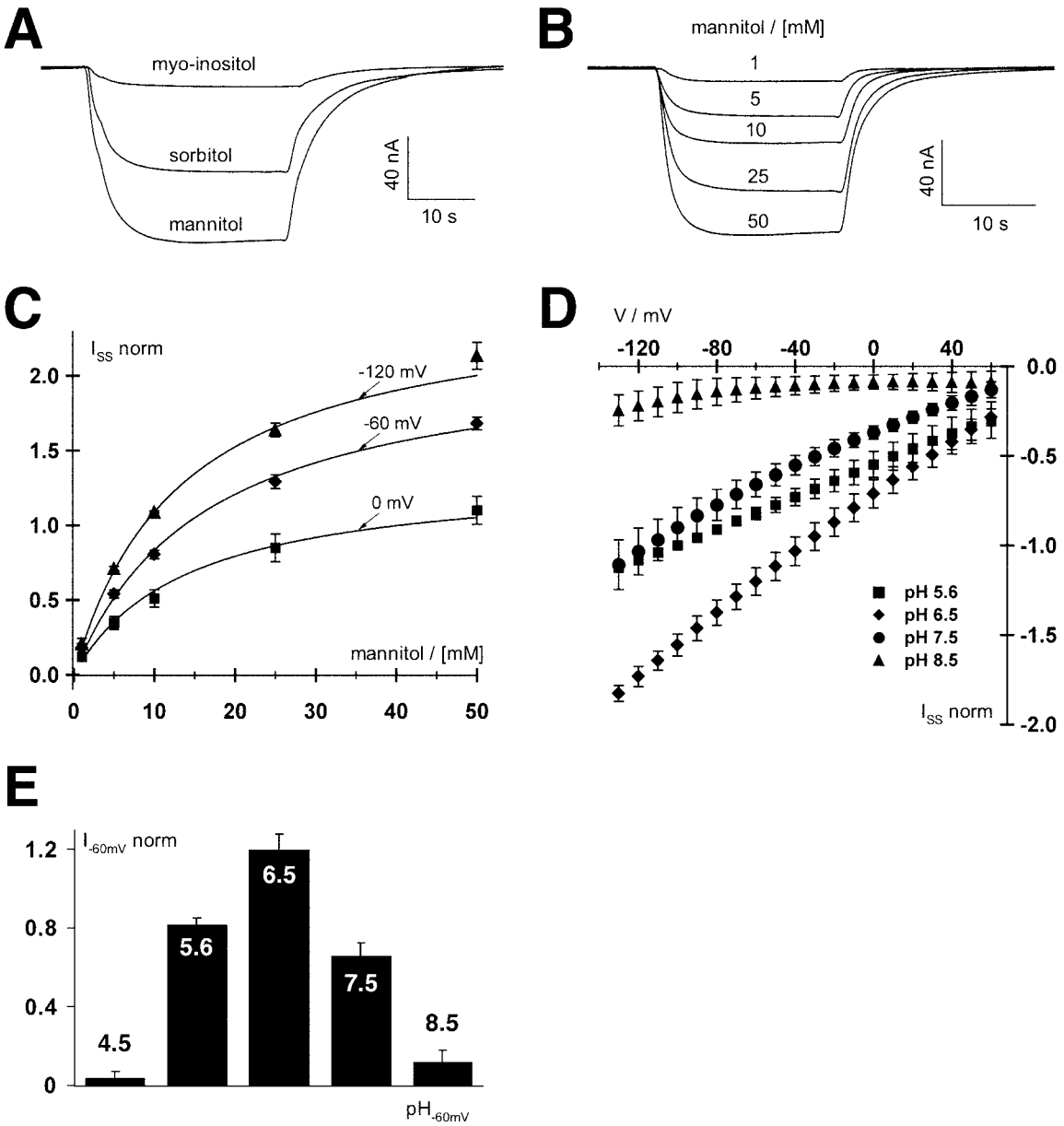

Figure 6. Biophysical analyses of PmPLT1 expressed in *Xenopus* sp. oocytes with the double-electrode voltage clamp technique. A, Polyol-induced inward H^+ currents mediated by *PmPLT1*-injected oocytes. Increase in H^+ currents in response to 30-s pulses of 30 mM mannitol, sorbitol, or myoinositol at pH 5.6 at a holding potential of −60 mV. Only mannitol and sorbitol but not myoinositol are substrates of PmPLT1. Relative currents were: $I_{mannitol} = 1$, $I_{sorbitol} = 0.66 \pm 0.11$, and $I_{myoinositol} = 0.12 \pm 0.05$ (data points were normalized to the currents at −60 mV in 30 mM mannitol; mean ± SD, $n = 4$). B, Whole-cell currents through PmPLT1 in response to a stepwise increase of mannitol concentration at pH 5.6 and a holding potential of −60 mV. C, Michaelis-Menten kinetics of PmPLT1 for mannitol at membrane potentials of 0, −60, and −120 mV. Data points represent the mean ± SD of five independent experiments normalized to the currents at −100 mV in 10 mM mannitol. Currents in the absence of mannitol were subtracted for leak correction. Continuous lines show the best nonlinear regression fits of the data points to the Michaelis-Menten equation. D and E, Mannitol-induced currents of oocytes injected with *PmPLT1* cDNA are voltage and pH dependent. D, Whole-cell steady-state currents (I_{SS}) in response to 10-mV voltage steps from 60 to −130 mV were recorded upon perfusion with 10 mM mannitol solutions at varying pH values from 4.5 to 8.5, as indicated. E, The pH optimum of PmPLT1 (determined at −120 mV) is 6.5. Data points represent mean ± SD, $n = 4$. Normalization to the currents at −100 mV in pH 5.6 and leak subtraction were performed as in C.

to sections of MRYs2 cells. Vice versa, anti-PmPLT2 antiserum binds only to sections of yeast strain MRYs2 but not to sections of MRYs1 cells. This shows the specificity of both antisera that were now used for immunolocalization of PmPLT1 and PmPLT2 in sections of common plantain source leaves.

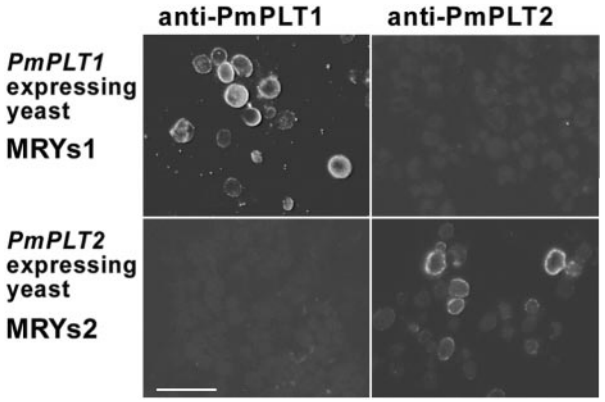

Figure 7. Specificity of anti-PmPLT1 and anti-PmPLT2 antisera. Sections of the *PmPLT1*-expressing yeast strain MRYs1 and the *PmPLT2*-expressing strain MRYs2 were fixed and embedded under the same conditions that were used for common plantain leaf material (Fig. 7). Sections were treated with anti-PmPLT1 or anti-PmPLT2 antiserum. Binding of antibody was detected under a fluorescence microscope by decoration with a fluorescent goat anti-rabbit IgG antiserum. Scale bar = 10 μm.

Figure 8A shows a cross section through a common plantain leaf treated with anti-PmPLT1 antiserum and decorated with Alexa Fluor 488-conjugated goat anti-rabbit IgG. Green fluorescence is clearly visible in individual cells of the phloem, suggesting that the labeled cells are part of the SE/CCC. Similar results were obtained with anti-PmPLT2 antiserum (data not shown). For a further characterization of the precise cell type, sections were double-stained with anti-PmPLT1 antiserum and with the monoclonal anti-PmSUC2 1A2 (Stolz et al., 1999). For detection of antibody binding, sections were decorated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (green fluorescence, localization of PmPLT1 or PmPLT2) and with Alexa Fluor 546-conjugated goat anti-mouse IgG (red fluorescence, localization of PmSUC2). PmSUC2 has previously been documented as a companion cell-specific Suc transporter by immunodetection (Stadler et al., 1995a) and can thus be used as an internal standard for the common plantain vascular system.

Figure 8, B and C, clearly shows that PmSUC2 and PmPLT1 are localized within the very same cell type of the common plantain phloem. In contrast to the vascular bundle shown in Figure 8A, this section is from a larger bundle, where phloem is already seen on both sides of the xylem. The green fluorescence in Figure 8B results from the immunodetection of PmPLT1 and is identical to the PmSUC2-specific red fluorescence in Figure 8C. The identical result was obtained in double labeling-analyses with anti-PmPLT2 and anti-PmSUC2 antisera (Fig. 8, D and E). The green fluorescence in Figure 8D results from the immunodetection of PmPLT2 and is identical to the PmSUC2-specific red fluorescence in Figure 8E. These data show that the PLT genes *PmPLT1* and *PmPLT2* are expressed in the companion cells of the common plantain phloem.

In our immunohistochemical analyses, we found that sometimes, especially in smaller and medium-sized common plantain vascular bundles, not all companion cells that were labeled with the anti-PmSUC2 antiserum were also labeled with the antisera raised against PmPLT1 or PmPLT2 (Fig. 9). Typically, companion cells that were labeled only by the monoclonal anti-PmSUC2 antibody seem to have higher levels of PmSUC2 protein than the other companion cells (i.e. stronger red fluorescence). Moreover, these "PmSUC2-only companion cells" are more frequently seen in the vicinity of the xylem, whereas the "PmSUC2-plus-PmPLT companion cells" are concentrated on the side of the phloem adjacent to the mesophyll (Fig. 9, B and D). One example for such a "PmSUC2-only" companion cell is also seen in the medium-sized vascular bundle shown in Figure 8D, where part of the strong PmSUC2-specific fluorescence is seen even with the filter for the green PmPLT2 signal.

DISCUSSION

This paper describes the molecular cloning and functional characterization of two higher plant sorbitol transporters and for the first time, to our knowledge, the immunohistochemical localization of the corresponding proteins. Moreover, the presented immunohistochemical data provide the first direct evidence for the existence of different types of companion cells in smaller veins during phloem development.

The model plant used for these analyses was common plantain, a dicot plant that allows simple isolation of pure vascular tissue (Gahrtz et al., 1994) and that is known to transport Suc and sorbitol in its phloem (Wallart, 1981; Lohaus and Fischer, 2002). The common plantain Suc transporters PmSUC1, PmSUC2, and PmSUC3 have been characterized (Gahrtz et al., 1994, 1996; Barth et al., 2003), and PmSUC2 was the first plant Suc transporter that has been localized on the cellular level (Stadler et al., 1995a). In the present paper, the analysis of the common plantain phloem is extended by the detailed characterization of two sorbitol transporters, PmPLT1 and PmPLT2. These two transporters differ in several functional properties, such as their K_m, their sensitivity to PCMBS, their substrate specificity, or their response to Glc, from the previously described PLTs PcSOT1 and PcSOT2 from cherry (Gao et al., 2003).

PmPLT1 and PmPLT2 Are Low-Affinity H^+ Symporters

Functional analyses of PmPLT1 and PmPLT2 in the yeast expression system clearly demonstrate that both transporters catalyze the transport of sorbitol, the polyol transported in the common plantain phloem (Fig. 2). Both transporters can also mediate

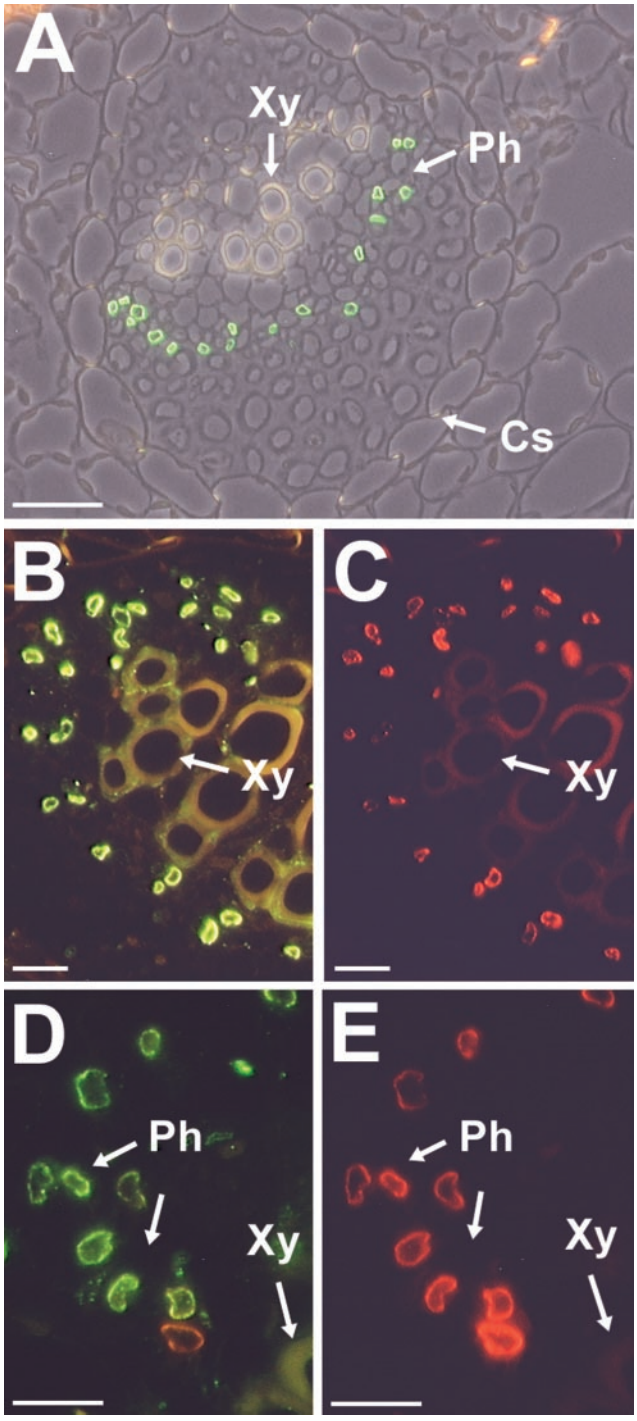

Figure 8. Immunodetection of PmPLT1 and PmPLT2 proteins in sections from common plantain source leaves. A, The section was labeled with anti-PmPLT1 antiserum and with fluorescent goat anti-rabbit IgG antiserum. The resulting green fluorescence is found only in cells of the common plantain phloem. For this figure, a fluorescence image was superposed on a photo taken under white light. Similar results were obtained with sections treated with anti-PmPLT2 antiserum and with fluorescent goat anti-rabbit IgG antiserum (data not shown). B and C, The presented section was double labeled with anti-PmPLT1 antiserum (B) and with the monoclonal anti-PmSUC2 antiserum 1A2 (C). Binding of antibodies was visualized under a fluorescent microscope after simultaneous incubation with fluorescent goat anti-rabbit IgG antiserum (B; green fluorescence, PmPLT1 localization) and fluorescent goat anti-mouse IgG antiserum (C; red fluorescence, PmSUC2 localization in companion cells). D and E, The presented section was double labeled with anti-PmPLT2 antiserum in D and with the monoclonal anti-PmSUC2 antiserum 1A2 in E. Binding of antibodies was visualized under a fluorescent microscope after simultaneous incubation with fluorescent goat anti-rabbit IgG antiserum (D; green fluorescence, PmPLT2 localization) and fluorescent goat anti-mouse IgG antiserum (E; red fluorescence, PmSUC2 localization in companion cells). The weak orange signal in one of the companion cells in D results from the extremely strong PmSUC2 signal of this cell, which is seen in E. Xy, Xylem; Ph, phloem; Cs, Casparian stripes. Scale bars = 25 μm in A, 10 μm in B and C, and 10 μm in D and E.

the uptake of mannitol (data not shown; Fig. 3) and, therefore, it seems sensible to name these genes PLT1 and PLT2, although their physiological function is the transport of sorbitol. A low specificity for polyols was also described for the AgMAT1 mannitol transporter from celery (Noiraud et al., 2001), whereas a high specificity was found for the sour cherry sorbitol transporters (Gao et al., 2003).

In contrast to all previously described PLTs with K_m values below 1 mM, PmPLT1 has a K_m value for its physiological substrate sorbitol of 12 mM (Fig. 5), and the K_m value for PmPLT2 seems to be even higher, because no saturation was observed over the concentration range analyzed (Fig. 5). Similar results were obtained for mannitol (yeast data not shown; Fig. 6). These substrate affinities are in the same order of magnitude as the K_m values described for sorbitol transport in apple (*Malus domestica*) tissue (35–55 mM; Berüter, 1997) and in the same range like the K_m value for phloem loading with sorbitol calculated from modeled carbon fluxes in a mature peach leaf (Moing et al., 1994). This not only shows that the common plantain sorbitol transporters have a more than 40- to 100-fold lower affinity than the proteins encoded by the previously cloned PLT cDNAs, but it also suggests different mechanism for the regulation of phloem loading with mannitol in celery and with sorbitol in common plantain. In celery, mannitol synthesis stays constant under stress, and the increase in root mannitol concentrations of stressed celery plants results from reduced mannitol catabolism (Everard et al., 1994; Stoop and Pharr, 1994a, 1994b). If AgMAT1 is responsible for phloem loading, maximal transport rates are reached already at 1 mM mannitol in the apoplast (Noiraud et al., 2001), which is ideal for a system where polyol synthesis is constant under stressed and unstressed conditions. In common plantain, however, a set of two transporters could respond with increased phloem loading rates to increasing apoplastic sorbitol concentrations between 0 and 100 mM. Therefore, it will be interesting to investigate whether in common plantain leaves the synthesis of sorbitol and its supply to the apoplastic space is increased under stress conditions.

Both common plantain transporters are highly sensitive to uncouplers of proton gradients (Fig. 4), both

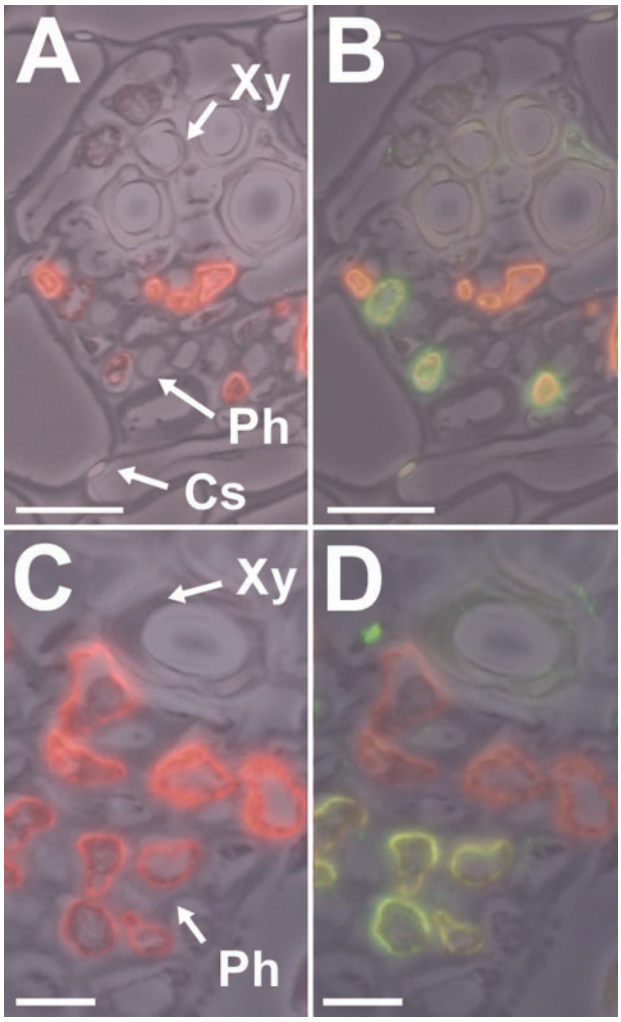

Figure 9. Immunodetection of PmPLT1 and PmPLT2 proteins in medium-sized veins of common plantain source leaves. A, Immunolocalization of PmSUC2 Suc transporter protein (red fluorescence) by immunodetection with the anti-PmSUC2 monoclonal antibody 1A2 in a small vein from a common plantain leaf. B, Additional labeling of the section shown in A with anti-PmPLT1 antiserum (green fluorescence). C, Immunolocalization of PmSUC2 Suc transporter protein (red fluorescence) by immunodetection with the anti-PmSUC2 monoclonal antibody 1A2 in a small vein from a common plantain leaf. D, Additional labeling of the section shown in C with anti-PmPLT2 antiserum (green fluorescence). Xy, Xylem; Ph, phloem; Cs, Casparian stripes. For the presented figures one (A and C) or two (B and D) fluorescence images were superposed on a photo taken under white light. Scale bars = 10 μm in A and B, and 5 μm in C and D.

show increased transport rates, when Glc is added to activate the yeast plasma membrane ATPase in the recombinant yeast cells MRYs1 and MRYs2 (Fig. 2), and PmPLT1 was shown to accumulate sorbitol more than 20-fold inside the yeast strain MRYs1 (Fig. 3). A similar accumulation of sorbitol inside MRYs2 cells could be shown for PmPLT2 (data not shown). Taken together, these data suggest that both common plantain sorbitol transporters mediate an energy-dependent H^+ sorbitol symport.

Direct proof for a H^+ symport mechanism was obtained by expressing *PmPLT1* in *Xenopus* sp. oocytes (Fig. 6). The observed inward currents were only obtained with mannitol and sorbitol, increased at higher substrate concentrations and with increasing membrane potentials, and did not respond to variations in the Na^+ concentration. This demonstrates that the uptake of polyols depends on the electrical potential ($\Delta\psi$) and on the protongradient (ΔpH) across the plasma membrane.

Noiraud et al. (2001) and Gao et al. (2003) found a strong inhibition of polyol transport by D-Glc and D-Fru. This unexpected observation could not really be explained, and the authors speculated that the inhibition of AgMAT1 or of PcSOTs by D-Glc does not result from Glc uptake via the mannitol transporter and may thus rather be an artifact of the yeast system (Noiraud et al., 2001; Gao et al., 2003). The effect of D-Glc is totally different for the common plantain sorbitol transporters PmPLT1 and PmPLT2. Sorbitol transport (Fig. 2) and mannitol transport (data not shown) are significantly enhanced in the presence of D-Glc. This is identical to previous analyses of plant Suc transporters in the yeast system (e.g. Gahrtz et al., 1994; Barth et al., 2003).

PmPLT1 has a consensus sequences for *N*-glycosylation at Asn_{334} (Fig. 1). This sequence is located in the predicted extracellular loop between the transmembrane helices 7 and 8 and is not conserved in PmPLT2 or in any of the other cloned PLTs. During secretion, it might be exposed to the lumen of the endoplasmic reticulum and be glycosylated. However, western-blot analyses with plasma membrane extracts from the recombinant yeast strains MRYs1 and MRYs2 showed no difference between the apparent M_r of PmPLT1 and PmPLT2, suggesting that this site in PmPLT1 is not used for *N*-glycosylation (data not shown).

PmPLT1 Is Sensitive to PCMBS

An interesting observation is the inhibition of PmPLT1-driven but not of PmPLT2-driven sorbitol transport by PCMBS (Fig. 4). A comparison of the deduced protein sequences shows that there is one extra Cys residue in PmPLT1 that is exposed to the predicted extracellular side of the protein. The observed inhibition can only be explained by PCMBS binding to the unique Cys_{61} in the PmPLT1 protein. This finding is not only a confirmation for the predicted topology of these proteins but may also be used for future structure/function analyses.

Most importantly, however, this difference in PCMBS sensitivity shows that inhibition of phloem loading of sorbitol (and possibly also of other substrates) by PCMBS does not allow a prediction on the mechanism of loading. The PCMBS sensitivity or insensitivity of substrate import (including sorbitol) into leaf discs of several plant species has previously been used to predict an apoplastic or symplastic

loading mechanism for the different plant species (Flora and Madore, 1996). However, our data clearly show that the PCMBS sensitivity of sorbitol transport can vary between different transporters of the very same species and the very same cell type.

PmPLT1 and PmPLT2 Are Phloem-Specific Transporters

Although both sorbitol transporters from common plantain are highly similar (83% sequence identity on the amino acid level), it was possible to raise antisera against N- and C-terminal peptides that specifically recognize only one of the two proteins (Fig. 7). These antisera were used for immunohistochemical analyses of common plantain leaf sections. Both transporters reacted exclusively with cells located in the phloem of the vascular tissue (Fig. 8A). Double labeling of leaf sections with anti-PmPLT1 antiserum and with the monoclonal anti-PmSUC2 antibody 1A2 (Stolz et al., 1999) revealed that these cells are the companion cells of the common plantain phloem (Fig. 8, B and C). Moreover, double labeling of leaf sections with anti-PmPLT2 antiserum and with the monoclonal anti-PmSUC2 antiserum 1A2 (Stolz et al., 1999) revealed an identical localization (Fig. 8, D and E). These analyses were possible because the PmSUC2 Suc transporter had previously been located in the companion cells of the common plantain vascular tissue (Stadler et al., 1995a).

The localization of both common plantain PLTs in the companion cells demonstrates that phloem loading of both compounds transported in common plantain, sorbitol and Suc, occurs primarily in the phloem companion cells of this plant (this paper; Stadler et al., 1995a). This is supported by the observation that expression of the PLT genes is source specific. In vascular bundles of sink leaves, neither PmSUC2 nor PmPLT proteins could be identified (data not shown). Also in Arabidopsis, phloem loading is catalyzed by a companion cell-specific transporter (AtSUC2; Truernit and Sauer, 1995; Stadler and Sauer, 1996). This is what one would expect according to the anatomical facts in minor veins, where the relatively large companion cells surround the small sieve elements in the center and mediate the contact to the leaf mesophyll. Moreover, the importance of companion cell-specific phloem loading is supported by the observation that a knock out-mutant in the Arabidopsis *AtSUC2* gene (Gottwald et al., 2000) shows a severe phenotype and can hardly survive under normal growth conditions. Nevertheless, solanaceous plants, which represent the only group of plants that has been analyzed besides common plantain and Arabidopsis, seem to behave differently. All phloem Suc transporters that have been studied in different members of this family (potato, tomato, and tobacco) so far were found in the phloem sieve elements (Kühn et al., 1997; Barker et al., 2000; Weise et al., 2000). It will, therefore, be important to study the cellular localization e.g. of the celery mannitol and Suc transporters (Noiraud et al., 2000, 2001) for a better understanding of the physiological basis for these differences in the cell-specific expression of phloem loaders in different plant species.

Only recently, Barth et al. (2003) showed that common plantain has also a transport protein in its sieve elements. The Suc transporter PmSUC3 could be localized in common plantain sieve elements. Interestingly, this sieve element-specific transporter has a lower affinity to its substrate Suc than its companion cell-specific partner PmSUC2, and expression of *PmSUC3* is also seen in the sink phloem (Barth et al., 2003). It was discussed that this might be a mechanism to regulate release and retrieval of Suc along the transport phloem and in apoplastically unloading sinks.

Smaller Veins Possess Different Types of Companion Cells

Most interestingly, not all companion cells seem to have the identical physiological function. As shown in Figure 9, certain companion cells seem to be specialized on the phloem loading of Suc, whereas others, with lower levels of PmSUC2 protein, seem to catalyze the simultaneous loading of Suc plus sorbitol. These different types of companion cells are only seen in smaller vascular bundles. Larger veins, as shown in Figure 8, or mature veins with bicollateral phloem (not shown) possess only or almost exclusively (Fig. 8, B–D) companion cells with both types of transporters. These data are likely to reflect different steps during phloem development. Obviously, the youngest SE/CCCs (next to the xylem) start with the expression of *PmSUC2*, and expression of both *PmPLT* genes is initiated only at later developmental stages. Moreover, this might be a mechanism to modulate the supply of Suc and/or sorbitol to different sink organs.

Differences between individual companion cells of the same vascular bundle have previously been described by Haritatos et al. (2000). In this paper, tobacco plants were analyzed expressing the *GUS* reporter gene under the control of the galactinol synthase promoter from melon (*Cucumis melo*). GUS histochemical staining was observed only in some of the companion cells and was absent from others, suggesting differences in the transcriptional activity for this transgene. Our data support this interpretation and represent the first direct proof for differences in the physiological functions of individual companion cells.

Similar Transporters Are Found in Other Plant Species That Do Not Translocate Polyols in Their Phloem

Database searches revealed a group of six sequences highly homologous to PmPLT1 and PmPLT2 also in the Arabidopsis genome (Munich Information

Center for Protein Sequences nos. AtPLT1, At2g16120; AtPLT2, At2g16130; AtPLT3, At2g18480; AtPLT4, At2g20780; AtPLT5, At3g18830; and AtPLT6, At4g36670). Due to sequence identities of 50% to 66% (on the amino acid level) between these transporters and the so-far characterized PLTs (Noiraud et al., 2001; this paper), these genes are likely to encode PLTs. It will be interesting to study the physiological roles and the physiological substrates of these proteins in Arabidopsis, a plant that does not transport polyols in the phloem. Similarly, two homologous transporter genes (GenBank accession nos. U64902 and U64903) were found in sugar beet. The function of these transporters might be necessary during local polyol synthesis in specific cell types or under certain environmental conditions. The identification of the physiological roles of these genes but also the detailed physiological roles of phloem localized sorbitol transporters, such as PmPLT1 and PmPLT2, will have to be analyzed further in the future. This will include analyses in plants that do normally not translocate polyols, such as Arabidopsis, but also mutants of polyol transporting plant species.

MATERIALS AND METHODS

Strains

Common plantain (*Plantago major*) wild-type plants were grown in potting soil in the greenhouse under ambient conditions. For cloning in *Escherichia coli*, we used strain DH5a (Hanahan, 1983). Yeast (*Saccharomyces cerevisiae*) expression was performed with strain SEY2102 (Emr et al., 1983). For the preparation of genomic DNA, leaves of celery (*Apium graveolens*) were purchased on a local market.

Cloning of PmPLT1 and PmPLT2 cDNAs

A common plantain cDNA library in λ-gt10 that had previously been used for the cloning of the Suc transporter cDNAs *PmSUC1* and *PmSUC2* (Gahrtz et al., 1996) was screened with a radiolabeled probe derived from the *AgMAT1* mannitol transporter gene of celery. To this end, genomic DNA was isolated from celery leaves, and a 1,700-bp *AgMAT1* genomic fragment was isolated by PCR (AgMAT1–5′ primer, 5′-AAG TAT GCT TTT GCT TGT GCT C-3′; AgMAT1–3′primer, 5′-AGC CTG TTG GAA GAA ATG AAT AC-3′). The radiolabeled probe was used to screen 120,000 pfu of the λ-gt10 library at a density of 7,000 pfu plate^{-1}. Phage-DNA was transferred to nitrocellulose filters, prehybridized, hybridized, and washed as described (Sauer et al., 1990). From more than 200 positive signals obtained after exposure of the filters to x-ray films (Kodak X-Omat AR, Eastman Kodak, Rochester, NY), 25 plaques were isolated and rescreened, and their *Eco*RI inserts were cloned into pGEM-T-easy (Promega, Mannheim, Germany) and sequenced. The obtained sequences turned out to result from two different common plantain mRNAs. For further analyses, the longest cDNAs for each mRNA were cloned into the *Eco*RI site of pUC19, yielding the plasmids pMR7 (*PmPLT1*) and pMR8 (*PmPLT2*). The corresponding genes were named *PmPLT1* (insert from λ-phage 5B) and *PmPLT2* (insert from λ-phage 9B).

Analysis of 5′-Flanking Sequences by 5′-RACE

The complete 5′-flanking regions of the *PmPLT1* and *PmPLT2* cDNAs were determined using the 5′/3′-RACE kit of Roche Diagnostics (Mannheim, Germany). The nested primers PmSBT1-SP1 (5′-GAG CTT TCC GGC ATC ACC GGA G-3′), PmSBT1-SP2 (5′-CTG GTG ATC AGC AGT CAT AGT TG-3′), PmPSBT1-SP3 (5′-GGT TTA ATT GAC TAG CTA GC-3′), PmSBT2-SP1 (5′-CGT CTG GCT TTT GAG ACT TAC-3′), PmSBT2-SP2 (5′-GCC ACC GGA GTT ATG GTG TTC AC-3′), and PmSBT2-SP3 (5′-GTG TGA GCC TAC TTG TGT GTT TGG C-3′) were used to generate full-length 5′ sequences from both cDNA clones after in vitro polyadenylation of their very 5′ ends. All treatments were performed according to the manufacturer's protocol.

Functional Expression of the cDNAs in Bakers' Yeast

For the functional expression in bakers' yeast, the *Eco*RI inserts from pMR7 and pMR8 were cloned into the unique *Eco*RI site of the yeast/*E. coli* shuttle vector NEV-E (Sauer and Stolz, 1994) in sense and antisense orientation. The four resulting plasmids were used to transform into yeast. In a second approach, the primers SBT1-5 (5′-AGT CTG CTT GAA TTC AAC TAT GAC TGC TGA TCA CCA GA-3′), SBT1-3 (5′-TCA GCA CAT AAG AAT TCT TAG GTA GCT TCA GAA CCA GT-3′), SBT2-5 (5′-ACA CTT GTT GAA TTC CCT AAC CAT CAT GAA TAG TGA AC-3′), and SBT2-3 (5′-CTC ACT ACT GAA TTC TTA GGC ACC ATC AGT ACC ACT CC-3′) were used to generate cDNAs lacking their 5′- and 3′-flanking sequences. Again, the four plasmids resulting from cloning of these cDNAs into NEV-E were used to transform yeast. In a third approach, cDNA clones were generated with a modified 5′-flanking sequence using the primers SBT1–5Eco (5′-CTC CGG AAT TCA AGC TTG TAA AAG AAA TGA CTG CTG ATC ACC AGA AGT CAA G-3′), SBT1–3, SBT2–5Eco (5′-CTC CGG AAT TCA AGC TTG TAA AAG AAA TGA ATA GTG AAC ACC ATA ACT CC-3′), and SBT2–3. Ligation into NEV-E gave the plasmids NEV::PLT1s (*PmPLT1* in sense orientation), NEV::PLT1as (*PmPLT1* in antisense orientation), NEV::PLT2s (*PmPLT2* in sense orientation), and NEV::PLT2as (*PmPLT2* in antisense orientation). These plasmids were used to transform the yeast strain SEY2102. The resulting strains were named MRYs1 (expressing *PmPLT1* in sense orientation), MRYas1 (expressing *PmPLT1* in antisense orientation), MRYs2 (expressing *PmPLT2* in sense orientation), and MRYas2 (expressing *PmPLT2* in antisense orientation).

Transport Measurements in Transgenic Yeast Cells

Uptake of radiolabeled substrates and analyses of inhibitor sensitivities and K_m values were performed in 50 mM Na-PO_4 buffer, pH 5.5, as described (Sauer et al., 1990).

Thin-Layer Chromatography

Yeast cells (A_{600} was 20) were incubated in 50 mM Na-PO_4 buffer, pH 5.5, in the presence of 0.1 mM ^{14}C-sorbitol for 60 min. At this point, 1 mL of yeast cells was harvested, washed extracted with 80% (v/v) ethanol, and subjected to thin-layer chromatography as described (Gahrtz et al., 1994). Radioactivity was determined by exposure to x-ray films (Kodak X-Omat AR, Eastman Kodak).

Immunohistochemical Techniques

The anti-PmPLT1 and anti-PmPLT2 antisera used in this paper were raised against mixtures of two protein-specific oligopeptides (PmPLT1, MTADHQKSSVA and KKTGSEAT; PmPLT2, MNSEHHNSGGLA and KRSGTDGA) that were used to immunize two rabbits and one guinea pig after coupling to a protein carrier (Pineda, Antikörper-Service, Berlin).

Common plantain tissue and yeast cells were prepared, fixed in methacrylate, sectioned, and transferred to adhesion microscope slides (Linaris, Wertheim-Bettingen, Germany) as previously described (Stadler and Sauer, 1996). Methacrylate was removed by incubation of the slides for 3 min in acetone. Sections were rehydrated by sequential incubation in ethanol of decreasing concentrations (100%, 95%, 80%, 60%, and 30% [v/v]) and blocked for 1 h (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% [w/v] skim milk powder). After overnight incubation with affinity-purified anti-PmSUC3 antiserum (diluted 1:10 in blocking buffer) and/or monoclonal anti-PmSUC2 antiserum (Stolz et al., 1999; diluted 1:2), sections were washed five times with blocking buffer. For detection of bound anti-PmPLT1 or anti-PmPLT2 antisera, sections were incubated for 1 h with a 1:300 dilution of Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Leiden, Netherlands). For double stainings of sections with polyclonal anti-PmPLT1 or anti-PmPLT2 antisera and with monoclonal anti-PmSUC2 antiserum, Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes) was used in addition (diluted 1:100). After five final washes with blocking

buffer, the slides were rinsed with water and mounted in ProLong Antifade kit (Molecular Probes). Photographs were taken on a fluorescence microscope (Zeiss, Göttingen, Germany) with appropriate excitation light.

For antibody-peptide competition experiments, a conjugate of the specific peptide with ovalbumin was used. Before immunolocalization, the affinity-purified antiserum was incubated for 2 to 3 h at room temperature with 200 μg mL^{-1} conjugate or pure ovalbumin, respectively.

Heterologous Expression in *Xenopus* sp. Oocytes

For functional analysis, *PmPLT1* cRNA was prepared using the mMESSAGE mMACHINE RNA Transcription Kit (Ambion, Austin, TX). Oocyte preparation and cRNA injection have been described elsewhere (Becker et al., 1996). In two-electrode voltage-clamp studies, oocytes were perfused with 30 mM K^+ gluconate-containing solutions, based on Tris/MES buffers for pH values from 5.6 to 8.5 or citrate/Tris buffers for pH 4.5. The standard solution contained 10 mM MES/Tris, pH 5.6, 30 mM K^+ gluconate, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. In addition, 20 mM $BaCl_2$ and 30 mM TEA-Cl were used to reduce cationic background conductances. Osmolarity was adjusted to 220 mOsmol using Suc. The content of polyols and the pH values are indicated in the figure legends. Steady-state currents (I_{SS}) were recorded with single-pulse protocols to 500-ms test voltages from 60 to −130 mV from a holding potential (V_H) of 0 mV.

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