Identification and Characterization of AtSTP14, a Novel Galactose Transporter from Arabidopsis

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AtSTP14, a new Arabidopsis sugar transporter, was identified and characterized on the molecular and physiological level. Reverse transcriptase–PCR analyses and reporter plants demonstrate high AtSTP14 expression levels in the seed endosperm and in cotyledons, as well as in green leaves. Thus, unlike previously characterized monosaccharide transporters, AtSTP14 is expressed in both source and sink tissues and represents the first monosaccharide transporter in the female gametophyte. Heterologous expression in yeast revealed that AtSTP14 does not transport glucose or fructose, but is the first plant transporter specific for galactose. Interestingly, AtSTP14 expression is regulated by factors which also induce cell wall degradation such as extended dark periods or changes in the sugar level, i.e. AtSTP14 is induced 3-fold by 24 h darkness and repressed 3-fold by 2% glucose and 2% sucrose. Two independent AtSTP14 mutant lines were identified, but no effect on seed development or other differences during growth under normal conditions could be observed. A putative role for AtSTP14 in the recycling of cell wall-derived galactose during normal conditions could be observed. A putative role for AtSTP14 in the recycling of cell wall-derived galactose during different developmental processes is discussed.

Keywords: Arabidopsis thaliana • AtSTP14 • Cell wall recycling • Galactose transporter • Sugar transport • Saccharomyces cerevisiae.

Abbreviations: CCCP, carbonyl cyanide-m-chlorophenyl-hydrazone; CLSM, confocal laser scanning microscopy; GFP, green fluorescent protein; GUS, β-glucuronidase; HPAE-PAD, high performance anion-exchange chromatography with pulsed amperometric detection; MS medium, Murashige and Skoog medium; MST, monosaccharide transporter; 3-OMG, 3-O-methylglucose; ORF, open reading frame; PCMBS, p-chloromercuribenzenesulfonic acid; STP, sugar transport protein.

Introduction

The allocation of carbohydrates is an important aspect in plant metabolism. For long-distance as well as subcellular distribution of sugars, transport steps across several membranes are necessary. At the site of photosynthesis, sugars are transported at the subcellular level for transient storage in the chloroplast or vacuole. On the other hand, photosynthetically inactive sink tissues are supplied with carbohydrates via the phloem and, in many plants species including Arabidopsis, the main transport sugar is sucrose, which can be taken up into sink cells by SUC/SUT-type transporters (Sauer 2007). Alternatively, sucrose can be broken down by an invertase in the apoplastic space and the generated monosaccharides glucose and fructose are taken up by monosaccharide transporters (MSTs) (Büttner and Sauer 2000). The Arabidopsis genome comprises a superfamily of MST-like transporter genes consisting of seven subgroups (Büttner et al. 2000, Büttner and Sauer 2000, Büttner 2007). The family of sugar transport proteins (AtSTPs) consists of 14 genes, eight of which have been investigated quite extensively (Büttner 2007).

Most of the AtSTP genes characterized to date show ‘sink’-specific expression patterns (Truernit et al. 1996, Truernit et al. 1999, Sherson et al. 2000, Schneidereit et al. 2003, Scholz-Starke et al. 2003, Stadler et al. 2003, Schneidereit et al. 2005). Surprisingly, five of these genes, AtSTP2, AtSTP4, AtSTP6, AtSTP9 and AtSTP11, are expressed in pollen during different developmental stages. AtSTP2 is expressed early during pollen maturation (Truernit et al. 1999), and both AtSTP2 mRNA and AtSTP2 protein are found during callose degradation and microspore release from the tetrads (Truernit et al. 1999). AtSTP6 expression is confined to the latest stage in pollen development (flower stages 11 and 12; Bowman 1993) during formation of the exine when the pollen grain is fully developed (Scholz-Starke et al. 2003). In contrast to AtSTP2 and AtSTP6, a different regulation was found for AtSTP4, AtSTP9 and AtSTP11. While the mRNA of these AtSTP genes was already found during early pollen development, the AtSTP proteins could be detected by specific antibodies only after pollen hydration and germination in the pollen grains and/or tubes (Schneidereit et al. 2003, Schneidereit et al. 2005). Such pre-loading of pollen with transcripts of genes essential for pollen germination and/or pollen tube growth has been described earlier (Mascarenhas 1989). However, the large number of functionally redundant MSTs in pollen is most probably the reason why mutants in single AtSTP genes are not impaired in pollen development, germination or fertility. While five AtSTPs have been found in pollen,
none of the AtSTPs has been shown to play a role in female gametophyte development so far.

AtSTP expression in other sinks has been found for AtSTP1 in germinating seeds (Sherson et al. 2000), roots (Sauer et al. 1990, Sherson et al. 2003) and guard cells (Stadler et al. 2003), for AtSTP4 in root tips (Truernit et al. 1996) and for AtSTP13 in the vascular tissue of petals (Norholm et al. 2006). The only AtSTP showing expression in source tissues under normal conditions is AtSTP3, which is found in all green leaves (Büttner et al. 2000). Certain conditions can also induce AtSTP expression in leaves, for instance AtSTP3 and AtSTP4, which are responsive to wounding and pathogen attack (Truernit et al. 1996, Büttner et al. 2000, Fotopoulos et al. 2003) or AtSTP13, which is induced during programmed cell death upon treatment with a fungal toxin or pathogen (Norholm et al. 2006).

All known AtSTPs transport a broad spectrum of monosaccharide substrates including the hexoses glucose, galactose and mannose, and the pentose xylose (but not ribose) as well as the non-metabolized 3-0-methylglucose (3-OMG). Interestingly, fructose, which is generated to equimolar amounts to glucose during the cleavage of sucrose, is transported only by AtSTP6 (Scholz-Starke et al. 2003) and AtSTP13 (Norholm et al. 2006), and to a lesser extent by AtSTP1 (Sauer et al. 1990; Boorer et al. 1994). Notably, the only AtSTP not accepting a broad range of substrates is AtSTP9, which is highly specific for glucose (Schneiderereit et al. 2003).

Plants partition their fixed carbon into storage pools such as plastidic starch or vacuolar sugars. However, another major resource for carbohydrates is the recycling of the structural carbohydrate contents in the cell wall. A recent analysis of the sugar composition of cell wall polysaccharides in Arabidopsis leaves showed that the main monosaccharide components are glucose, xylose, galactose, rhamnose, arabinose, mannose and fucose (Lee et al. 2007). During certain circumstances such as prolonged darkness, sugar starvation or leaf senescence, these cell wall sugars are re-mobilized by the activity of glycosyl hydrolases such as β-galactosidase (Lee et al. 2007) and thus can be re-imported into the cell for further metabolism. However, no hexose transporter potentially mediating the uptake of cell wall-derived galactose has been identified so far.

Here, we report the molecular identification and physiological characterization of AtSTP14, a previously undescribed member of the AtSTP family, which is different from the other AtSTPs due to its specificity for galactose and its ubiquitous expression in source and sink tissues, with the highest levels in the endosperm. A potential role for AtSTP14 in the recycling of the cell wall is discussed.

**Results**

The AtSTP family is the best investigated subgroup within the MST (-like) genes in Arabidopsis (Büttner and Sauer 2000, Büttner et al. 2000, Büttner 2007). Previous screenings have identified 14 highly homologous AtSTP genes, several of which have been characterized in detail (reviewed in Büttner 2007).

One of these genes, AtSTP14, was identified on chromosome 1 (coordinates 29,013,833–29,015,984 bp, reverse; BacT14N5), and database annotation (TAIR, The Arabidopsis Information Resource) suggested an AtSTP14 open reading frame (ORF) of 1,512 bp, interrupted by three putative introns. To investigate this putative transporter further, we amplified the full-length AtSTP14 cDNA by reverse transcription–PCR (RT–PCR) using total RNA from leaves and gene-specific primers. DNA sequencing of the 1,675 bp product ruled out PCR mistakes and confirmed the position of the three predicted introns. According to the computational analysis (TAIR; UniProtKB), the encoded AtSTP14 protein consists of 504 amino acids, for which a mol. wt of 55385.8 and an isoelectric point of 8.8 can be calculated. Furthermore, the AtSTP14 protein contains one consensus sequence motif for potential N-glycosylation at position 73 (NET), located in the first extracellular loop of the predicted 2D structure. The high degree of sequence conservation of the AtSTP family (57–94% similarity and 41–87% identity) and the intron positions of AtSTP14, which are also conserved within the AtSTPs, are depicted in Supplementary Fig. S1. According to the InterPro database (http://www.ebi.ac.uk/interpro), the AtSTP14 protein contains the domains IPR003663 (Sugar/insolot transporter), IPR005828 (General substrate transporter) and IPR007114 (Major facilitator superfamily). The determined AtSTP14 cDNA sequence (GenBank accession No. AJ344334.1) is confirmed by the predicted annotation for the AtSTP14 gene locus At1g77210.

**AtSTP14 expression pattern in Arabidopsis**

To investigate the AtSTP14 gene expression in different parts of the plant, RT–PCR was performed using AtSTP14-specific primers and cDNA from RNA of several Arabidopsis tissues. As shown in Fig. 1, a specific AtSTP14 transcript is detectable in all tissues tested, most prominent in leaves.

**Fig. 1** Quantitative RT–PCR analysis of AtSTP14 expression in different tissues. AtSTP14-specific RT–PCR products were obtained with cDNA preparations from Arabidopsis source leaves, roots and stems of 4-week-old plants, and green silique and flowers of 6-week-old plants. Bars represent mean values (three independent samples ±SD) relative to the AtSTP14 expression in leaves, which was set to 1. The AtSTP14 transcript level was normalized against the transcript level of the reference gene LDL3 in each sample.
and siliques. In order to determine the AtSTP14 expression sites in more detail, we generated transgenic Arabidopsis lines expressing the green fluorescent protein (GFP) under the control of the AtSTP14 promoter. As shown in Fig. 2, GFP fluorescence in these lines was only detectable in the seed endosperm (Fig. 2I, J), most prominent in the outer cell layer (Fig. 2K).

To detect AtSTP14 promoter activity also in the other tissues identified by RT–PCR, we used the β-glucuronidase (GUS) gene as a more sensitive reporter. Transgenic Arabidopsis lines expressing the GUS gene under the control of the AtSTP14 promoter displayed reporter activity in seeds and rosette leaves as well as in cotyledons and trichomes (Fig. 2A–F).

Fig. 2 Analyses of AtSTP14 promoter activity in different tissues and subcellular localization of an AtSTP14–GFP fusion protein. Expression of GUS and GFP reporter genes under the control of the AtSTP14 promoter. (A) An inflorescence stem with GUS histochemical staining showing GUS expression in seeds starting from floral stage 14 (according to Bowman, 1993). (B) A silique at higher magnification at floral stage 16–17 showing GUS staining in seeds. (C) A 3-day-old seedling with strong GUS staining in cotyledons. (D) A young plant (six-leaf stage) grown on a plate showing GUS expression in leaves. (E) A rosette leaf of a 4-week-old plant. (F) Close-up of GUS staining in trichomes. (G) Roots showing no GUS expression except in the root tip (H). (I) A fertilized ovule at flower stage 14–15 showing GFP fluorescence in the endosperm photographed with white and blue excitation light (merged) or with excitation light only (J). (K) Confocal image showing GFP expression in the outer layers of the endosperm. Plasma membrane localization of the AtSTP14–GFP fusion transiently expressed in N. benthamiana (L, M) and A. thaliana (N) by CLSM (red, Chl autofluorescence; green, GFP fluorescence). Scale bars: 2 mm in A, B and E; 1 mm in C and D; 500 µm in F and G; 100 µm in H–J; 40 µm in K–M; 5 µm in (N).
Deviating from Genvestigator data, which indicate a weak but ubiquitous AtSTP14 expression in roots, we observed GUS activity only in the root tip (Fig. 2G, H). Taken together, a nearly ubiquitous expression of AtSTP14 was verified, with the highest levels in leaves and siliques, demonstrating that AtSTP14 is not, like most other AtSTP genes, expressed exclusively in sink tissues.

### Subcellular localization of AtSTP14

To exclude a possible localization of AtSTP14 in internal membranes, we transiently expressed an AtSTP14–GFP fusion (pGP14-1) in Nicotiana benthamiana and Arabidopsis thaliana leaves. Confocal laser scanning microscopy (CLSM) analysis revealed that in both plant species the Chl autofluorescence of the chloroplasts (red) is found inside the ring of GFP fluorescence (green), clearly demonstrating plasma membrane localization of the AtSTP14 protein (Fig. 2L–N).

### Functional characterization of AtSTP14 by heterologous expression in yeast

To determine the AtSTP14 transport properties, we cloned the AtSTP14 cDNA into the vector NEV-N (Sauer and Stolz 1994) and expressed it in the hexose transport-deficient yeast mutant EBY.VW4000 (Wieczorke et al. 1999). Since all AtSTPs characterized so far are glucose/H⁺ symporters, we also tested the AtSTP14-expressing strain BHY3s for the ability to accumulate [14C]glucose. However, glucose uptake was only marginally higher compared with the control strain expressing the empty vector (data not shown). Interestingly, when we tested other monosaccharide substrates, a strict preference for galactose was observed, while glucose and fructose are barely accepted (Fig. 3A, B). In addition, we could not detect significant inhibition of galactose uptake by adding a 10-fold excess of mannose, ribose or 3-OMG (data not shown). This distinguishes AtSTP14 from the other AtSTPs, which can transport a broad spectrum of monosaccharides (Büttner 2007).

The Km of AtSTP14 for galactose was measured to be 529 µM (Fig. 3A inset) and the transport rate was significantly decreased by the proton uncoupler CCCP (carbonyl cyanide-m-chlorophenylhydrazone) but not by the sulfhydryl agent PCMBS (p-chloromercuribenzenesulfonic acid) (Fig. 3B), indicating that AtSTP14, like all other tested AtSTPs, is not sensitive to disulfide bond cleavage, and galactose uptake via AtSTP14 is driven by a proton gradient across the plasma membrane. This localization is further supported by a recent study analyzing the phosphoproteome, clearly identifying AtSTP14 as a plasma membrane protein (Benschop et al. 2007).

Consistent with the uptake experiments, growth of the AtSTP14-expressing strain BHY3s (expressing the vector control; open circles) using an initial outside concentration of 100 µM galactose at pH 5.5, inset, the uptake rates for increasing concentrations of [14C]galactose were determined 2 min after substrate addition and used to calculate the Km value according to Lineweaver–Burk. The plot of a typical Km determination is presented. In six independent measurements, a Km value of 529 µM and a maximum uptake rate (Vmax) of 4.1 µmol h⁻¹ ml⁻¹ packed cells (p.c.) was determined for the galactose uptake driven by the AtSTP14 transporter. (B) Relative uptake rates of glucose (Glc) and fructose (Fru) at an initial outside concentration of 100 µM and inhibition of galactose (Gal) uptake by the uncoupler CCCP (carbonyl cyanide-m-chlorophenylhydrazone; 50 µM) and the sulfhydryl agent PCMBS (p-chloromercuribenzenesulfonic acid; 50 µM) in AtSTP14-expressing yeast cells were determined. Data represent average values of three independent transport tests (± SD). Expression in yeast demonstrates that AtSTP14 is a galactose-specific transporter, strongly inhibited by a protonophore.

### Growth complementation of yeast mutant strain EBY.VW4000 by AtSTP14

The hexose transport-deficient baker’s yeast mutant EBY.VW4000 carrying the NEV-vector (control) does not grow on galactose, while the AtSTP14-expressing strain BHY3s (AtSTP14) regained growth on both 0.2 and 2.0% galactose as the sole carbon source. A 10 µl aliquot of yeast cells of OD600 = 1 and of three consecutive 1/10 dilutions were spotted and grown at 29°C for 4 d.
Regulation of AtSTP14 expression

Since galactose is an important component of the xyloglucans in the primary cell wall, we analyzed the regulation of AtSTP14 expression under conditions when cell wall recycling occurs (Lee et al. 2007). AtSTP14 transcript levels were determined in rosette leaves at different time points during the day/night cycle (short day) by quantitative PCR. As depicted in Fig. 5, AtSTP14 expression strongly decreases at the onset of light (8:30 h), slowly increases during the light phase, and again rises drastically upon the onset of darkness (16:30 h). To examine further whether AtSTP14 expression is directly induced by darkness, we put plants into the dark for 3 h in the middle of the photoperiod (12:30 h). Dark-incubated plants showed about 2-fold increased AtSTP14 expression as compared with plants kept under normal light conditions (Fig. 5B). In addition, we compared transcript levels in plants grown under a normal day/night cycle (16 h light/8 h dark) with those from plants grown in darkness for 24 h (extended night). After a 24 h dark period, AtSTP14 expression in the middle of the light phase (13:00 h) was increased about 3-fold (Fig. 5B). Since prolonged darkness during extended nights leads to sugar starvation conditions (Usadel et al. 2008), we also tested AtSTP14 transcriptional regulation by sugars. As shown by RT–PCR analysis, both sugars, glucose and sucrose, reduce AtSTP14 expression about 3-fold (Fig. 5C). Our expression studies indicate that AtSTP14 is regulated by factors such as extended darkness and sugars, which also lead to cell wall degradation. Therefore, we examined a possible co-regulation of two genes coding for β-galactosidases, At5g63800 (MUM2) and At3g52840 (Gal2), which are involved in cell wall degradation (Dean et al. 2007, Gantulga et al. 2008). As shown in Fig. 6, MUM2 is up-regulated 13-fold after a 24 h dark period (Fig. 6A), and expression of Gal2 is about 4-fold increased after both 3 and 24 h darkness (Fig. 6B). This could indicate a functional connection of the cell wall-degrading β-galactosidases and the galactose transporter AtSTP14 during (extended) darkness.

Identification and analysis of AtSTP14 mutant lines

To identify a T-DNA insertion line, a population of Arabidopsis mutants (Knockout Facility at the University of Wisconsin, Madison, WI, USA) was screened with AtSTP14-specific primers. A second, independent T-DNA insertion line was obtained from the Torrey Mesa Research Institute (San Diego, CA, USA). For both lines, WiscCSJ4172-33 and Garlic_354_A02, plants homozygous for the Atst4 mutant allele were identified by genomic analysis or RT–PCR (Supplementary Fig. S2). However, both mutants exhibited no phenotype with respect to their growth under normal conditions (long or short day). Also seed germination on inhibitory concentrations of galactose or glucose was indistinguishable from that of wild-type plants (also compared with galactose-insensitive Atstp1 mutants; Sherson et al. 2000), and by light microscopic analysis we could not detect any effect on seed development (data not shown), suggesting that the lack of AtSTP14-driven galactose transport in the mutants can be compensated by other members of the AtST family. A good candidate is AtSTP4, which can also

Fig. 5 Regulation of AtSTP14 expression by light and sugars. AtSTP14 mRNA transcript levels were determined by quantitative real-time RT–PCR with total RNA preparations from 4-week-old rosette leaves. Actin-2 was used to normalize AtSTP14 expression levels. (A) AtSTP14 expression during the photoperiod and the light–dark and dark–light transition (n = 2 ±SE). (B) Transcriptional regulation of AtSTP14 by darkness (n = 4 ±SE). (C) AtSTP14 repression by 2% glucose and 2% sucrose (n = 3 ±SE).

Fig. 6 Induction of the β-galactosidase genes At5g63800 (MUM2) and At3g52840 (Gal2) by darkness. MUM2 (A) and Gal2 (B) transcript levels were determined by quantitative real-time RT–PCR. First, relative expression levels after 3 h darkness (during the normal light period; ‘dark’) were compared with those after 3 h light (‘normal’). Secondly, relative expression levels after 24 h darkness (representing an extended night; ‘dark’) were compared with those after a regular 8 h night (‘normal’). LDL3 expression was used to normalize expression levels (n = 3 ±SD).
transport galactose and, according to our quantitative RT–PCR analyses, shows increased expression in the mutant lines Garlic_354_A02 and WiscCSJ4172-33 by factors of 2.71 ± 0.71 and 2.60 ± 0.87 (mean ± SD; n = 3), respectively.

To demonstrate further the in planta function of AtSTP14, we tested galactose uptake of the Atstp14 mutant lines in comparison with the wild type. To this end, plant leaves were harvested at the end of the dark phase (when AtSTP14 expression is high) and further incubated in the dark (to keep AtSTP14 expression high) in buffer with or without 15 mM galactose. After 6 h, the leaves were rinsed extensively and galactose uptake was determined by high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) of sugars. As seen in Fig. 7, in both the wild type and Atstp14 mutant lines galactose accumulation was detectable after 6 h incubation. However, in the mutant lines Garlic_354_A02 and WiscCSJ4172-33, galactose uptake was significantly reduced to 33.5 ± 5.0% (mean ± SE n = 9) and 56.3 ± 7.3% (mean ± SE; n = 5), respectively.

Discussion

The MST (-like) gene family in Arabidopsis comprises 53 homologous members. The first MSTs that were identified and characterized belong to the STP family (sugar transport proteins) which consists of 14 members (Büttner 2007). So far, eight AtSTPs have been studied in detail and were shown to be proton symporters of the plasma membrane, accepting a broad spectrum of monosaccharide substrates (except AtSTP9). AtSTP genes have sink-specific expression patterns (except AtSTP3) and function in the supply of sink cells with the cleavage products of apoplastic sucrose, namely glucose and fructose. Due to their functional redundancy, no phenotype could be observed under physiological conditions for mutants lacking one of these transporters. Here, we present the molecular and functional characterization of a new member of this family, AtSTP14. Different from the other AtSTPs, AtSTP14 is galactose specific and is also expressed in source tissues.

Heterologous expression in baker's yeast allowed extensive analysis of the transport properties of AtSTP14. Surprisingly, unlike all other AtSTPs, AtSTP14 does not transport glucose and also does not accept a variety of monosaccharide substrates. In contrast, AtSTP14 is a proton symporter of the plasma membrane specific for galactose.

Promoter–reporter fusions demonstrated that AtSTP14 is the first MST identified in the female gametophyte. GUS reporter plants displayed strong AtSTP14 promoter activity in seeds, which could be further narrowed down to the endosperm by the use of a GFP reporter line. This finding is consistent with microarray data from the GEO Series GSE12404, describing expression data from Arabidopsis seed compartments at five discrete stages of development. According to this study, AtSTP14 is expressed throughout seed development, with the highest levels in the micropylar endosperm surrounding the embryo during the pre-globular stage and in the entire endosperm (chalazal, micropylar and peripheral) during the globular stage. In addition, AtSTP14 expression also increases in the general seed coat during the linear cotyledon stages. This reflects the abundance of the GFP reporter in our transgenic lines and could indicate a role for AtSTP14 in sugar supply for the developing embryo (early stages) and the forming endosperm (later stages) from the seed coat apoplast. Although sucrose, glucose and fructose (and in later stages also raffinose and stachyose) represent the most abundant soluble sugars in Arabidopsis seeds, we could measure significant amounts of galactose in developing and mature seeds (data not shown). Possibly, free galactose is generated during cell wall reconstruction and/or formation of mucilage (a pectinaceous carbohydrate) in seed coat maturation (Haughn and Chaudhury 2005) and AtSTP14 could play a role in the retrieval of this apoplastic galactose.

In addition to its occurrence in the endosperm, we found AtSTP14 also to be expressed in green source tissues such as rosette and cauline leaves (and stems). In leaves, expression was increasing with age and, especially in young leaves, was more pronounced in trichomes as determined by GUS reporter lines. This finding is supported by two recent studies examining the trichome-specific transcriptome (Jakoby et al. 2008, Marks et al. 2008). In these studies, AtSTP14 transcripts were found to be >4-fold increased in isolated trichomes as compared with leaves when trichomes were removed, and expression was reduced to about 50% in the mutants glabra3 and triptychon, which show impaired trichome morphology and physiology (Jakoby et al. 2008).

So far, expression in source tissues has only been found for AtSTP3, which on the other hand does not transport galactose (Büttner et al. 2000). Thus, AtSTP14 expression in source leaves as well as the specificity for galactose point towards a function for AtSTP14 in the recycling of the cell wall. Galactose is
a component of primary cell wall polysaccharides such as xyloglucan, galactoglucomannan and cell wall arabinogalactan proteins (O’Donoghue et al. 2009). The galactose residues of xyloglucan are essential to maintain the mechanical strength of the primary cell walls during growth (Pena et al. 2004). However, the plant cell wall is a dynamic structure which can be changed under certain conditions. Internal stimuli such as growth-induced cell expansion or senescence as well as external factors such as stress or darkness lead to transient or final degradation of cell wall components (Lee et al. 2007, Lers 2007). Under these conditions, galactose is liberated by the activity of β-galactosidases and other cell wall-degrading enzymes such as pectate lyases. However, senescence can also occur prematurely if triggered by external factors including drought stress (Quirino et al. 2000). AtSTP14 is induced by both senescence (19-fold; NASCARRAYS-30) and drought stress (4-fold; GEO Series GSE10643), which in turn could allow the uptake of liberated cell wall galactose into the cell for further metabolism. This putative role of AtSTP14 in the retrieval of cell wall-derived galactose is further supported by its transcriptional regulation. We showed by quantitative RT–PCR analysis that AtSTP14 is induced by darkness (or repressed by light). A short dark phase (3 h) during the normal photoperiod as well as 24 h dark induction increase AtSTP14 expression 2- and 3-fold, respectively. This finding is also supported by NASCARRAYS-194 showing a strong induction after 3 h darkness in seedlings. Similarly, Gibon et al. (2006) showed strong AtSTP14 induction by a prolonged night, which leads to sugar starvation (Usadel et al. 2008) and in turn to cell wall degradation (Lee et al. 2007). Accordingly, AtSTP14 is repressed by the sugars glucose and sucrose, which control the cell’s energy homeostasis via sugar sensing (Rolland and Sheen 2005). Low cellular sugar levels indicate a low energy status signaling a demand for the import of sugars from the cell wall and in turn an up-regulation of the corresponding transporter genes. Interestingly, AtSTP14 is induced >10-fold by the overexpression of AtKIN10, a central regulator of plant stress and energy management. AtKIN10-activated (and sugar-repressed) genes represent a variety of major catabolic pathways including cell wall degradation that provide alternative sources of energy and metabolites (Baena-Gonzalez et al. 2007). According to this study, AtSTP14 expression is strongly enhanced by sucrase starvation, starvation-induced senescence, carbon deprivation during an extended night and darkness-induced senescence, and significantly repressed by 3% glucose, low [CO₂] (<50 p.p.m.) and 1% sucrose (Baena-Gonzalez et al. 2007), further supporting a role for AtSTP14 in cell wall sugar recycling.

In addition to our expression analyses, we searched the GeneCat webpage (http://genecat.mpg.de/cgi-bin/Ainitiator.py) for genes significantly co-expressed with AtSTP14. In accordance with its postulated role during cell wall recycling, AtSTP14 displayed a high degree of co-expression (Pearson correlation coefficient) with two β-galactosidase genes (At5g63800, MUM2 and At3g52840, Gal2). We could demonstrate that the expression of At3g52840 (Gal2) is significantly increased after 3h darkness, and both genes, At3g52840 (Gal2) and At5g63800 (MUM2), are strongly up-regulated after an extended night, conditions that also induce AtSTP14 expression. Furthermore, some glycosyl hydrolases of the cell wall are also induced by sugar starvation, which in turn leads to cell wall degradation (Lee et al. 2007) and again fits with the sugar repression of AtSTP14.

Two independent Atstp14 mutant lines were identified, but no differences in seed development or growth under normal conditions compared with the wild type could be observed. Possibly, another AtSTP can complement for the lack of AtSTP14-mediated galactose transport in the mutant lines. AtSTP3 is expressed in all green leaves but does not transport galactose (Büttner et al. 2000) and AtSTP1 does accept galactose, but its expression in green tissues is restricted to guard cells (Stadler et al. 2003). One conceivable candidate is AtSTP4, which shows only marginal expression in source leaves under normal conditions (Truernit et al. 1996), but is induced by stress conditions such as wounding and pathogen attack (Truernit et al. 1996, Fotopoulos et al. 2003). AtSTP4 can also transport galactose, and we found a 2-4-fold induction of AtSTP4 expression in both Atstp14 mutant lines. Thus, AtSTP4 might compensate for the loss of AtSTP14 in these knockout lines, at least under certain conditions. Alternatively, an as yet uncharacterized AtSTP could compensate for the loss of AtSTP14 function. This is in perfect agreement with our transport studies, where we see significantly reduced galactose uptake in leaves of Atstp14 mutant lines, and the remaining galactose transport activity could be due to AtSTP4 up-regulation.

**Materials and Methods**

**Strains and growth conditions**

*Escherichia coli* strain DH5α (Hanahan 1983) was used for cloning. Yeast transformation was as described (Gietz et al. 1992). For characterization of transport properties AtSTP14 was expressed in *Saccharomyces cerevisiae* strain EBY.VW4000 (kindly provided by E. Boles, University of Düsseldorf, Germany). *Arabidopsis thaliana* ecotype Col-0 was grown in potting soil in the greenhouse or on agar medium in growth chambers under short day (8 h light/16 h dark) or long day (16 h light/8 h dark) conditions at 22°C and 55% relative humidity, as indicated. Arabidopsis transformation was performed with *Agrobacterium tumefaciens* strain GV3101 (Holsters et al. 1980) using the floral dip method (Clough and Bent 1998).

**Isolation of AtSTP14 full-length cDNA clone**

To investigate this putative transporter further, we used the partial sequences of the expressed sequence tag (EST) clone APZ26f03 available at GenBank (5′, AV442769; and 3′, AV441077) to design primers for PCR amplification and cloning of the AtSTP14 cDNA. The full-length AtSTP14 cDNA was amplified by RT–PCR using total RNA from leaves and primers...
Functional characterization of AtSTP14 by heterologous expression in baker’s yeast

A K/N/K linker (5'-CCTTATGGCAGCGGTTTAC-3') was introduced into the KpnI site of pBH1, allowing the transfer of a 1,721 bp NotI fragment containing the complete AtSTP14 ORF into the yeast expression vector NEV-N (Sauer and Stolz 1994) in the sense orientation, yielding construct pBH1s. Transformation of the hexose transport-deficient baker’s yeast strain EBY.VW4000 (Wieczorke et al. 1999) resulted in the AtSTP14-expressing strain BHY3s. Transport assays with transgenic yeast strains were performed as described (Schneidereit et al. 2003).

Plant treatments for RT–PCR analyses

To analyze the effect of darkness on the AtSTP14 expression level, 6-week-old plants were wrapped in aluminum foil. After 3 and 24 h, leaves from dark-induced and control plants (kept under normal long day conditions) were harvested and frozen immediately in liquid N₂. The effect of external sugars on AtSTP14 expression was determined by incubation of 4-week-old rosette leaves in liquid Murashige and Skoog (MS) medium containing no sugar, 2% glucose or 2% sucrose for 6 h under constant temperature and light conditions. All experiments were started in the middle of the light period.

RNA isolation and RT–PCR analysis

Total RNA was isolated as described by Schneidereit et al. (2003) and RT–PCR was performed according to the supplier’s instructions (MBI Fermentas). Copy- DNA (0.5 µl) and primers AtSTP14c+1338f (5'-TTG TCA CAT GAA ATT CGG ACT ATT-3') and AtSTP14–5' EcoRI (5'-GGA AGG AC-3') were used to PCR-amplify AtSTP14-specific products from the indicated tissues. In parallel, LDL3 (Czechowski et al. 2005) and/or Actin-2 were amplified as reference genes as indicated using primers AtLDL3f-RT (5'-TAC TGG TAA ACA GAG GCA GAA ACA-3') and AtLDL3-r RT (5'-TTG TTG TAA ACC TGT TAT GC-3') and AtACT2g+886f (5'-ATT CAG ATG CCC AGA AGT CTT GGT-3') and AtACT2g+1243r (5'-GGA GAT CCA CAT CTT CGG TGA TGT-3'), respectively. Further, Gal2 and MUM2 gene expression was determined by quantitative RT–PCR with cDNAs from treated and control plants and primers AtbGal2c+1182f RT (5'-TGA TTT GCC TCC TTG TGC TGT TA-3') and AtbGal2c+1312r RT (5'-GGA GAA GGG CCT CTT TCG TTG TA-3') and AtMUM2c+1904f RT (5'-GGT GGA ACA AAG TTT GGA AGG AC-3') and AtMUM2c+1035r RT (5'-GCA GAT TGG CAG AGG ATT TT-3'), respectively. Expression of AtSTP4 was measured by quantitative RT–PCR using cDNA from mature leaves of Col-0 wild-type plants and Atstp4 knockout plants and primers AtSTP4c+1337f RT (5'-TTG TCA CAT GAA ATT CGG ACT ATT-3') and AtSTP4c+1435r RT (5'-ACA TCT TGT TCA TCT CTA ATT-3') as a reference gene we used LDL3 as indicated above. For quantitative real-time PCR we used the SYBR Green I dye and the RotorGene 2000 system (Corbett Research, Mortlake, Australia). The PCR amplification was performed with 1.0 µl of cDNA, 0.5 µM of each primer as indicated and 2x Quantitect SYBR Green RT–PCR mix (Qiagen). Relative transcript abundance was determined using the comparative ΔΔCt method with the RotorGene software version 5.

AtSTP14 promoter–reporter plants

To generate a GFP reporter line, a 2,022 bp region upstream of the AtSTP14 ORF was amplified by PCR using primers STP14prom5 (5'-TTG TTG TTT TGA AAA CAG GCC ACC-3') and STP14prom3 (5'-TAG GCC TCC TTC TTC ATC GGT AAG AGC-3') and genomic DNA as template. The promoter was cloned into the vector pGEM®-T Easy (Promega), to give construct pBP43. After verification of the correct sequence, a 2,009 bp Ncol fragment from pBP43 was transferred in front of the GFP ORF in the vector pAF1 (GFP-containing pUC19), yielding construct pS0142. From here, a 2,902 bp HindIII–SacI fragment containing the AtSTP14 promoter and the GFP gene was used to replace the GUS cassette in the plant expression vector pGPTV-Bar (Becker et al. 1992), yielding pS0142. To generate a GFP reporter line, a 2,034 bp Psil–Ncol fragment from pS0142 was transferred into pSR1 (pUC19-based plasmid harboring the GUS reporter gene) digested with Psil and Ncol, yielding construct pS0143. From here, a 3,866 bp Psil/SacI fragment containing the AtSTP14 promoter and the GUS gene was transferred into pGPTV-BAR digested with Sdal (PstI-compatible) and SacI (removing the internal GUS gene sequence) to give pS0144. Arabidopsis plants were transformed with Agrobacterium harboring constructs pS0142 or pS0144 by the floral dip method (Clough and Bent 1998), resulting in 35 (GFP) and 30 (GUS) independent basta-resistant transformants, respectively. GFP fluorescence or β-glucuronidase activity was studied in all transgenic lines in the T₁ generation.

Transient expression of AtSTP14–GFP in N. benthamiana and A. thaliana

AtSTP14 cDNA was amplified by PCR using AtSTP14-specific primers AtSTP14c+1f GW (5'-CAC CAT GGC CGG TGG AGC TCT TAC CG-3') and AtSTP14c+1482r STOP (5'-TTG ATC AAC ATC TTC GGC ATC ATA TCT CCA-3') and leaf cDNA as template, and cloned into pENTR/D-TOPO (Invitrogen). To generate a construct for subcellular localization, a Gateway LR reaction was performed transferring the AtSTP14 coding sequence into the destination vector pK7GW2.0 (Karimi et al. 2002) in front of the GFP sequence, yielding vector pGP14-1. For transient expression, N. benthamiana and A. thaliana leaves were infiltrated with an Agrobacterium culture (strain C58C1; Deblaere et al. 1985) harboring pGP-14-1 and analyzed after 2 d by CLSM.
Identification of T-DNA-tagged Arabidopsis knockout lines for AtSTP14

Line WiscCSJ4172-33 was identified by screening a population of 60,400 T-DNA insertion lines at the Arabidopsis Knockout Facility (University of Wisconsin Biotechnology Center; http://www.biotech.wisc.edu/Arabidopsis) following the method of Krysan et al. (1996). PCRs 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 AtSTP14g-42f (5′-AGA CCA GAA TAT TCC TTC TTT GTG CTT CA-3′) and AtSTP14g+2185r (5′-TTC GTA CTT CGG ATT GTA CTA AGA CAT TT-3′). AtSTP14-specific products were identified by Southern blot analysis using a 2,227bp probe (region –42 to +2,185 of the AtSTP14 gene). The exact T-DNA insertion site was determined at position +1,616 downstream of the start ATG in exon 4 (correlates to position +1,187 of the cDNA) by sequencing of the PCR products of the T-DNA border primer JL-270 (5′-TTC CTC CAT ATT GAC CAT CAT ACT TG-3′). Line Garlic_354_A02 (insertion at position +51) was obtained from the Torrey Mesa Research Institute (San Diego, CA, USA). Plants homozygous for both Atstp14 alleles were identified by PCRs with genomic DNA and primers flanking the insertion site. In addition, the absence of the AtSTP14 transcript in these knockout lines was verified by RT–PCR analyses with total RNA and AtSTP14-specific primers STP14cs5′ (5′-ATT TTA AAG CCA TGG CCG GTG GAC-3′) and AtSTP14g+1851r (5′-AAC AGA TAA CAC AAG CCC ACC ATC-3′), which span each insertion site.

Galactose uptake assay and ion chromatography

To assay galactose uptake into leaves, mature rosette leaves from 4-week-old Col-0 wild type and Atstp14 mutants were taken at the end of an extended night (18 h darkness) and after additional incubation for another 6 h in darkness in MS liquid medium containing 15 mM galactose. Leaves were washed three times with sterile water, briefly dried and immediately frozen in liquid nitrogen. Frozen plant material was incubated for 1 h at 80°C with 80% ethanol. After centrifugation, the supernatant was vacuum dried and the pellet redissolved in water. These samples were used to quantify soluble sugars by HPAE-PAD in an ICS-3000 system (Dionex) with a CarboPac PA1 column and 15–300 mM NaOH (Fluka) in HPLC water (VWR) as eluent. Quantitative calculation of sugars was performed using the Chromleon software 6.7 (Dionex).

Supplementary data

Supplementary data are available at PCP online.

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