Arabidopsis thaliana POLYOL/MONOSACCHARIDE TRANSPORTERS 1 and 2: fructose and xylitol/H⁺ symporters in pollen and young xylem cells

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Abstract

The genome of Arabidopsis thaliana contains six genes, AtPMT1 to AtPMT6 (Arabidopsis thaliana POLYOL/MONOSACCHARIDE TRANSPORTER 1–6), which form a distinct subfamily within the large family of more than 50 monosaccharide transporter-like (MST-like) genes. So far, only AtPMT5 [formerly named AtPLT5 (At3g18830)] has been characterized and was shown to be a plasma membrane-localized H⁺-symporter with broad substrate specificity. The characterization of AtPMT1 (At2g16120) and AtPMT2 (At2g16130), two other, almost identical, members of this transporter subfamily, are presented here. Expression of the AtPMT1 and AtPMT2 cDNAs in baker’s yeast (Saccharomyces cerevisiae) revealed that these proteins catalyse the energy-dependent, high-capacity transport of fructose and xylitol, and the transport of several other compounds with lower rates. Expression of their cRNAs in Xenopus laevis oocytes showed that both proteins are voltage-dependent and catalyse the symport of their substrates with protons. Fusions of AtPMT1 or AtPMT2 with the green fluorescent protein (GFP) localized to Arabidopsis plasma membranes. Analyses of reporter genes performed with AtPMT1 or AtPMT2 promoter sequences showed expression in mature (AtPMT2) or germinating (AtPMT1) pollen grains, as well as in growing pollen tubes, hydathodes, and young xylem cells (both genes). The expression was confirmed with an anti-AtPMT1/AtPMT2 antiserum (αAtPMT1/2) raised against peptides conserved in AtPMT1 and AtPMT2. The physiological roles of the proteins are discussed and related to plant cell wall modifications.

Key words: AtPLT, AtPMT, fructose, monosaccharide transport, plasma membrane, polyol transport, xylem, xylitol.

Introduction

In 2001, the first higher plant polyol transporter was characterized from Apium graveolens (celery; AgMAT1; Noiraud et al., 2001) and it was suggested that this transporter is involved in the loading of mannitol into the phloem of this polyol translocating plant (family Apiaceae). In the following years, additional polyol transporters were cloned and characterized from species of other plant families known to represent sorbitol or mannitol-translocating plants, such as Prunus cerasus (sour cherry, PsSOT1 and PsSOT2; Rosaceae; Gao et al., 2003), Plantago major (common plantain; PmPLT1 and PmPLT2; Plantaginaceae; Ramsperger-Gleixner et al., 2004), Malus domestica (apple; MdSOT3–MdSOT5; Rosaceae; Watari et al., 2004), Olea europaea (olive, OeMAT1; Oleaceae; Conde et al., 2007), and again from celery (AgMAT2; Juchaux-Cachau et al., 2007). All of these transporters were shown or discussed to be responsible for the loading of the acyclic polyols sorbitol or mannitol into the phloem (Noiraud et al., 2001; Ramsperger-Gleixner et al., 2004) or for the transfer of these compounds from the phloem to
different sinks (Gao et al., 2003; Juchaux-Cachau et al., 2007). Immunohistochemical analyses performed by Ramsperger-Gleixner et al. (2004) localized PmPLT1 and PmPLT2 to the companion cells of the Plantago phloem, the cell type mediating phloem loading with the disaccharide sucrose in most species (Stadler et al., 1995; Stadler and Sauer, 1996; Schmitt et al., 2008). Moreover, these authors demonstrated that PmPLT1 and PmPLT2 expression was confined to mature companion cells, whereas the gene of the sucrose transporter, PmsUC2, was already expressed in young companion cells. This observation fitted in with earlier data showing that the onset of sucrose export or mannitol export from celery source leaves is differentially regulated (Davis et al., 1988). A quite different cell-specificity was published by Juchaux-Cachau et al. (2007) for the celery AgMAT2 protein, which was identified in all phloem cell types of celery leaf petioles, i.e. in companion cells, sieve elements, and phloem parenchyma cells. This localization was interpreted as being related to the complex sugar fluxes in an organ involved in both mannitol accumulation and in mannitol transfer from source to sink.

When Reinders et al. (2005) and Klepek et al. (2005) published the characterization of the first polyol transporter from Arabidopsis, a different physiological role was expected for this protein, as Arabidopsis translocates sucrose and minor amounts of raffinose but no polyols (Haritatos et al., 2000). Nevertheless, highly variable and often strong expression of AtPLT5 was observed in the leaf vasculature (Klepek et al., 2005), but due to the lack of phenotypic differences between AtPLT5 wild-type plants (WT) and an Atplt5 T-DNA insertion mutant (Klepek et al., 2005) the physiological role of this transporter is still elusive and will need further investigation. After these first publications on Arabidopsis polyol transporters in 2005, it was found that, in 2004, the abbreviation PLT had already been used for the PLETHORAI (PLT1) and PLETHORAI2 (PLT2) genes (Aida et al., 2004). To avoid confusion, the Arabidopsis POLYOL TRANSPORTER (PLT) gene family has been renamed as POLYOL/MONOSACCHARIDE TRANSPORTER (PMT). The abbreviations AtPMT1 to AtPMT6 will, therefore, be used in the rest of this publication.

Functional analyses characterized AtPMT5 as a low-specificity H⁺-symporter that mediates the energy-dependent uptake of hexoses, pentoses, linear polyols of various chain lengths (3–6 carbons), and of inositol across the plasma membrane (Klepek et al., 2005; Reinders et al., 2005). In fact, detailed analyses of all functional data on plant polyol transporters revealed that the related proteins of the above-mentioned sorbitol or mannitol translocating species have similarly low substrate specificities (Noiraud et al., 2001; Gao et al., 2003; Watari et al., 2004; Klepek et al., 2005; Juchaux-Cachau et al., 2007). Nevertheless, based on their predicted roles in the long-distance transport of these compounds these proteins could be characterized as sorbitol or mannitol transporters.

To identify the physiologically relevant substrate(s) of AtPMT5, Reinders et al. (2005) used the Xenopus laevis expression system to compare the proton currents associated with a large number of potential substrates. By contrast, Klepek et al. (2005) performed competition analyses in Saccharomyces cerevisiae (baker’s yeast). In both analyses AtPMT5 was found to transport sorbitol and glucose with similar rates and comparable Kᵣₐ₉-values (Klepek et al., 2005; Reinders et al., 2005). Nevertheless, it seems quite unlikely that sorbitol or glucose represent physiological substrates of AtPMT5 for the following reasons: (i) noteworthy concentrations of sorbitol have never been reported in Arabidopsis and (ii) glucose is likely to be transported by one of the 14 members of the much more specific, plasma membrane-localized hexose transporters of the AtSTP subfamily. The latter are found in most Arabidopsis cells and tissues and have 50–100-fold lower Kᵣₐ₉-values for glucose than AtPMT5 (Büttner and Sauer, 2000; Büttner, 2007).

The situation is similar for inositol, another molecule transported by AtPMT5 in both heterologous expression systems (Klepek et al., 2005; Reinders et al., 2005). In planta, highly specific inositol transporters have been identified (AtINT2 and AtINT4; Schneider et al., 2006, 2007) that might catalyse the uptake of inositol more efficiently than AtPMT5. Taken together, these results demonstrate that, despite a very intense characterization of the AtPMT5 transporter, the physiology of PMT-type proteins is much more complex in non-polyol-translocating species such as Arabidopsis than in sorbitol- and mannitol-translocating plants.

The characterization of two new members of the Arabidopsis AtPMT family is reported here. AtPMT1 and AtPMT2 share 93.6% identity on the amino acid level, are encoded by neighbouring genes that run in opposite directions on chromosome 2, and reporter gene analyses performed with the AtPMT1 and AtPMT2 promoters showed (i) that both genes are expressed and (ii) that they have quite similar and largely overlapping expression patterns. Functional characterizations of the proteins encoded by the individual cDNAs in bakers’ yeast or of cRNAs in Xenopus oocytes were performed. Thereby, the substrate specificities and the energy dependence of AtPMT1 and AtPMT2-driven transport were characterized, and the symported ion was identified. The subcellular localization of the proteins was studied with AtPMT1-GFP (green fluorescent protein) and AtPMT2-GFP fusions. Finally, an Atpmt1 T-DNA insertion line and an RNAi-line were characterized. The data presented suggest that, in Arabidopsis, AtPMT1 and AtPMT2 might represent xylitol and fructose transporters in pollen and young xylem.

Materials and methods

Strains and growth conditions

Arabidopsis thaliana plants (Col-0 and SALK_035269) were grown in growth chambers on potting soil under a 16/8 h light/dark regime at 22 °C and 60% relative humidity or in the greenhouse under ambient conditions. For heterologous expression of AtPMT1 and AtPMT2 cDNAs in yeast, strain EBY.VW-4000
was used (Wiczorke et al., 1999). Escherichia coli strain DH5α (Hanahan, 1983) was used for all basic cloning steps. Transformation of Arabidopsis was performed using Agrobacterium tumefaciens strain GV3101 (Holsters et al., 1980).

cDNA cloning and constructs for yeast expression

AtPMT1 and AtPMT2 cDNAs were amplified from whole plant (Col-0) total RNA with gene specific primers [for AtPMT1: AtPUT1-5 (5′-GAG ACA CGG GCC GCA AGC TTG TAA AAG AAA TGA ATT CCT CGG GAG TTG AAC A-3′) and AtPUT1-3 (5′-GAG ACA CGG GCC GCA TTG CGT TTA AAG AAA TGA TGT CAG GAG AAG AAC G-3′) and for AtPMT2: AtPUT2-5 (5′-GAG ACA CGG GCC GCA TTG CGT TTA AAG AAA TGA TGT CAG GAG AAG AAC G-3′) and AtPUT2-3 (5′-GAG ACA CGG GCC GCT CAT TGT TCA ACT TGT TGT-3′)] binding to the very 5′-ends (including the start ATG) or the very 3′-ends (including the stop codon) of the cDNAs. These PCR reactions added the 15-nucleotide sequence aag ctt gta aaa gaa to either the 5′- or 3′-ends of the PCR product. These PCR products were used as templates for amplification of two restriction sites by PCR after the last amino acid of the respective ORF. These second NcoI sites replaced the stop codons of the original sequences. These modified ORFs were inserted into the unique NcoI cloning site representing the start ATG of the GFP ORF in the pSO35e plasmid. The continuous ORFs were confirmed by sequencing.

The resulting constructs were used for transient expression of AtPMT1/GFP or AtPMT2/GFP in Arabidopsis protoplasts (polyethylene glycol transformation; Abel and Theologis, 1994) or in tobacco epidermis cells (particle bombardment; Klepek et al., 2002).

T-DNA and RNAi lines

The position of the T-DNA insertion in SALK_035269 was determined using the left border-specific primers LBal (5′-TGG TTC ACG TAG TGG GCC AT-3′) and LBbl (5′-GCC TGG ACC GCT TGC TGC AAC T-3′) and with the AtPMT1-specific primer AtPMT1p3.

For production of an RNAi construct in phANNIBAL (Welsh et al., 2001) a 472 bp fragment corresponding to the region from 710 to 1191 in the AtPMT1 cDNA (96.6% sequence identity with the corresponding fragment of AtPMT2) was amplified with the primers AtPMT1c+719f (5′-GAC ACA CTC GAG TCT AGA ATG ACA TCA AAC GCC GAC CAG T-3′) and AtPMT1c-1191r (5′-CTG TGT GGT ACC AAG CTT ACA AAA GTC ATC ACC GTC GT-3′) that added two restriction sites to each end. The fragment was cloned (i) between the XbaI and HindIII sites of pHANNIBAL and (ii) between the KpnI and XhoI sites of phANNIBAL. The RNAi construct was then inserted into pAF16 yielding pMVRNAiA1.

Expression levels of AtPMT1, AtPMT2, and ACTIN2 were determined by RT-PCR using the primers AtPMT1c11f (5′-GGG AGT TGA ACA AGC TTT GTG TA-3′) and AtPMT1c1253r (5′-ACA GGG AAT ATG TCT CAG GAA-3′), AtPMT2c550f (5′-GGG AGT TGA ATG TTA GGT ATT TTT GAT-3′) and AtPMT2c1253r (5′-ACG GAA GAA ATC ATG TGA GC-3′), and AtAct2g+846f (5′-ATG CAG ATG CCC AGA AGTCTT CTG-3′) and AtAct2g+1295r (5′-GAA ACA TTT CTC GTG AAC CAT TCTC-3′).

Immunohistochemical techniques and Western blot analyses

For production of an anti-AtPMT1/AtPMT2 antisera (αAtPMT1/2) two AtPMT1-derived peptides that were identical or almost identical in the AtPMT2 protein were synthesized and used to immunize two rabbits (Pineda Antikörper Service, Berlin, Germany). Pre-immune sera were taken from each rabbit prior to the first immunization. Sera from bleedings performed 90 d after the first immunization were used without further purification (unpurified antiserum), sera from bleedings performed 120 d after the first immunization were affinity-purified with column-bound peptides (affinity-purified antisera). This purification was performed by Pineda Antikörper Service (Berlin, Germany).

Binding of αAtPMT1/2 or of preimmune serum (dilutions shown in the figure legends) to microtome sections of fixed and embedded yeast cells or plant material (prepared as described by Ransperger et al., 2004) was visualized after incubation with anti-rabbit IgG-fluorescein isothiocyanate (FITC)-isomer 1-conjugate (1:100; Sigma-Aldrich, Deisenhofen, Germany) as described by Stadler and Sauer (1996). Microscopic slides were mounted in anti-fading medium (ProLong Antifade Kit; Molecular Probes, Invitrogen, Carlsbad, CA).
Detection of GFP fluorescence and GUS histochemical staining

Images of GFP fluorescence were made with an epifluorescence microscope (Zeiss Axioskop, Carl Zeiss Jena GmbH, Jena, Germany) with an excitation wavelength of 460–500 nm. Emitted fluorescence was monitored at detection wavelengths longer than 510 nm.

Standard protocols were used for GUS histochemical staining of plant tissues (100 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.5 mg/ml 1% X-Gluc). All stainings were performed for a minimum of 24 h or longer. For analyses of flower stalk sections, stained tissue was fixed for 2 h at room temperature in 2.5% glutaraldehyde, washed with water (3 times), cleared with 70% ethanol, dehydrated (90% and 100% ethanol), and cut with razor blades.

Results

Cloning of AtPMT1 and AtPMT2 cDNAs

In silico analyses of the Arabidopsis genome revealed five open reading frames (ORFs) for transporters with significant homology to the previously characterized Arabidopsis plasma membrane polyol transporter AtPMT5 (At3g18830; Klepek et al., 2005). Two of these genes, AtPMT1 (At2g16120) and AtPMT2 (At2g16130), are located on the same chromosome (2) with their start ATGs being separated by 6881 nucleotides. Both genes are interrupted by 2 introns, and in both genes these introns are inserted at identical positions (after nucleotides 127 and 474 of the open reading frame) yielding exons that encode identical numbers of amino acids (Fig. 1). The first of these two introns is conserved in all AtPMT genes, the second only in AtPMT1, AtPMT2, and AtPMT5. The high degree of similarity in the coding regions of AtPMT1 and AtPMT2 (93.6% identity on the amino acid level) is conserved also within their intron sequences that are 83.3% (1st intron; 91 bp in AtPMT1 and 94 bp in AtPMT2) and 68.7% identical (2nd intron; 88 bp in AtPMT1 and 92 bp in AtPMT2). Moreover, the first 500 nucleotides of the 5′-flanking sequences share 76.60% identity. Together, these data suggest that AtPMT1 and AtPMT2 are the result of a gene duplication in the recent history of Arabidopsis evolution. As a result of this high degree of sequence similarity, microarrays do not discriminate between AtPMT1 and AtPMT2 expression. They do show, however, that AtPMT1 and AtPMT2 are the PMT genes with the lowest expression levels in Arabidopsis (https://www.geneinvestigator.com/).

Expression of AtPMT1 and AtPMT2 cDNAs in Saccharomyces cerevisiae

To determine the functional properties of the two transporters, their cDNAs were cloned into the unique NotI site of the yeast expression vector NEV-N (Sauer and Stolz, 1994). Constructs harbouring the cDNAs in sense or antisense orientation [pMV-Y (AtPMT1 sense), pMV-Y-as (AtPMT1 antisense), pYK34 (AtPMT2 sense) and pYK35 (AtPMT2 antisense)] were used to transform yeast strain EBY.VW-4000 (Wieczorke et al., 1999) that has no background of monosaccharide transporter activity. The resulting strains were named VMY15 (AtPMT1 sense), VMY21 (AtPMT1 antisense), YKY6 (AtPMT2 sense) and YKY7 (AtPMT2 antisense).

In a first approach, VMY15 and YKY6 cells were used for competition analyses. To this end, the transport of 14C-sorbitol was tested first, and as sorbitol was transported by both cell lines, the uptake rates for 14C-sorbitol (0.1 mM) in the presence of several potential competitors, all at a 100-fold molar excess (=10 mM; Fig. 2A) were determined. In both strains, uptake of sorbitol was strongly reduced in the presence of xylitol, several pentoses, inositol, erythritol, inositol, and unlabelled sorbitol, with xylitol being the best competitor in both cases. Fructose was also an excellent competitor of AtPMT1-driven sorbitol uptake; mannitol and several other compounds were only poor or no competitors. This result suggested that, like AtPMT5 and

Fig. 1. Comparison of the six Arabidopsis PMT proteins. Schematic alignment of the deduced protein sequences (black bars) of AtPMT1 to AtPMT6 based on the intron positions (arrows) in the respective genes. Grey vertical bars (I–XII) indicate the positions of the predicted transmembrane helices, thin lines show two small gaps in the AtPMT4 sequence. Numbers of amino acids encoded by the different exons are indicated (white).
PMT-type transporters from polyol-translocating species, AtPMT1 and AtPMT2 are broad-spectrum sugar/polyol transporters with a preference for xylitol and fructose.

Direct transport assays were performed next with a selected set of radiolabelled substrates, including sorbitol, glucose, galactose, fructose, xylose, and xylitol (Fig. 2B, C). Ribose and inositol (not shown). All transport tests were performed at an initial substrate concentration of 0.1 mM. Although the maximal transport rates determined for AtPMT2 in YKY6 cells (Fig. 2C) were about 50% lower...
than those of AtPMT1 in VMY15 cells (Fig. 2B), the relative transport rates were quite similar for both transporters. The relative permeability sequence (xylitol>fructose>sorbitol or glucose>ribose>inositol>xylose>galactose) demonstrated that xylitol and fructose represented the preferred substrates of AtPMT1 and AtPMT2. Uptake of the different substrates was also studied in the antisense yeast strains VMY21 and YKY7 (antisense controls are better than untransformed yeast cells, as they carry the same gene defect. Antisense controls are better than empty vector controls as the multicopy sense and antisense plasmids have identical sizes, which excludes that differences result from plasmid copy numbers). The rates determined in these strains were negligible (Fig. 2B, C).

pH-dependence and uncoupler-sensitivity of AtPMT1 and AtPMT2 appeared similar. Transport rates increased with decreasing pH-values, but the pH-dependence was steeper for AtPMT1 than for AtPMT2 (Fig. 2D). In agreement with this, a strong sensitivity to carbonyl cyanide-m-chlorophenylhydrazone (CCCP), an uncoupler of proton gradients, was found for both transporters. The CCCP effect on AtPMT1 was more pronounced than on AtPMT2 (Fig. 2D). Both sets of experiments suggested that AtPMT1 and AtPMT2 function as H+-symporters.

When determining the $K_m$-values of AtPMT1 and AtPMT2 for their putatively best substrate xylitol, almost identical affinities were obtained, with 0.14±0.02 for AtPMT1 and 0.18±0.01 for AtPMT2 (Fig. 3). In addition, the $K_m$ of AtPMT1 was determined for sorbitol [0.77±0.12 mM (graph not shown)] and the $K_m$ of AtPMT2 was determined for glucose [1.25±0.4 mM (graph not shown)]. These results confirm that xylitol is the best substrate of both transporters.

Expression of AtPMT1 and AtPMT2 cDNAs in X. laevis oocytes

*Xenopus laevis* oocytes had been used for the successful expression of cRNA of the plasma membrane H+-symporter AtPMT5 (Klepek et al., 2005; Reinders et al., 2005) and of several other H+-symporters (Aoshima et al., 1993; Schneider et al., 2006, 2007). Therefore, this system was used to analyse the putatively electrogenic transport mechanisms of AtPMT1 and AtPMT2. Following the injection of *AtPMT1* and *AtPMT2* cRNAs into *Xenopus* oocytes, xylitol-dependent ion currents were monitored. These currents were not affected by K+, Na+ or N-methylglucamine (NMG+) ions in the bath medium (Fig. 4A, C) pointing to protons as the symported ion species. No xylitol-induced currents were detected in water-injected controls (not shown).

Currents induced by the polyol appeared strongly voltage-dependent. Steps in membrane potentials from 0 mV to -40 mV and -90 mV (Fig. 4B, D) elicited gradually increasing inward currents. Thereby, membrane hyperpolarization increased the electrochemical force for protons to enter the transporter and together with the polyol released the co-transported solutes into the cytoplasm. Together these data demonstrate that AtPMT1 and AtPMT2 catalyse the electrogenic, voltage-dependent symport of protons with xylitol.

Transient expression of AtPMT1-GFP and AtPMT2-GFP fusion-constructs

Although the transport activities of AtPMT1 and AtPMT2 in yeast and *Xenopus* plasma membranes (Figs 2, 3, 4) provided indirect evidence that these proteins might be transporters of the plasma membrane, targeting of AtPMT1 and AtPMT2 to other membranes in planta cannot be excluded. To identify the subcellular localization of both transporters in *Arabidopsis*, the open reading frame (ORF) for green fluorescent protein (GFP) was attached to the 3′-ends of *AtPMT1* or *AtPMT2* cDNAs and the resulting constructs were used for transient expression in *Arabidopsis* mesophyll protoplasts or in intact *Arabidopsis* epidermal cells. All cells transformed with these constructs (pMV-L for *AtPMT1*-GFP expression; Fig. 5A, B; pYK26 for *AtPMT2*-GFP expression; Fig. 5C) showed strong and specific labelling of their cell surfaces. Chloroplasts (recognized by their red autofluorescence) are clearly located inside this labelled structure. This localization supports the plasma membrane as the origin of the signal rather than the tonoplast or the endoplasmic reticulum.
Analysis of the tissue-specific expression in AtPMT1 promoter/reporter and AtPMT2 promoter/reporter plants

For each gene two separate reporter gene constructs with the ORFs of GUS (β-glucuronidase) or GFP were generated. Promoter fragments were obtained from PCR amplifications that introduced HindIII cloning sites at the 5'-ends and NcoI cloning sites at the transcriptional start sites. Promoter/reporter cassettes were generated, inserted into pAF16 (Stadler et al., 2005), and used to transform Arabidopsis plants. For each construct (pAtPMT1/GFP; pAtPMT1/GUS; pAtPMT2/GFP; pAtPMT2/GUS) 20–30 transformants were analysed.

During the analysis of 30 pAtPMT1/GUS plants, GUS staining was found in the hydathodes and in pollen grains germinating on stylar papillae or on agar medium (Fig. 6B, C) in seven plants. No staining was detected in pollen grains inside the anthers (Fig. 6A) or in released but yet ungerminated pollen grains (Fig. 6B, C). One pAtPMT1/GUS plant showed weak staining in the vasculature of its flower stalks. Analysis of embedded cross-sections showed that this staining resulted from very low GUS activity in the cambium and from slightly stronger GUS activity in young xylem cells (Fig. 6D–F).

Analysis of 30 pAtPMT2/GUS plants confirmed the already described GFP expression in pollen (Fig. 7A, B). In contrast to the GUS staining observed in pAtPMT1/GUS plants, the hydathodes and the pollen grains were not stained. No staining was detected in the anthers, but weak staining was found in the vasculature of some flower stalks.
pollen (Fig. 6B, C), however, this staining (i) was much stronger and (ii) was detected in pollen grains of still closed anthers (Fig. 7B). Moreover, our analyses also revealed GUS activity in hydathodes and (weakly) in minor veins of several plants (Fig. 7D). Finally, one pAtPMT2/GUS plant showed staining in the vasculature of the flower stalks. The latter signal resulted from low GUS activity in the cambium and from stronger GUS activity in young xylem cells (Fig. 7E).

While GFP fluorescence could not be detected in any of the pAtPMT2/GFP plants (not shown), 100% of the pAtPMT2/GFP plants showed intense fluorescence in their mature pollen grains (Fig. 7C). This fluorescence was detected only during the late stages of pollen development, i.e. in still closed anthers of just opening flowers. The signal increased further with ongoing anther dehiscence. No other sites of GFP fluorescence were detected. This GFP signal was absent from wild-type (WT) pollen (insert of Fig. 7C).

Immunohistochemical analyses of AtPMT1 and AtPMT2 localization

The observed expression of AtPMT2 in pollen is supported by data from publicly accessible microarray analyses that show strong expression of AtPMT2 exclusively in mature pollen (stage 12). However, as already mentioned, these microarray data show identical data for AtPMT1, as they do not discriminate between these two almost identical genes (https://www.genevestigator.com/; http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Moreover, the data presented do not show expression of AtPMT1 or AtPMT2.
in any other tissue, which could result from weak promoter activities in only a limited number of cells of a given organ, for example, in young xylem cells of the flower stalk.

To test this possibility and to confirm or confute the GUS stainings observed in flower stalks, antisera were raised against a mixture of two peptides (peptide 1: NH2-KYLKDTSNTKKEAISR-COOH; peptide 2: NH2-SYTANKKNNSMVDNENEV-COOH) predicted to represent immunogenic regions in AtPMT1. These peptides corresponded to the regions 223–238 (central cytoplasmatic loop) and 491–507 (C-terminus) of AtPMT1. The respective regions of AtPMT2 are either identical (peptide 1) or very similar (peptide 2: NH2-YSANKKNVMGSKGKV-COOH), and resulting antibodies were, therefore, not expected to discriminate between these proteins. Peptides for the production of potentially monospecific antibodies could not be identified.

The quality of the antisera was first tested in AtPMT1-expressing or AtPMT2-expressing yeast cells. Sera from two immunized rabbits (αAtPMT1/2-R1 and αAtPMT1/2-R2) recognized AtPMT1 in detergent extracts of enriched plasma membranes from yeast cells expressing the AtPMT1 cDNA (Fig. 8A). Most importantly, however, immunolocalizations with sections from yeast cells expressing AtPMT1 or AtPMT2 either in a sense or in an antisense orientation (i) labelled AtPMT1 and AtPMT2 equally well in sense but not in antisense sections, and (ii) labelled the cell surface exclusively, most likely the plasma membranes of these cells (Fig. 8B). This demonstrated that, as expected, the sera do not discriminate between AtPMT1 and AtPMT2. The sera, however, appeared to be suitable to detect these proteins in immunohistochemical analysis of plant tissue.

Based on these results, immunohistochemical analyses of cross-sections from Arabidopsis WT flower stalks were performed with unpurified αAtPMT1/2-R1 antiserum. Figure 9A shows that this serum labelled the same cell layers that were stained by the GUS histochemical stainings shown in Fig. 6D–F (pAtPMT1) and in Fig. 7D (pAtPMT2). No fluorescence was detected, when preimmun serum was used for these analyses (Fig. 9B, C). The use of affinity-purified αAtPMT1/2-R1 improved the signal intensity, but did not alter the specificity of the labelling (Fig. 9D, E). This confirmed the observed GUS staining that pointed to pAtPMT1 and pAtPMT2 activities in young xylem cells (Figs 6D–F, 7E) although this staining was only seen in a very few transgenic plants.

Analysis of mutant plants with potentially reduced AtPMT1 and/or AtPMT2 expression levels

In publicly available T-DNA insertion libraries (http://signal.salk.edu/cgi-bin/tdnaexpress) mutants with T-DNA insertions in an exon or intron of AtPMT1 or AtPMT2 could not be found, but four lines (SALK_035269, SALK_035929, GABI_141A06, GABI_204C03) were identified that had insertions in the intergenic region between the AtPMT1 and AtPMT2 start ATGs. In three of these lines, the predicted insertions seemed to be too far upstream from the translational start sites to affect the expression of AtPMT1 or AtPMT2. Therefore, only one mutant (Salk_035269), that was predicted to carry an insertion near the translational start ATG of the AtPMT1 gene, was analysed. Figure 10A demonstrates that the insertion in this Atptm1-1 mutant was 559 bp upstream from the ATG, a distance that might still be long enough to allow expression of the downstream AtPMT1 coding region. In fact, when the AtPMT1 mRNA levels were compared in three WT and three Atpmt1-1 plants (insert of Fig. 10A) by RT-PCR, similar levels were found in all the plants analysed.

In a second approach, an RNAi construct was used that was designed to reduce AtPMT1 and AtPMT2 mRNA levels simultaneously. This construct carried the region with the highest sequence conservation between AtPMT1 and AtPMT2 (nucleotides 719–1191 in the two ORFs; 96.6% identity) in opposite directions under the control of the 35S promoter. After transformation of Arabidopsis plants with this construct, a large number of independent transgenic

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**Analysis of mutant plants with potentially reduced AtPMT1 and/or AtPMT2 expression levels**

In publicly available T-DNA insertion libraries (http://signal.salk.edu/cgi-bin/tdnaexpress) mutants with T-DNA insertions in an exon or intron of AtPMT1 or AtPMT2 could not be found, but four lines (SALK_035269, SALK_035929, GABI_141A06, GABI_204C03) were identified that had insertions in the intergenic region between the AtPMT1 and AtPMT2 start ATGs. In three of these lines, the predicted insertions seemed to be too far upstream from the translational start sites to affect the expression of AtPMT1 or AtPMT2. Therefore, only one mutant (Salk_035269), that was predicted to carry an insertion near the translational start ATG of the AtPMT1 gene, was analysed. Figure 10A demonstrates that the insertion in this Atptm1-1 mutant was 559 bp upstream from the ATG, a distance that might still be long enough to allow expression of the downstream AtPMT1 coding region. In fact, when the AtPMT1 mRNA levels were compared in three WT and three Atpmt1-1 plants (insert of Fig. 10A) by RT-PCR, similar levels were found in all the plants analysed.

In a second approach, an RNAi construct was used that was designed to reduce AtPMT1 and AtPMT2 mRNA levels simultaneously. This construct carried the region with the highest sequence conservation between AtPMT1 and AtPMT2 (nucleotides 719–1191 in the two ORFs; 96.6% identity) in opposite directions under the control of the 35S promoter. After transformation of Arabidopsis plants with this construct, a large number of independent transgenic
lines was identified (pMV-RNAi1 plants). The AtPMT1 and AtPMT2 mRNA levels in these plants were then compared with the corresponding mRNA levels in WT plants. Thereby AtPMT2 expression was found down-regulated or absent from most of these plants, whereas that of AtPMT1 was not affected (Fig. 10B). Therefore, neither the Atpmt1-1 mutant nor the pMV-RNAi1 plants were used for further analyses.

Discussion

This paper presents the comparative analysis of AtPMT1 and AtPMT2, two almost identical proteins that are encoded by adjacent genes (At2g16120 and At2g16130) separated by an intergenic region of 6602 nucleotides. AtPMT1 and AtPMT2 belong to one of seven subfamilies of the Arabidopsis MST-like superfamily. PMT-type proteins are found in all higher plants. The Arabidopsis PMT subfamily has six members, of which only one, AtPMT5, has been characterized to date (Klepek et al., 2005; Reinders et al., 2005). All other PMT-type proteins were characterized mainly from polyol-translocating species, and the respective proteins were characterized as sorbitol or mannitol transporters. In Arabidopsis, the transmembrane transport of sorbitol and mannitol plays no or only a minor role, and the physiology of AtPMT proteins thus remained unclear. A detailed functional characterization of the Arabidopsis AtPMT1 and AtPMT2 proteins after expression of their cDNAs in baker’s yeast and Xenopus oocytes is presented here. The proteins were characterized as voltage-dependent, plasma membrane-localized H⁺-symporters for polyols, cyclitols, and monosaccharides with a clear preference for xylitol and fructose. Promoter/reporter gene analyses and immunolocalization studies demonstrate that these AtPMT1 and AtPMT2 transport activities occur in mature or germinating pollen grains and in young xylem cells.

Interestingly, in heterologous expression systems both proteins also transport sorbitol with \( K_m \)-values quite similar to those of SOT proteins from sorbitol-translocating species. This demonstrates that PMT-type transporters from polyol-translocating species and from non-polyol-translocating species have almost identical functional properties. Moreover, this suggests that polyol-translocating species are very likely to have PMT-type proteins with physiological functions similar to those of the Arabidopsis PMT proteins, i.e. PMT-type proteins that are not involved in phloem translocation.
**AtPMT1 and AtPMT2 are plasma membrane-localized transporters for xylitol and fructose**

AtPMT1 and AtPMT2 were functionally characterized as electrogenic H⁺-symporters by expression of their cDNAs or cRNAs in baker’s yeast or *Xenopus* oocytes (Figs 2, 3, 4). Immunolocalization of the proteins in recombinant yeast cells (Fig. 8B) and the subcellular localization of GFP fusions in *Arabidopsis* (Fig. 5) demonstrate that both proteins are plasma membrane localized.

Previously characterized transporters of the *Arabidopsis* MST-like superfamily are mostly specific for only a few different substrates and accept other compounds only if these are structurally very similar [e.g. the members of the AtSTP subfamily with high specificity for certain hexoses (Sauer et al., 1990; Scholz-Starke et al. 2003; Schneider et al., 2003) or the members of the AtINT subfamily with high specificity for different inositol epimers (Schneider et al., 2006, 2007, 2008)]. In contrast, the list of substrates sympported by AtPMT1 and AtPMT2 is complex. It contains structurally quite different molecules, such as linear polyols with 4 (erythritol), 5 (xylitol), or 6 (sorbitol) carbon atoms, the cyclic hexitol inositol, plus pentoses (ribose, arabinose, xylose) and hexoses (fructose, glucose, mannose; Fig. 2A–C). One may speculate that this spectrum does not reflect the list of physiological substrates that are transported by AtPMT1 and AtPMT2 in planta. Aldohexoses and inositols, for example, might be taken up by the aldohexose-specific and inositol-specific AtSTP and AtINT proteins that, in addition, have higher affinities for their substrates, and that are present in the plasma membranes of the very same cells as AtPMT1 and AtPMT2 (Scholz-Starke et al., 2003; Schneider et al., 2006, 2007).

This leaves polyols, pentoses, and the ketohexose fructose as potential physiological substrates. In fact, competition analyses (Fig. 2A) revealed that xylitol, fructose, and pentoses are the best competitors of sorbitol uptake. Direct transport analyses with radiolabelled substrates revealed that, at substrate concentrations of 0.1 mM, xylitol transport is about 5-fold (AtPMT1) or 9-fold (AtPMT2) faster than the transport of xylose (Fig. 2B, C). In agreement with the very good competition data, fructose seems to represent the second best substrate for both carriers. Interestingly, a comparison of this result with competition data published for the celery AgMAT2 protein (Juchaux-Cachau et al., 2007) shows that uptake of radiolabelled mannitol by AgMAT2 is also inhibited significantly by fructose and xylitol than by unlabelled mannitol, suggesting that xylitol and fructose might be better substrates for this protein.

Finally, *Kₘ* measurements yielded quite similar values of 0.14 mM (AtPMT1) and 0.18 mM (AtPMT2) for xylitol (Fig. 3). These are the lowest *Kₘ* values found for a PMT-type transporter so far. The *Kₘ* values for sorbitol or mannitol in most SOT and MAT proteins from polyol translocating species are between 0.65 mM (AgMAT2; Juchaux-Cachau et al., 2007) and 3 mM (MdSOT5; Watari et al., 2004), and in *Plantago*, PnPLT1 and PnPLT2 proteins have even higher *Kₘ* values for sorbitol (12 and 20 mM, respectively). Interestingly, the *Kₘ* values for sorbitol determined for AtPMT1 (0.77 mM, Fig. 3) or AtPMT5 [0.5 mM in yeast; Klepek et al., 2005; 0.86–2.6 mM (voltage-dependent) in *Xenopus*; Reinders et al., 2005] are in the same range as those of the related proteins from polyol translocating plants.

In summary, these data demonstrate that PMT-type proteins from polyol-translocating species (MATs, SOTs, PLTs) and from non-polyol translocating species do not just share sequence homologies, but also functional properties: (i) All of these proteins accept a wide range of substrates, (ii) their *Kₘ* values for the phloem-translocated hexitols mannitol and sorbitol are similar, and (iii) compounds other than mannitol or sorbitol might be even better transport substrates.

**AtPMT1 and AtPMT2 are expressed in pollen, hydathodes, and in young xylem cells**

Expression analyses of plants transformed with pAtPMT1 reporter or pAtPMT2/reporter constructs (Figs 6, 7) revealed promoter activities in pollen, hydathodes and, in rare cases, also in young xylem cells, immediately adjacent to the cambium. Microarray analyses that show consistently low levels of expression for both genes did not discriminate between AtPMT1 and AtPMT2 mRNA levels and these data could, therefore, not be assigned to the expression levels of a specific gene.

Our data demonstrate that both genes are expressed and that their expression patterns are similar, however, they also revealed differences. The anther-specific (tricellular pollen) expression shown in all microarray analyses (https://www.genevestigator.com/ and http://www.bar.utoronto.ca/efp/) is clearly due to the activity of pAtPMT1 (Fig. 7A–C). No activity of pAtPMT1 was detected in anthers at any developmental stage (Fig. 6). By contrast, AtPMT1 expression was observed in germinating pollen grains and in pollen tubes (Fig. 6B, C). Moreover, both AtPMT1 (not shown) and AtPMT2 (Fig. 7D) are expressed in hydathodes, and for both genes one plant was found with GUS activity in young xylem cells (Figs 6D–F, 7E). Most importantly, this expression in the xylem which found only for one pAtPMT1-GUS line and only for one pAtPMT2-GUS line could be confirmed in immunohistochemical analyses with the αAtPMT1/2-R1 antiserum that specifically recognizes AtPMT1 and AtPMT2 in yeast cells (Fig. 8), and that decorates AtPMT1 and/or AtPMT2 in the young xylem cells of the vasculature (Fig. 9). The reason for the low frequency of plants with GUS-staining in the xylem is unclear. The strong signals obtained with αAtPMT1/2-R1 (Fig. 9) suggest high expression levels in these cells. Possibly strong promoter activities cause xylem-specific silencing in the majority of the GUS reporter lines.

**The physiological roles of AtPMT1 and AtPMT2**

What are the physiological roles of the H⁺/xylitol or H⁺/fructose symporters AtPMT1 and AtPMT2 in *Arabidopsis*?
pollen/pollen tubes and young xylem cells? Both cells and tissues are rapidly expanding, non-green sinks and depend on the supply with organic carbon to drive their metabolism and energy supply. A first possible function of AtPMT1 and AtPMT2 might, of course, be the retrieval of molecules lost from certain cells by passive diffusion. Developing xylem cells are important sites of cell wall biosynthesis, and cellulose, lignin, and glucuronoxylans are the main components of their secondary cell walls (Pena et al., 2007). However, in plants, the xylose residues necessary for biosynthesis of xylose-containing cell wall oligosaccharides are synthesized by nucleotide sugar interconversion as UDP-xylose by decarboxylation of UDP-glucuronic acid (Harper and Bar-Peled, 2002) and immediately transferred to the different acceptor molecules via xylosyltransferases (Moore et al., 1991; Bolwell, 2000). Similarly, UDP-arabinose is synthesized from UDP-xylose by an epimerase (Burget et al., 2003). Thus, the amount of free pentoses that might leak out of developing xylem cells or out of pollen grains is most likely small, and high cellular concentrations of xylitol have never been reported for Arabidopsis.

A second function of AtPMT1 and AtPMT2 might be the feeding of cells with fructose. It is well known that germinating pollen and growing pollen tubes hydrolyse extracellular sucrose (Ylstra et al., 1998), that this hydrolysis is mediated by an extracellular invertase, and that a loss of this invertase can impair pollen development and may even cause male sterility (Goetz et al., 2001). Similarly, from several invertase genes identified in poplar (Populus alba L.×grandidentata Michx., one, PaxgINV2, appears to encode an enzyme that is involved in phloem unloading and in providing developing xylem cells with energy and carbon skeletons (Canam et al., 2008). So far, several pollen-specific monosaccharide transporters that might be responsible for the uptake of these products of sucrose hydrolysis have been described (Ylstra et al., 1998; Schneidereit et al., 2003; Scholz-Starke et al., 2003). However, these transporters are rather specific for aldohexoses (including glucose), whereas the ketohehose fructose is not a substrate or only a poor substrate for these transporters. A function of PMT-type proteins in rapidly growing tissues like pollen, pollen tubes or young xylem cells might, therefore, be the uptake of fructose produced by extracellular invertases and not transported by classical STP-type monosaccharide transporters.

Functions of PMT-type proteins in polyol-translocating species

The finding that the physiological substrate of AtPMT1 and AtPMT2 might be fructose and, to a lesser extent, possibly also pentoses, and the observation that AtPMT1 and AtPMT2 can transport sorbitol with $K_m$ values similar to those found in SOT proteins, although sorbitol is most probably not their physiological substrate, might be relevant for further analyses of PMT-type transporters in polyol-translocating species.

Firstly, the fact that all higher plants (also non-polyol-translocating species) do possess PMT-type proteins that can transport mannitol and/or sorbitol and that even the moss Physcomitrella patens has a related protein (accession number: XP_001755457), may provide an insight into the evolution of polyol translocation. When polyol-translocating species evolved, the transporters necessary for phloem loading were probably already available, although their main function might not have been the transport of sorbitol or mannitol. The main challenge might have been to redirect the expression of one of these genes to the phloem companion cells, i.e. to modify cis-regulatory sequences in the promoter. Such an evolution on the promoter level has been demonstrated for C₄-plants, where the allocation of cis-elements led to an altered cell-specificity of genes already available in C₃ plants (Akyildiz et al., 2007; Engelmann et al., 2008).

Secondly, it is quite likely that polyol-translocating species also have to deal with similar transport processes (e.g. with fructose transport) in cells and tissues outside their phloem that are handled by AtPMT proteins like AtPMT1 and AtPMT2 in Arabidopsis. Polyol-translocating species will, therefore, (i) either have independent PMT-type proteins for these functions that are not involved in phloem transport, or (ii) their phloem-loading proteins take over these functions and are also expressed in cells or tissues different from the phloem. The identification of a PMT-type protein in a sorbitol-translocating or mannitol-translocating plant will, therefore, not be sufficient to describe the respective protein as SOT or MAT. Without additional localization data as provided by Ramsperger-Gleixner et al. (2004) for the Plantago PmPMT1 and PmPMT2 proteins or by Juchaux-Cachau et al. (2007) for the celery AgMAT2 protein, the identified proteins might have functions similar to the Arabidopsis AtPMT proteins.

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