

Functional Characterization and Expression Analyses of the Glucose-Specific AtSTP9 Monosaccharide Transporter in Pollen of Arabidopsis¹

Alexander Schneidereit, Joachim Scholz-Starke, and Michael Büttner*

Molekulare Pflanzenphysiologie, Universität Erlangen-Nürnberg, Staudtstrasse 5, D-91058 Erlangen, Germany

A genomic clone and the corresponding cDNA of a new Arabidopsis monosaccharide transporter AtSTP9 were isolated. Transport analysis of the expressed protein in yeast showed that AtSTP9 is an energy-dependent, uncoupler-sensitive, high-affinity monosaccharide transporter with a K_m for glucose in the micromolar range. In contrast to all previously characterized monosaccharide transporters, AtSTP9 shows an unusual specificity for glucose. Reverse transcriptase-polymerase chain reaction analyses revealed that *AtSTP9* is exclusively expressed in flowers, and a more detailed approach using *AtSTP9* promoter/reporter plants clearly showed that *AtSTP9* expression is restricted to the male gametophyte. *AtSTP9* expression is not found in other floral organs or vegetative tissues. Further localization on the cellular level using a specific antibody revealed that in contrast to the early accumulation of *AtSTP9* transcripts in young pollen, the AtSTP9 protein is only found weakly in mature pollen but is most prominent in germinating pollen tubes. This preloading of pollen with mRNAs has been described for genes that are essential for pollen germination and/or pollen tube growth. The pollen-specific expression found for *AtSTP9* is also observed for other sugar transporters and indicates that pollen development and germination require a highly regulated supply of sugars.

Pollen development and pollen tube growth represent an essential stage in plant reproduction (Zheng and Yang, 2000). Because pollen tissue is a photosynthetically inactive "sink," a constant supply with carbohydrates has to be provided during these processes. The main transport form of photoassimilates, Suc, is synthesized in green "source" tissues, loaded into the phloem stream by a specific Suc/H⁺ symporter (Riesmeier et al., 1992; Stadler et al., 1995; Stadler and Sauer, 1996; Kühn et al., 1997) and is then delivered to the various sink organs. Here, the Suc is symplastically unloaded from the phloem into the surrounding mesophyll cells and from there into the apoplastic space. The pollen itself represents a symplastically isolated sink without plasmodesmatal connections to the surrounding tissue. Therefore, the sugars have to be taken up from the apoplast by specific transporters either directly as Suc or, after cleavage by an apoplastic invertase, as hexoses. Gene products involved in this sink-loading process have already been isolated from different plants. Pollen-specific Suc transporters have been identified in tobacco (*Nicotiana tabacum*; Lemoine et al., 1999) and Arabidopsis (Stadler et al., 1999). In *Petunia* sp., a putative monosaccharide transporter, *Pmt1*, was found to be expressed specifically in the male ga-

metophyte, and mRNA levels accumulate in mature and germinating pollen (Ylstra et al., 1998). At the same time, the presence of cell wall-bound invertase activity was found in an in vitro germination assay (Ylstra et al., 1998). Furthermore, the expression of a putative apoplastic invertase in potato (*Solanum tuberosum*) was shown to be restricted to pollen by promoter/ β -glucuronidase (GUS) analysis (Maddison et al., 1999).

The recent completion of the Arabidopsis genome revealed the existence of more than 50 genes homologous to sugar transporters. Within this superfamily, we have identified a family of 14 highly homologous monosaccharide transporters called AtSTPs (Büttner et al., 1999; Büttner and Sauer, 2000). Most of the so far characterized AtSTPs show a sink-specific expression profile (Truernit et al., 1996, 1999; Sherson et al., 2000; Stadler et al., 2003), and three of them, *AtSTP2*, *AtSTP4*, and *AtSTP6*, are expressed in pollen. The high-affinity monosaccharide transporter AtSTP2 was localized in *AtSTP2* promoter-GUS Arabidopsis plants showing reporter activity during pollen maturation (Truernit et al., 1999). Further analysis using in situ hybridization and immunolocalization revealed that both *AtSTP2* mRNA and AtSTP2 protein are first seen at the beginning of callose degradation and microspore release from the tetrads and are no longer detectable after the mitotic divisions and the gametophyte formation or in mature pollen grains or pollen tubes (Truernit et al., 1999). *AtSTP6* expression is confined to the latest stage in pollen development (Scholz-Starke et al., 2003). *AtSTP4* was also found to

¹ This work was supported by the Deutsche Forschungsgemeinschaft (grant no. Bu 973/4-1).

* Corresponding author; e-mail mbuettne@biologie.uni-erlangen.de; fax 49-9131/852-8751.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.026674.

be expressed in pollen by promoter/GUS analysis (Truernit et al., 1996), but in contrast to *AtSTP2* and *AtSTP6*, *AtSTP4* mRNA and *AtSTP4* protein are not detectable at the same time during pollen development. *AtSTP4* mRNA was found during early pollen development by in situ hybridization, whereas a specific antibody could detect *AtSTP4* protein only in mature pollen grains and after pollen germination in the pollen tube (R. Stadler, unpublished data). *AtSTP1*, the first monosaccharide transporter isolated from higher plants (Sauer et al., 1990), shows a low substrate specificity in heterologous expression systems (baker's yeast; Sauer et al., 1990; *Xenopus* sp. oocytes; Boorer et al., 1992, 1994). In accordance, a broad spectrum of monosaccharide substrates was determined for *AtSTP2*, *AtSTP4*, and *AtSTP6* by heterologous expression (Truernit et al., 1996, 1999; Scholz-Starke et al., 2003).

Here, we present the characterization of a new Arabidopsis monosaccharide transporter *AtSTP9*, which is expressed specifically in pollen and shows an unusual selectivity for Glc. Potential functions of monosaccharide transporters in pollen development and germination are discussed.

RESULTS

Isolation of *AtSTP9* Sequences

Isolation of the Genomic Clone

Screening 250,000 plaque forming units of a genomic Arabidopsis library with a radiolabeled *AtSTP9*-specific 204-bp PCR fragment led to the isolation of six positive clones. Southern analyses using the same 204-bp probe identified a 3,386-bp *EcoRI* fragment in all six positive clones (data not shown). This 3,386-bp *EcoRI* fragment (of the 13-kb phage insert) was cloned into the vector pUC19 yielding the clone pAS91. Determining the complete sequence of the 3,386-bp fragment identified the *AtSTP9* gene containing 1,095 bp of the *AtSTP9* promoter region and the complete *AtSTP9* gene sequence (2,123 bp). This sequence is identical to the chromosome locus At1g50310. Computer analysis for splice site predictions (GENSCAN; NetGene2) and comparison with other *AtSTPs* suggest an *AtSTP9* open reading frame of 1,551 bp, interrupted by three putative introns.

Isolation of the cDNA Clone

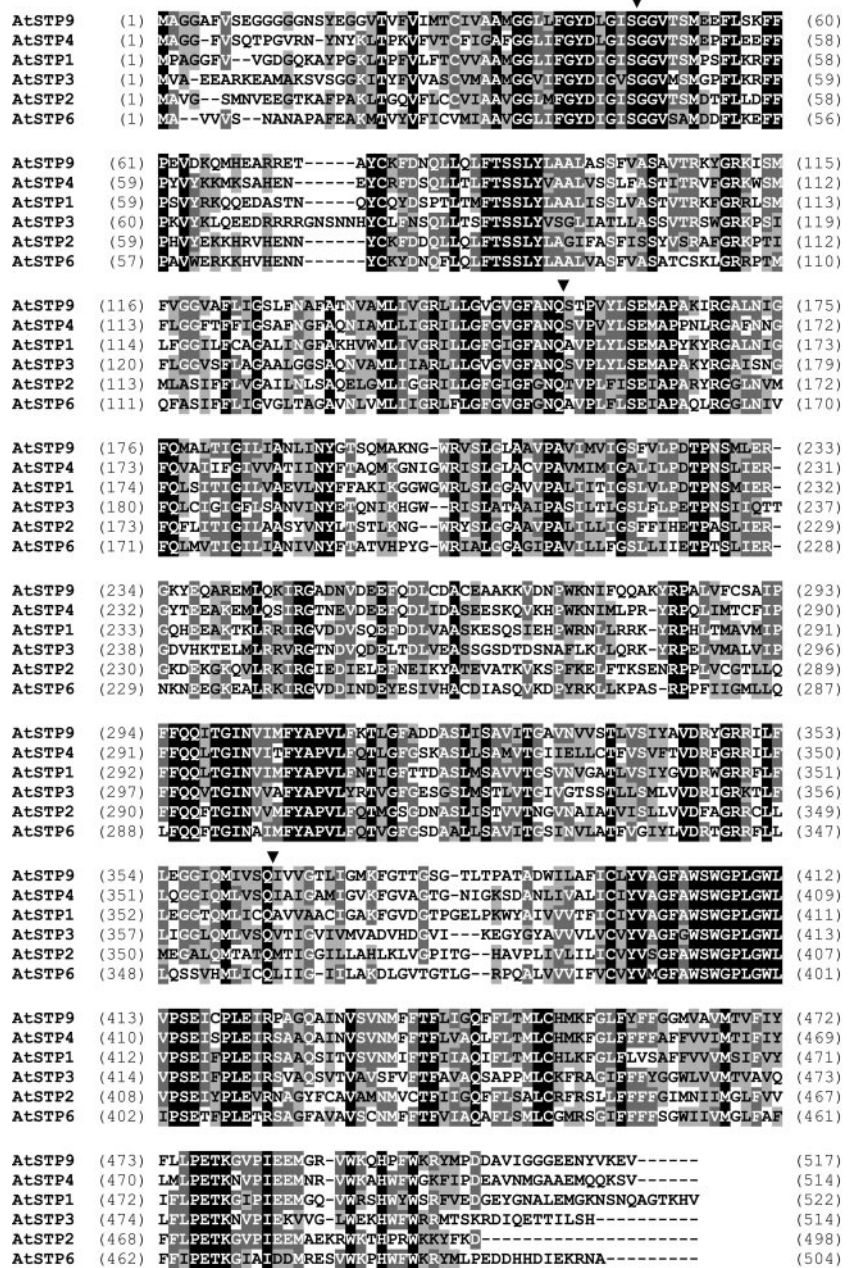
The sequence information obtained with the isolation of the genomic *AtSTP9* clone was used to generate specific primers directed against the N- and C-terminal sequences of the *AtSTP9*-coding region. By reverse transcriptase (RT)-PCR using these primers and total RNA from flowers of different developmental stages, a 1,550-bp fragment was isolated and subcloned into pGEM-T easy (Promega, Madison, WI), yielding the construct pLEX13. Sequencing of

the 1,550-bp *AtSTP9* fragment in pLEX13 and comparison with the genomic sequence reconfirmed the absence of PCR artifacts and the position of the three introns. The encoded *AtSTP9* protein is 517 amino acids long and has a calculated molecular mass of 56.3 kD and an pI of 6.4. Furthermore, the *AtSTP9* protein sequence has two consensus sequence motifs for potential N-glycosylation at positions 153 to 156 (NQST) and 430 to 433 (NVSV). As shown in Figure 1, the *AtSTP9* protein shows a high degree of homology to the so far characterized Arabidopsis monosaccharide transporters *AtSTP1*, *AtSTP2*, *AtSTP3*, *AtSTP4*, and *AtSTP6* (65%–76% similarity and 49%–64% identity). In addition, the positions of introns are strongly conserved (marked in Fig. 1) with *AtSTP6* having introns at positions 1 and 5, *AtSTP3* at positions 1 and 2, and *AtSTP1*, *AtSTP2*, *AtSTP4*, and *AtSTP9* at positions 1, 2, and 5 (Büttner and Sauer, 2000). The determined *AtSTP9* cDNA sequence (GenBank accession no. AJ344336) verifies the predicted annotation for the *AtSTP9* gene locus At1g50310 on chromosome one.

Functional Characterization of *AtSTP9* by Heterologous Expression in Yeast

The sequence of *AtSTP9* shows a high degree of homology to the other members of the Arabidopsis *AtSTP* family. Therefore, we wished to investigate whether *AtSTP9* is also a functional monosaccharide transporter by expressing the *AtSTP9* gene in the heterologous yeast system and studying the transport characteristics. To this end, we cloned the cDNA sequence of *AtSTP9* into the yeast expression vector pEX-Tag (Meyer et al., 2000) in both sense and antisense orientation, yielding clones pACH91s and pACH91a, respectively. With these constructs, the yeast mutant strain EB.Y.VW4000 (Wieczorke et al., 1999) was transformed. EB.Y.VW4000 is a *hxt* null mutant, which does no longer grow on monosaccharides because all genes for hexose transporters (*Hxt1*–*17*) and for the Gal transporter (*Gal2*) are deleted but can be grown on maltose. Expression of the *AtSTP9* gene in antisense orientation in this null mutant background (ScLEX12) did not restore growth on Glc-containing medium (data not shown) nor the uptake of [¹⁴C]Glc (Fig. 2A). However, expression of the *AtSTP9* sense construct (ScLEX8) restored growth on 0.2% and 2% (w/v) Glc (data not shown). Furthermore, ScLEX8 regained the ability to accumulate [¹⁴C]Glc (concentration equilibrium was 0.07 nmol μL^{-1} packed cells), which was not seen in the antisense control ScLEX12 (Fig. 2A). ScLEX8 was further used to determine the substrate specificities for *AtSTP9*. All *AtSTPs* characterized so far (*AtSTP1*, *AtSTP2*, *AtSTP3*, *AtSTP4*, and *AtSTP6*) transport a broad spectrum of monosaccharides at comparable rates. In contrast, *AtSTP9* shows an unusual preference for Glc, whereas other monosaccharides are

Figure 1. Comparison of Arabidopsis monosaccharide transporter sequences. The protein sequence of AtSTP9 (At1g50310) is compared with protein sequences of the previously characterized Arabidopsis monosaccharide transporters AtSTP1 (At1g1260), AtSTP2 (At1g07340), AtSTP3 (At5g61520), AtSTP4 (At3g19930), and AtSTP6 (At3g05960). Shading of the boxes represents identity of all AtSTPs (white letter on black background), identity of more than 50% (white letter on dark gray background), or similarity of more than 50% (black letter on light gray background). Conserved intron positions 1, 2, and 5 found in AtSTPs are marked with black triangles. The *AtSTP9* cDNA sequence has been submitted to the EMBL database with the accession number AJ344336.



transported with much lower rates if at all (Gal, 25%, and Man and Xyl below 5% with respect to Glc uptake; Fig. 2B). The K_m of AtSTP9 for Glc was measured to be $84 \pm 2.5 \mu M$ (Fig. 2A, inset) and is similar to the values determined for previously characterized AtSTPs (Sauer et al., 1990; Truernit et al., 1996, 1999; Büttner et al., 1999; Scholz-Starke et al., 2003). Glc accumulation was maximal at pH 5.5, which is in agreement with the pH optima determined for the other AtSTPs (data not shown). Furthermore, low concentrations of the proton uncouplers carbonyl cyanide m-chlorophenyl-hydrazone and 2,4-Dinitrophenol decrease Glc uptake significantly (Fig. 2B), suggesting that sugar uptake via

AtSTP9 is driven by a proton gradient across the plasma membrane. Taken together, heterologous expression in the yeast system suggests that AtSTP9 is an energy-dependent, high-affinity monosaccharide/ H^+ symporter specific for Glc.

Tissue-Specific Expression of the *AtSTP9* Gene

The majority of the AtSTPs characterized so far in Arabidopsis are specifically expressed in sink tissues (Sauer et al., 1990; Truernit et al., 1996, 1999; Stadler et al., 2003). To determine the spatial and developmental expression profile of the *AtSTP9* gene, we first tried to detect the *AtSTP9* transcript in different

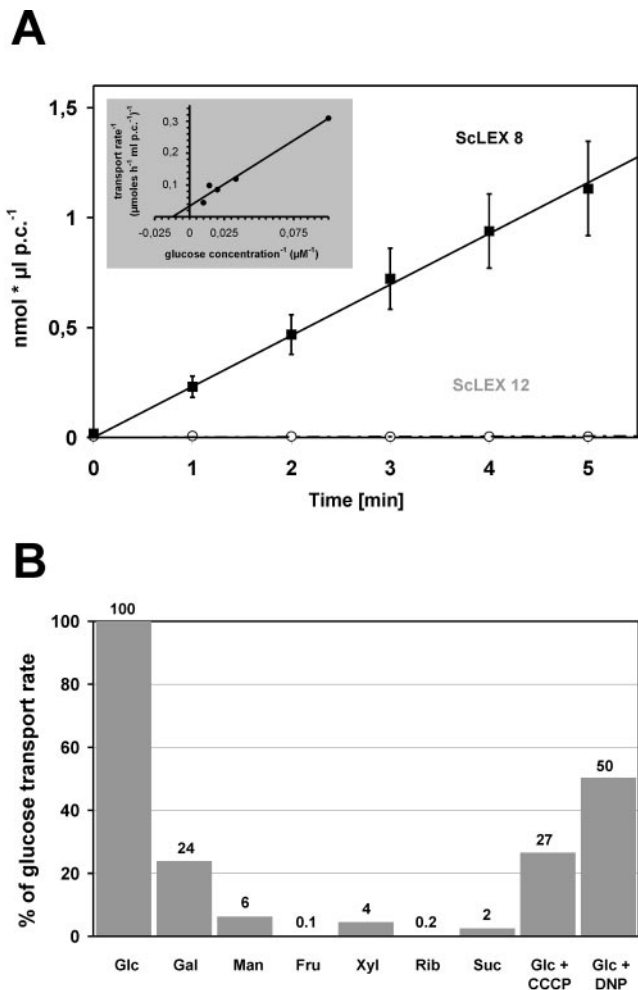


Figure 2. Transport activity of the AtSTP9 monosaccharide transporter. A, Uptake of $[^{14}\text{C}]\text{Glc}$ was measured in yeast strains ScLEX8 (expressing *AtSTP9* sense; ■) and ScLEX12 (expressing *AtSTP9* anti-sense control; ○) using an initial outside concentration of $100\ \mu\text{M}$ Glc at pH 5.5. Data of ScLEX8 and ScLEX12 represent mean values of four and three independent experiments, respectively (bars represent the SE); inset, the uptake rates for increasing concentrations of $[^{14}\text{C}]\text{Glc}$ were determined and used to calculate the K_m value according to Lineweaver-Burk. Data represent mean values of two independent measurements. The AtSTP9 transporter drives Glc-uptake with a K_m of $84 \pm 2.5\ \mu\text{M}$ and a maximum uptake rate (V_{max}) of $31 \pm 3.2\ \mu\text{mol h}^{-1}\ \text{mL}^{-1}$ packed cells (p.c.). B, Uptake rates of different monosaccharide substrates at an initial outside concentration of $100\ \mu\text{M}$ and inhibition of Glc-uptake by uncouplers (final concentration $50\ \mu\text{M}$) in *AtSTP9*-expressing yeast cells.

types of sink and source tissues using RT-PCR. Total RNA was isolated from 5- to 10-d-old seedlings, from leaves, stems, and roots of 3- to 6-week-old plants, and from flowers and fruits of mature plants. First-strand cDNA synthesis was performed using a poly(dT) primer and equal amounts of RNA from each tissue. With this cDNA, a PCR reaction was run with *AtSTP9*-specific primers. As shown in Figure 3A, only with cDNA from flowers an *AtSTP9*-specific PCR product be detected (confirmed by direct se-

quencing of the PCR product). As a control that equal amounts of cDNA were used, a PCR reaction with Actin1-specific primers was run in parallel (Fig. 3B). The fact that PCR products from contaminations with genomic DNA are much stronger for *AtSTP9* than for Actin is probably due to differences in the expression rates of both genes. *AtSTP9* is only weakly expressed, and 40 PCR cycles had to be run before an amplified product could be seen. Actin on the other hand is highly expressed in all tissues, and the majority of template is cDNA rather than genomic DNA.

The *AtSTP9* Promoter Drives Pollen-Specific Expression of Reporter Genes

To examine the *AtSTP9* expression profile in more detail, transgenic Arabidopsis plants were generated, which drive reporter gene expression under the control of the *AtSTP9* promoter. To this end, a fragment containing 1,085 bp of the *AtSTP9* promoter sequence and 1,147 bp of the *AtSTP9* structural gene sequence was cloned in front of the *uidA* gene (GUS) in the plant transformation vector pGPTV-BAR (Becker et al., 1992), yielding construct pLEX22 (Fig. 4A). The N-terminal part of the *AtSTP9* structural gene used in pLEX22 encodes 232 amino acids representing the first six transmembrane domains of AtSTP9, so that expression of this construct should result in a membrane-bound *AtSTP9*-GUS fusion protein with the GUS portion facing the cytosol. In parallel, a second construct was generated, in which the 1,085-bp fragment of the *AtSTP9* promoter without the *AtSTP9* structural gene sequence was cloned into a pGPTV-BAR derivative, in which the *uidA* gene was replaced by the green fluorescent protein (GFP) gene, yielding construct pLEX23 (Fig. 4B). Both constructs were used to transform Arabidopsis, and for each construct 30 independent lines were analyzed for reporter gene expression. As shown in Figure 5, GUS expression under the control of the *AtSTP9*

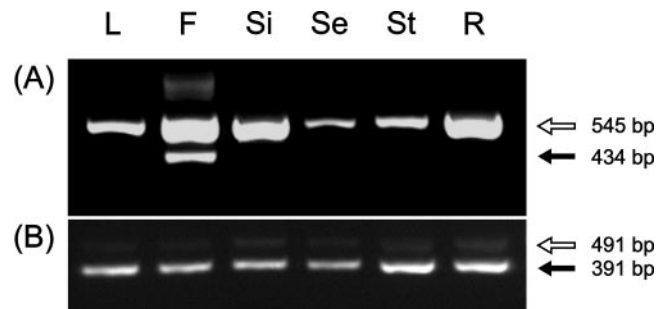


Figure 3. RT-PCR Analysis of *AtSTP9* expression in various tissues. A, *AtSTP9*-specific RT-PCR products in various Arabidopsis tissues (L, leaf; F, flower; Si, silique; Se, seedling; St, stem; R, root). Arrows indicate the size of PCR products derived from genomic DNA contaminations (white arrow) and from reverse-transcribed mRNA (black arrow). A specific product derived from *AtSTP9* cDNA can only be detected in RNA preparations from flowers. B, Control with Actin1-specific primers.

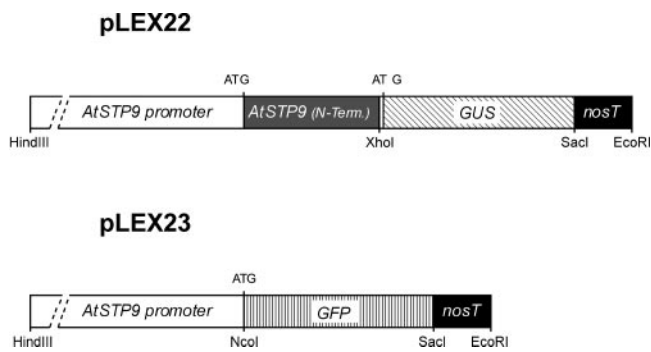


Figure 4. *AtSTP9* promoter/reporter constructs in pGTV-BAR. Top, The 4,433-bp *HindIII*/*EcoRI* fragment of clone pLEX22, which contains the *AtSTP9* genomic sequence including the *AtSTP9* promoter region and part of the *AtSTP9* gene, which in turn is fused to the *GUS* reporter gene. Bottom, The 2,122-bp *HindIII*/*EcoRI* fragment of clone pLEX23, which contains the *AtSTP9* promoter region fused to the *GFP* reporter gene.

promoter was only found in pollen. *GUS* activity first appeared in flowers of stage 10 (Bowman, 1994) and was detectable throughout pollen maturation up to its germination on the stigma (Fig. 5C). An identical expression profile could be observed in the *AtSTP9* promoter-*GFP* plants (Fig. 5, D–J). *GFP* expression in pollen started when flowers reached the developmental stage 10 and could be detected in isolated pollen grains (Fig. 5H) and in germinating pollen on the stigma (Fig. 5G). To further investigate the *AtSTP9* promoter activity in germinating pollen, we used an in vitro pollen germination assay. Here, the *GFP* fluorescence was strongly visible in pollen grains and in pollen tubes (Fig. 5J).

Localization of *AtSTP9* Protein in Pollen with an *AtSTP9*-Specific Antibody

To detect the *AtSTP9* protein on the cellular level, a specific antibody was raised against the last 31 amino acids of the *AtSTP9* protein, representing one of the most variable regions within the *AtSTP* proteins (Fig. 1). To this end, the *AtSTP9* C terminus was expressed in *Escherichia coli* as a fusion to the maltose-binding protein and was used to inject rabbits. Finally, the generated antiserum was affinity-purified against *AtSTP9* full-length protein expressed in yeast. To exclude cross-reactivity with other *AtSTP* proteins, the purified anti-*AtSTP9* antiserum was tested for specificity on western blots. As shown in Figure 6, a specific antibody reaction can only be observed in extracts from yeast cells expressing *AtSTP9* in sense orientation. The observed 43-kD band differs in size from the calculated mass of 56 kD for the *AtSTP9* protein, which is due to the hydrophobic nature of membrane proteins and has also been seen for previously characterized sugar transport proteins (Beyreuther et al., 1980; Sauer and Tanner, 1984; Sauer and Stadler, 1993; Truernit et al., 1999). No signals can be seen in extracts from yeast cells ex-

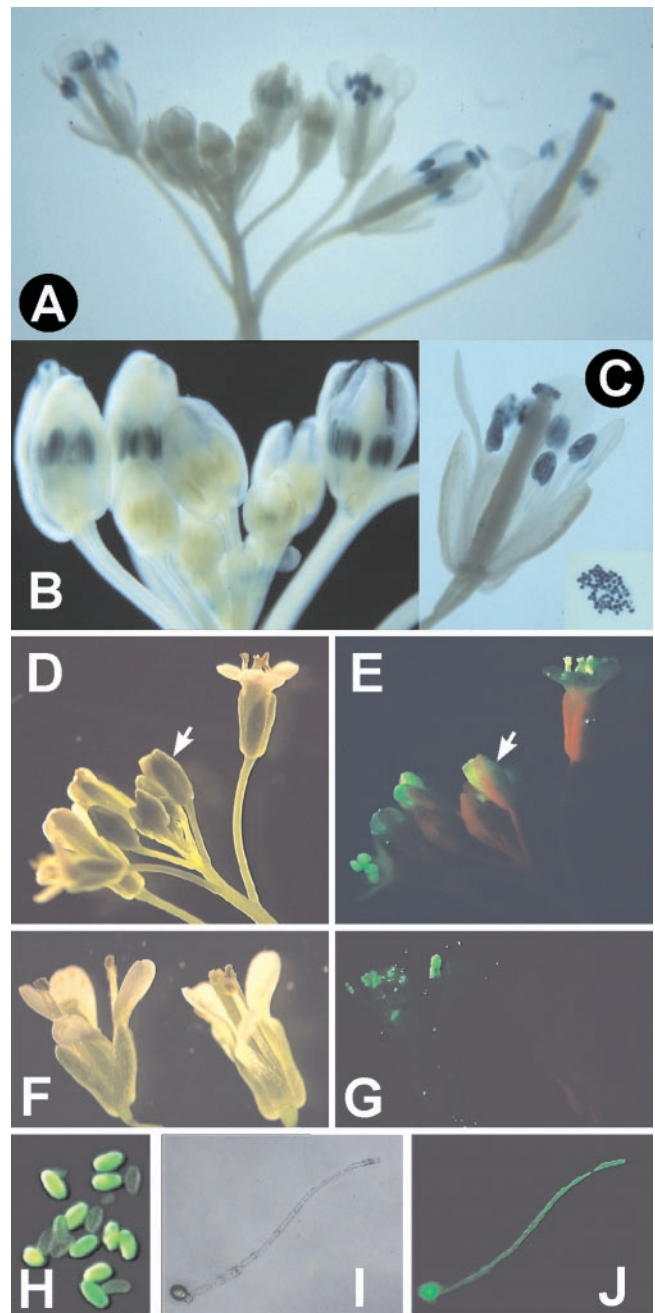


Figure 5. Expression of *GUS* and *GFP* reporter genes under the control of the *AtSTP9* promoter. A, Inflorescence stem with *GUS* histochemical staining. B, Inflorescence at higher magnification showing *GUS* expression starting from flower stage 10 (according to Bowman [1994]). C, Single flower showing *GUS* histochemical staining within anthers and on the stigma. Inset, When pollen grains and empty anthers are stained separately, *GUS* staining can be seen only in pollen grains. D, Inflorescence stem in white light. E, Inflorescence stem under blue excitation light. Arrows indicate a stage 10 flower, when *GFP* reporter expression is first seen. F, *AtSTP9* promoter/*GFP* plant (left) and wild-type Arabidopsis (right) in white light. G, *AtSTP9* promoter/*GFP* plant (left) and wild-type Arabidopsis (right) under blue excitation light. H, Isolated pollen grains under blue excitation light. I, In vitro germinated pollen in white light. J, In vitro germinated pollen under blue excitation light.

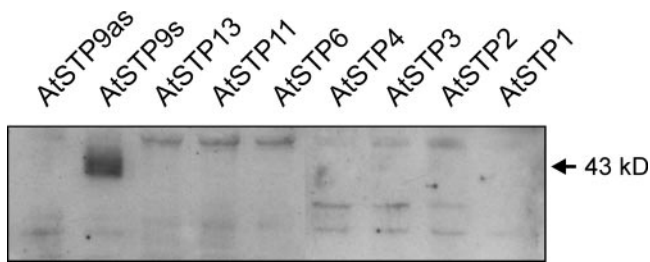


Figure 6. Test for cross-reactivity of the anti-AtSTP9 antiserum by western-blot analysis with total membrane preparations of yeast strains expressing AtSTPs. Membrane proteins from yeast cells expressing various AtSTPs were prepared, and 20 μ g of each preparation was separated on SDS-PAGE, blotted onto nitrocellulose, and incubated with anti-AtSTP9 antiserum. A specific 43-kD band was detected only in cells expressing *AtSTP9* in sense orientation (s) but was absent in cells expressing *AtSTP9* in antisense orientation (as) or other AtSTPs.

pressing *AtSTP9* in antisense orientation or other AtSTP genes (including all AtSTP genes expressed in pollen) in sense orientation (Fig. 6), reconfirming that the purified antiserum specifically recognizes AtSTP9 protein. This antiserum was further used to detect AtSTP9 protein in flowers of various developmental stages. As shown in Figure 7, AtSTP9 protein is only found in germinating pollen with the strongest signals in the pollen tube. The staining in cross sections of pollen tubes clearly demonstrates that the AtSTP9 monosaccharide transporter localizes to the plasma membrane and not to internal membranes (Fig. 7B, inset). No AtSTP9 protein was detectable in other flower organs. In some cases, we could also see AtSTP9-specific immunofluorescence in mature pollen grains attached to the stigma. Presumably, this pollen had already germinated, and the appendant pollen tube could not be seen in the thin section; or pollen germination was just initiated. However, AtSTP9 protein was absent in pollen of stage 10 flowers at the time when *AtSTP9* promoter activity started in GUS and GFP reporter plants. This suggests that pollen grains are preloaded with *AtSTP9* mRNA during maturation but that translation does not start until the pollen germinates on the stigma.

Identification and Analysis of a T-DNA Insertion Mutant of *AtSTP9*

To further analyze the physiological role of AtSTP9 in Arabidopsis pollen development, a knockout mutant line was isolated from a T-DNA mutagenized population of Arabidopsis (Knockout Facility at the University of Wisconsin, Madison). By DNA sequencing of PCR-amplified fragments, the insertion site of the T-DNA was determined at position +1,631 within the *AtSTP9* gene. Plants homozygous for the *Atstp9* allele were identified by PCR, and the absence of *AtSTP9* transcript was verified by RT-PCR (data not shown). We extensively analyzed this homozygous *AtSTP9* knockout line but could not find any

differences from wild-type plants. The *AtSTP9* knockout mutants were fertile and produced normal seeds, and light microscopic analyses could not detect any differences in pollen development, pollen size or pollen number, and in vitro pollen germination when compared with wild-type anthers (data not shown), suggesting that the *Atstp9* mutation does not interfere significantly with pollen viability, with pollen tube growth, or with pollen fertility.

DISCUSSION

In the results presented here, we describe the functional characterization and in planta localization of AtSTP9, a new member of the Arabidopsis monosaccharide transporter family. We have isolated the genomic clone and the cDNA of *AtSTP9*, which enabled us to study AtSTP9 transport activity in the

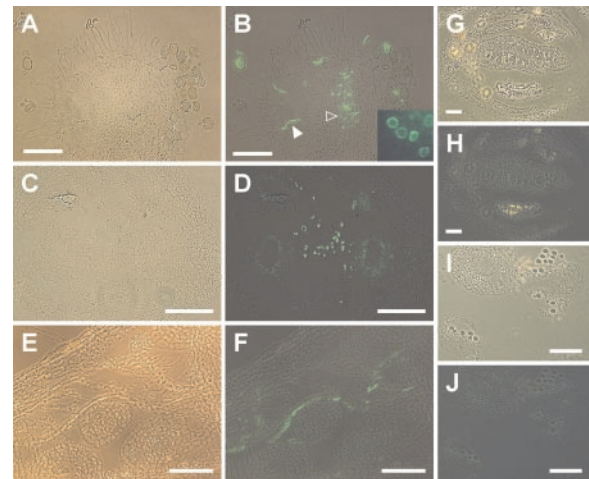


Figure 7. Immunolocalization of AtSTP9 protein in pollen tubes of Arabidopsis wild-type plants using affinity-purified anti-AtSTP9 antiserum. A, Cross section through the upper part of a pollinated style photographed under white light. B, Same section as in A stained with anti-AtSTP9 antiserum/fluorescein isothiocyanate (FITC) and photographed under blue excitation light. AtSTP9 protein is detected in pollen tubes that are either cut lengthwise (black arrowhead) or crosswise (white arrowhead) in this section. In some cases, AtSTP9 is also present in the pollen grain, presumably after the pollen germinates; inset, cross sections of pollen tubes. C, Cross section through the middle part of a pollinated pistil photographed under white light. D, Same section as in C stained with anti-AtSTP9 antiserum/FITC and photographed under blue excitation light. Stained pollen tubes can be seen in the central tissue. E, Longitudinal section of the lower part of a pollinated pistil photographed under white light. F, Same section as in E stained with anti-AtSTP9 antiserum/FITC and photographed under blue excitation light. AtSTP9 protein is detected in a longitudinally cut pollen tube. G, Longitudinal section through a flower bud photographed under white light. H, Same section as in G stained with anti-AtSTP9 antiserum/FITC and photographed under blue excitation light. I, Cross section through a flower showing open anthers with mature pollen grains photographed under white light. J, Same section as in I stained with anti-AtSTP9 antiserum/FITC and photographed under blue excitation light. No AtSTP9 protein is detected in pollen grains of early developmental stages (H) or in mature, not germinated pollen grains (J). Scale bars = 100 μ m.

heterologous yeast system and to follow *AtSTP9* expression in promoter/reporter plants. Considering the high degree of homology to the characterized AtSTPs (Fig. 1) and the similarities of their hydropathy profiles (data not shown), *AtSTP9* (At1g50310) clearly is a new member of the AtSTP family.

AtSTP9 Is a Putative Monosaccharide/H⁺ Symporter

Analysis of the transport activity in yeast demonstrates that *AtSTP9* is an active, energy-dependent monosaccharide transporter. The observed pH dependence as well as the inhibitory effect of uncouplers indicate that *AtSTP9* allows substrate accumulation by a proton symport mechanism. *AtSTP9* shows a K_m value for Glc of 84 μM , which is in the same micromolar range as the values found for the other characterized AtSTPs. However, *AtSTP1*, *AtSTP2*, *AtSTP3*, *AtSTP4*, and *AtSTP6* transport a broad spectrum of monosaccharides. In contrast, *AtSTP9* is the first Arabidopsis transporter that shows a high selectivity for Glc. It is not clear whether this Glc specificity of *AtSTP9* is of relevance for its in planta function during pollen development and/or germination.

AtSTP9 Is Expressed Exclusively in Pollen

The presented set of data clearly demonstrates that *AtSTP9* is exclusively expressed in pollen. RT-PCR analysis showed that the *AtSTP9* transcript is only found in flowers but not in other tissues like leaves (sink or source), stems, roots, or siliques or in seedlings. A more detailed expression profile was obtained by analyzing transgenic Arabidopsis plants expressing *AtSTP9*-promoter/GUS or *AtSTP9*-promoter/GFP fusions. In these plants, GUS activity or GFP fluorescence was exclusively found in pollen starting in stage 10 flowers and continuing until pollen germination. In an in vitro germination assay, GFP expression was also found in the pollen tube. Finally, to detect the *AtSTP9* protein, we generated an *AtSTP9*-specific antibody and examined thin sections of Arabidopsis flowers. In contrast to the early activity of the *AtSTP9* promoter found in promoter/reporter plants, the *AtSTP9* protein was not or only very weakly detectable in pollen grains but was very abundant in pollen tubes. A similar translational control has been found earlier for another monosaccharide transporter expressed in pollen, *AtSTP4* (R. Stadler, unpublished data). Also, there are other examples showing that mature ungerminated pollen contains a store of presynthesized mRNAs that are translated only upon germination (for review, see Mascarenhas, 1989). This store presumably comprises mRNAs that have to be available immediately at the rapid onset of metabolism upon contact with the papillae and rehydration. One example is a calcium-dependent calmodulin-independent protein kinase from maize (*Zea mays*; Estruch et al., 1994),

which was shown to be required for normal germination and pollen tube growth. Similarly, preloading of pollen with *AtSTP* mRNAs suggests an important function during the process of pollen germination. Like all *AtSTPs* characterized so far, *AtSTP9* is targeted to the plasma membrane. This was already suggested by the heterologous expression analyses in yeast and could be verified by immunolocalization in cross sections of pollen tubes (Fig. 7B, inset). Thus, the *AtSTP* family is clearly distinguished to the recently identified plastidic Glc transporter pGlcT (Weber et al., 2000), to which the *AtSTPs* show only little homology.

Possible Function of AtSTP9 during Pollen Development

Pollen development and maturation seem to require sugar supply during distinct phases. In early stages, *AtSTP2* facilitates the uptake of hexoses released during callose degradation within the pollen sac. Both *AtSTP2* mRNA and *AtSTP2* protein are only detectable at the beginning of callose degradation and microspore release from the tetrads and are no longer detectable after the mitotic divisions and the gametophyte formation or in mature pollen grains or pollen tubes (Truernit et al., 1999). *AtSTP6* expression is only found in stages 11 and 12 of pollen grain development (Scholz-Starke et al., 2003). Although the *AtSTP9* transcript can already be found in developing pollen grains, the *AtSTP9* protein is only detectable in pollen tubes, suggesting an important function of *AtSTP9* for the sugar supply of the growing pollen tube. Whether the Glc specificity of *AtSTP9* plays a role in germination or growth of pollen tube is not clear. However, in vitro germination experiments demonstrate that pollen germination is strictly dependent on Suc in the medium. Suc could directly be taken up by *AtSUC1*, a Suc/H⁺ symporter, which was also identified in pollen tubes (Stadler et al., 1999). Alternatively, the Suc can be cleaved by a pollen-specific invertase, and the resulting hexoses might be imported. Such a pollen-specific invertase was recently identified in tomato (*Lycopersicon esculentum*; Goetz et al., 2001). Interestingly, the addition of Glc to germinating pollen leads to an immediate burst of the pollen tubes (Lemoine et al., 1999). This could be due to a lack of stabilizing surrounding tissue but could also indicate that disaccharides and monosaccharides have different functions during germination, where one serves as a signal (e.g. in pollen tube guidance) and the other as metabolite. One example for such a regulation mediated by the supply of different sugars is the seed development in broad bean (*Vicia faba*). Here, it was shown that hexoses are required for the division phase, whereas Suc initiates the storage phase (Weber et al., 1997). In this respect, the expression of two monosaccharide transporters, *AtSTP4* and

AtSTP9, in pollen tubes suggest that sugar uptake into this cell type is a highly regulated process. The expression of several sugar transporter genes in pollen and the absence of phenotypes in single knockouts of these genes (*AtSTP6*; Scholz-Starke et al., 2003; *AtSTP9*; this work) further stress the importance of sugar supply during this essential stage in plant reproduction. Future experiments involving the analysis of double/multiple knockout mutants will be directed toward unraveling the distinct role of monosaccharide transporters during pollen development, germination, and pollen tube growth.

MATERIALS AND METHODS

Strains and Growth Conditions

Escherichia coli strain DH5 α (Hanahan, 1983) was used for cloning. Brewer's yeast (*Saccharomyces cerevisiae*) strain EBY.VW4000 (kindly provided by E. Boles, University of Düsseldorf, Germany) was used for heterologous expression. The *hxt* null mutant EBY.VW4000 was grown on maltose, and yeast transformation was performed as described (Gietz et al., 1992). Arabidopsis C24 was grown in the greenhouse in potting soil or on agar medium in growth chambers under a 16-h-light/8-h-dark regime at 22°C and 55% relative humidity as previously described (Truernit and Sauer, 1995). Transformations of Arabidopsis were performed with *Agrobacterium tumefaciens* strain GV3101 (Holsters et al., 1980).

Isolation of AtSTP9 Genomic and Full-Length cDNA Sequences

Screening of 250,000 plaque forming units of a genomic library from Arabidopsis (strain Columbia wild type in λ GEM11; generated by J.T. Mulligan and R.W. Davis, Department of Biochemistry, Stanford, CA; supplied by the European Economic Community Arabidopsis stock center, Köln, Germany) was performed using a radiolabeled *AtSTP9*-specific 204-bp PCR fragment encoding bases +1,222 to +1,425 of the coding region. The 3,386-bp *EcoRI* fragment of the Lambda insert containing the *AtSTP9* promoter and gene sequences was cloned into the vector pUC19 yielding the clone pAS91. A full-length *AtSTP9* cDNA clone was isolated by RT-PCR using primers STP9cs5' (5'-CTA GGA ATT CAT GGC TGG AGG AGC CTT TGT ATC AGA AGG-3') and STP9cs3' (5'-CTA GGC GGC CGC GGC TCT TCA AAC CTC CTT GAC G-3') and total RNA from flower tissues. The PCR product was cloned into the vector pGEM-T Easy (Promega), yielding the plasmid pLEX13.

Functional Characterization of AtSTP9 by Heterologous Expression

The insert of the *AtSTP9* cDNA clone pLEX13 was ligated into the unique *NotI* site of the Brewer's yeast/*E. coli* shuttle vector pEX-Tag (Meyer et al., 2000), in both sense and antisense orientation, yielding constructs pACH91s and pACH91a, respectively. Constructs pACH91s and pACH91a were then used for transformation of Brewer's yeast strain EBY.VW4000 that carries mutations in all 18 endogenous hexose transporter genes (Wieczorke et al., 1999), yielding strains ScLEX8 and ScLEX12, respectively. For uptake experiments with ¹⁴C-labeled sugars, all strains were precultured on maltose-casamino acids medium (0.67% [w/v] yeast nitrogen base, 1% [w/v] casamino acids, 0.002% [w/v] Trp, and 2% [w/v] maltose) to an A_{600} of 1, and transport tests were performed as described by Sauer and Stadler (1993).

RNA Isolation and RT-PCR

Total RNA was isolated as described by Sauer et al. (1990). RT-PCR was performed according to the supplier's instructions (MBI Fermentas, St. Leon-Rot, Germany). In brief, in a 12- μ L reaction volume, 5 μ g of total RNA and 0.5 μ g of oligo(dT)₁₂₋₁₈ were heated to 70°C for 10 min and quickly chilled on ice. After the addition of 4 μ L of 5 \times first-strand buffer, 2 μ L of 0.1 M dithiothreitol, and 1 μ L of 10 mM dNTP-Mix, the mixture was incubated at 42°C for 2 min, 1 μ L (200 units) of Moloney murine leukemia virus

reverse transcriptase was added, and after further incubation for 50 min at 42°C, the reaction was heat-inactivated at 70°C for 15 min. The cDNA (0.5 μ L) was then used as a template for amplification in PCR with primers STP9cs5' (CTA GGA ATT CAT GGC TGG AGG AGC CTT TGT ATC AGA AGG) and pas91-6 (GCA ACA ATC TAC CAA CGA TGA GC) to detect the *AtSTP9* transcript or with primers AtACT1-5' (GCG ATG AAG CTC AAT CCA AAC GAG G) and AtACT1-3' (GGT CAC GAC CAG CAA GAT CAA GAC G) to detect the Actin transcript.

Localization of AtSTP9 Expression by Reporter Gene Analyses

A 2,232-bp fragment containing 1,085 bp of the *AtSTP9* promoter sequence and 1,147 bp of the *AtSTP9* gene sequence was cloned in front of the *uidA* gene (GUS) in the plant transformation vector pGPTV-BAR (Becker et al., 1992), yielding construct pLEX22. To generate a promoter/GFP fusion construct, a PCR fragment containing 1,085 bp of the *AtSTP9* promoter upstream of the start ATG was amplified using primers STP9p5' (5'-CTA GGA ATT CGG ATC CCG ATG GTT AAT TAC G-3') and STP9startNcoI_r&c (5'-GGC TCC TCC AGC CAT GGT TAT TTA TTC TTC-3'). The PCR product was digested with *Bam*HI and *Nco*I and was cloned into pEP/pUC19 (Imlau et al., 1999) in which the *AtSUC2* promoter fragment had been removed, yielding plasmid pLEX7. After verification of the sequence, a 2,122-bp fragment containing the *AtSTP9* promoter and the GFP gene was used to replace the GUS gene sequence in the vector pGPTV-BAR (Becker et al., 1992), yielding plasmid pLEX23. Arabidopsis plants were transformed with *A. tumefaciens* harboring the constructs pLEX22 or pLEX23 using the floral dip method according to the protocol of Clough and Bent (1998). Transformation of Arabidopsis with pLEX22 and pLEX23 resulted in 30 and 35 independent basta-resistant transformants, respectively. Reporter gene activity was studied in all of these plants in the T₁ generation. For GFP analysis in pollen tubes, in vitro germination of pollen was as described by Stadler et al. (1999).

Computer Analysis

Splice site prediction was performed with the programs GENSCAN (<http://genome.dkfz-heidelberg.de/cgi-bin/GENSCAN/genSCAN.cgi>) and NetGene2 (<http://www.cbs.dtu.dk/services/NetGene2>) using default parameters.

Generation of Anti-AtSTP9 Antisera, Affinity Purification of the Serum, and Immunolocalization of AtSTP9 Protein

A 108-bp DNA fragment was PCR-amplified from plasmid pAS91 using the oligonucleotides STP9-Cter5E (5'-TGA AGA ATT CGG AAG AGT ATG GAA GCA ACA TC-3') and STP9cs32 (5'-CTA GGA ATT CGG CTC TTC AAA CCT CCT TGA CG-3'). The PCR product was cloned into the *EcoRI* restriction site of the pMal-c2 vector (New England Biolabs, Schwalbach/Taunus, Germany) resulting in a translational fusion of the maltose-binding protein and the last 31 amino acids of AtSTP9. Expression of this fusion protein was induced by the addition of 0.3 mM isopropylthiogalactosid for 30 min at 37°C. After separation of *E. coli* protein extracts on preparative polyacrylamide gels, the fusion protein band was excised, eluted, lyophilized, and used for the immunization of two rabbits (Pineda, Berlin). To test the quality of the antisera, western blots with protein extracts of ScLEX8 and ScLEX12 (antisense control) were performed (data not shown). ScLEX8 plasma membrane proteins (400 μ g) were blotted onto nitrocellulose, and the excised AtSTP9 protein band was used to affinity-purify 500 μ L of serum as described previously (Sauer and Stadler, 1993). For immunohistochemical analyses, semithin sections (2–4 mm) from methacrylate-embedded Arabidopsis flower tissue were incubated with affinity-purified anti-AtSTP9 antiserum and stained with anti-rabbit IgG-FITC-isomer 1-conjugate as described (Stadler et al., 1995).

Identification of a T-DNA-Tagged AtSTP9 Knockout Line of Arabidopsis

A population of 60,400 independent kanamycin-resistant T-DNA insertion lines was screened at the Arabidopsis Knockout Facility at the University of Wisconsin Biotechnology Center (<http://www.biotech.wisc.edu/Arabidop->

sis) following the method described by Krysan et al. (1996, 1999). PCR reactions were performed with the T-DNA border primer JL-202 (5'-CAT TTT ATA ATA ACG CTG CGG ACA TCT AC-3') and the gene-specific primers AtSTP9g+2239r (5'-ATA AGG CAG CAA GAT GTG AGA ATC TGC AG-3') and AtSTP9g-816f (5'-CTA TGT GTT CTT CGT CCA GCT CAT TGT TA-3'). PCR reactions were tested for *AtSTP9*-specific products by Southern analysis using a 3,055-bp probe (region -816 to +2239 of the *AtSTP9* gene). The sequence of hybridizing PCR products was determined using the T-DNA border primer JL-270 (5'-TTT CTC CAT ATT GAC CAT CAT ACT CAT TG-3') to reconfirm the T-DNA insertion. Plants homozygous for the *Atstp9* allele were identified because they did not show a PCR product with genomic DNA and primers flanking the insertion site (AtSTP9g-816f and AtSTP9g+2239r). To verify the absence of *AtSTP9* mRNA in these *AtSTP9* knockout lines, RT-PCR analysis was performed with total RNA from flowers of knockout and wild-type lines (ecotype Wassilewskija) using *AtSTP9*-specific primers AtSTP9cs5' (5'-CTA GGA ATT CAT GGC TGG AGG AGC CTT TGT ATC AGA AGG-3') and either AtSTP9c+695r (5'-CCA TGG CTT CGA GCA TCG AGT TTG GTG TAT CGG G-3') or AtSTP9g+2125r (5'-CCA TGG AAA CCT CCT TGA CGT AGT TTT CTT CAC-3').

ACKNOWLEDGMENTS

We thank Dr. Norbert Sauer for his continuous support, for helpful discussions, and for critical reading of the manuscript. We also thank Gudrun Steingraber and Silke Oppelt for excellent experimental help and Eckhard Boles for providing the yeast strain EBY.VW4000.

Received May 9, 2003; returned for revision June 10, 2003; accepted June 17, 2003.

LITERATURE CITED

- Becker D, Kemper E, Schell J, Masterson R (1992) New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol Biol* 20: 1195-1197
- Beyreuther K, Bieseler B, Ehring R, Griesser HW, Mieschendahl M, Müller-Hill B, Triesch I (1980) Investigation of structure and function of lactose permease of *Escherichia coli*. *Biochem Soc Trans* 8: 675-676
- Boorer KJ, Forde BG, Leigh RA, Miller AJ (1992) Functional expression of a plant plasma membrane transporter in *Xenopus* oocytes. *FEBS Lett* 302: 166-168
- Boorer KJ, Loo DDF, Wright EM (1994) Steady-state and presteady-state kinetics of the H⁺/hexose cotransporter (STP1) from *Arabidopsis thaliana* expressed in *Xenopus* oocytes. *J Biol Chem* 269: 20417-20424
- Bowman J, editor (1994) *Arabidopsis*, An Atlas of Morphology and Development. Springer-Verlag, New York
- Büttner M, Sauer N (2000) Monosaccharide transporters in plants: structure, function and physiology. *Biochim Biophys Acta* 1465: 263-274
- Büttner M, Truernit E, Baier K, Scholz-Starke J, Sontheim M, Lauterbach C, Huss VAR, Sauer N (1999) AtSTP3, a green leaf-specific, low affinity monosaccharide-H⁺ symporter of *Arabidopsis thaliana*. *Plant Cell Environ* 23: 175-184
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16: 735-743
- Estruch JJ, Kadwell S, Merlin E, Crossland L (1994) Cloning and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase. *Proc Natl Acad Sci USA* 91: 8837-8841
- Gietz D, St Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* 20: 1425
- Goetz M, Godt DE, Guivarc'h A, Kahmann U, Chriqui D, Roitsch T (2001) Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. *Proc Natl Acad Sci USA* 98: 6522-6527
- Hanahan D (1983) Studies on transformation of *E. coli* with plasmids. *J Mol Biol* 166: 557-580
- Holsters M, Silva B, van Vliet F, Genetello C, De Block M, Dhaese P, Depicker A, Inzé D, Engler G, Villarroel R et al. (1980) The functional organization of the nopaline *Agrobacterium tumefaciens* plasmid pTiC58. *Plasmid* 3: 212-230
- Imlau A, Truernit E, Sauer N (1999) Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. *Plant Cell* 11: 309-322
- Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell* 11: 2283-2290
- Krysan PJ, Young JC, Tax F, Sussman MR (1996) Identification of transferred DNA insertions within *Arabidopsis* genes involved in signal transduction and ion transport. *Proc Natl Acad Sci USA* 93: 8145-8150
- Kühn C, Franceschi VR, Schulz A, Lemoine R, Frommer W (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. *Science* 275: 1298-1300
- Lemoine R, Bürkle L, Barker L, Sakr S, Kühn C, Regnacq M, Gaillard C, Delrot S, Frommer WB (1999) Identification of a pollen-specific sucrose transporter-like protein NtSUT3 from tobacco. *FEBS Lett* 454: 325-330
- Maddison AL, Hedley PE, Meyer RC, Aziz N, Davidson D, Machray GC (1999) Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. *Plant Mol Biol* 41: 741-751
- Mascarenhas JP (1989) The male gametophyte of flowering plants. *Plant Cell* 1: 657-664
- Meyer S, Melzer M, Truernit E, Hümmer C, Besenbeck R, Stadler R, Sauer N (2000) AtSUC3, a gene encoding a new *Arabidopsis* sucrose transporter, is expressed in cells adjacent to the vascular tissue and in a carpel cell layer. *Plant J* 24: 869-882
- Riesmeier JW, Willmitzer L, Frommer WB (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. *EMBO J* 11: 4705-4713
- Sauer N, Friedländer K, Gräml-Wicke U (1990) Primary structure, genomic organization and heterologous expression of a glucose transporter from *Arabidopsis thaliana*. *EMBO J* 9: 3045-3050
- Sauer N, Stadler R (1993) A sink specific H⁺/monosaccharide cotransporter from *Nicotiana tabacum*: cloning and heterologous expression in baker's yeast. *Plant J* 4: 601-610
- Sauer N, Tanner W (1984) Partial purification and characterization of inducible transport proteins of *Chlorella*. *Z Pflanzenphysiol* 114: 367-375
- Scholz-Starke J, Büttner M, Sauer N (2003) AtSTP6, a new pollen-specific H⁺-monosaccharide symporter from *Arabidopsis*. *Plant Physiol* 131: 70-77
- Sherson SM, Hemmann G, Wallace G, Forbes S, Germain V, Stadler R, Bechtold N, Sauer N, Smith SM (2000) Monosaccharide/proton symporter AtSTP1 plays a major role in uptake and response of *Arabidopsis* seeds and seedlings to sugars. *Plant J* 24: 849-857
- Stadler R, Brandner J, Schulz A, Gahrz M, Sauer N (1995) Phloem loading by the PmSUC2 sucrose carrier from *Plantago major* occurs into companion cells. *Plant Cell* 7: 1545-1554
- Stadler R, Büttner M, Ache P, Hedrich R, Ivashikina N, Melzer M, Sherson SM, Smith SM, Sauer N (2003) Diurnal and light-regulated expression of *AtSTP1* in guard cells of *Arabidopsis*. *Plant Physiol* (in press)
- Stadler R, Sauer N (1996) The *Arabidopsis thaliana* AtSUC2 gene is specifically expressed in companion cells. *Bot Acta* 109: 299-306
- Stadler R, Truernit E, Gahrz M, Sauer N (1999) The AtSUC1 sucrose carrier may represent the osmotic driving force for anther dehiscence and pollen tube growth in *Arabidopsis*. *Plant J* 19: 269-278
- Truernit E, Sauer N (1995) The promoter of the *Arabidopsis thaliana* SUC2 sucrose-H⁺ symporter gene directs expression of β -glucuronidase to the phloem: evidence for phloem loading and unloading by SUC2. *Planta* 196: 564-570
- Truernit E, Schmid J, Eppe P, Illig J, Sauer N (1996) The sink-specific and stress regulated *Arabidopsis* STP4 gene: enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors and pathogen challenge. *Plant Cell* 8: 2169-2182
- Truernit E, Stadler R, Baier K, Sauer N (1999) A male gametophyte-specific monosaccharide transporter in *Arabidopsis*. *Plant J* 17: 191-201
- Weber A, Servaites JC, Geiger DR, Kofler H, Hille D, Groner F, Hebbeker U, Flügge UI (2000) Identification, purification, and molecular cloning of a putative plastidic glucose translocator. *Plant Cell* 12: 787-802
- Weber H, Borisjuk L, Heim U, Sauer N, Wobus U (1997) A role for sugar transporters during seed development: molecular characterization of a hexose and a sucrose carrier in fava bean seeds. *Plant Cell* 9: 895-908
- Wieczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg CP, Boles E (1999) Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Lett* 464: 123-128
- Ylstra B, Garrido D, Busscher J, van Tunen AJ (1998) Hexose transport in growing petunia pollen tubes and characterization of a pollen-specific, putative monosaccharide transporter. *Plant Physiol* 118: 297-304
- Zheng ZL, Yang Z (2000) The Rop GTPase switch turns on polar growth in pollen. *Trends Plant Sci* 5: 298-303