The Arabidopsis Trehalose-6-P Synthase *AtTPS1* Gene Is a Regulator of Glucose, Abscisic Acid, and Stress Signaling[1]

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In Arabidopsis (*Arabidopsis thaliana*), trehalose is present at almost undetectable levels, excluding its role as an osmoprotectant. Here, we report that overexpression of *AtTPS1* in Arabidopsis using the *35S* promoter led to a small increase in trehalose and trehalose-6-P levels. In spite of this, transgenic plants displayed a dehydration tolerance phenotype without any visible morphological alterations, except for delayed flowering. Moreover, seedlings overexpressing *AtTPS1* exhibited glucose (Glc)- and abscisic acid (ABA)-insensitive phenotypes. Transgenic seedlings germinated on Glc were visibly larger with green well-expanded cotyledonary leaves and fully developed roots, in contrast with wild-type seedlings showing growth retardation and absence of photosynthetic tissue. An ABA dose-response experiment revealed a higher germination rate for transgenic plants overexpressing *AtTPS1* showing insensitive germination kinetics at 2.5 μM ABA. Interestingly, germination in the presence of Glc did not trigger an increase in ABA content in plants overexpressing *AtTPS1*. Expression analysis by quantitative reverse transcription-PCR in transgenic plants showed up-regulation of the *ABI4* and *CAB1* genes. In the presence of Glc, *CAB1* expression remained high, whereas *ABI4*, *HXK1*, and *ApL3* levels were down-regulated in the *AtTPS1*-overexpressing lines. Analysis of *AtTPS1* expression in *HXK1*-antisense or *HXK1*-sense transgenic lines suggests the possible involvement of AtTPS1 in the hexokinase-dependent Glc-signaling pathway. These data strongly suggest that *AtTPS1* has a pivotal role in the regulation of Glc and ABA signaling during vegetative development.

Trehalose is a nonreducing disaccharide (α-D-glucopyranosyl-1, 1-α-D-glucopyranoside) that accumulates in a wide variety of organisms that withstand drought, salt, heat, or freeze stress. It is present in some ''resurrection plants'' such as *Selaginella lepidophylla*, where it works as osmoprotectant during desiccation stress (Adams et al., 1990), and in yeast (*Saccharomyces cerevisiae*), where it can serve as stress protectant and storage carbohydrate (Thevelein, 1984; Wiemken, 1990). There are at least three different pathways for trehalose biosynthesis. The most widely distributed and present in many bacteria, yeasts, and plants is a two-step process in which trehalose-6-P (T6P) synthase (TPS) synthesizes T6P from UDP-Glc and Glc-6-P, followed by dephosphorylation to trehalose by T6P phosphatase (TPP). Trehalase (TH) converts trehalose to two molecules of Glc (Elbein et al., 2003). Genetic analysis of bacteria and yeast led to the isolation and functional characterization of *TPS1* genes in *Escherichia coli*, yeast, and other microorganisms (Bell, et al., 1992; Luyten et al., 1993; Kaasen et al., 1994). Deletion mutants of the *TPS1* gene in yeast are not only unable to synthesize trehalose but also lack the ability to grow on rapidly fermentable sugars such as Glc, due to a deregulation of glycolysis leading to hyperaccumulation of sugar phosphates and depletion of ATP and inorganic phosphate (Thevelein and Hohmann, 1995). These data have supported the idea that TPS1 and/or T6P have an important role in controlling sugar metabolism through glycolysis regulation, for instance, at the level of hexokinase activity (Blázquez et al., 1993; Hohmann et al., 1993; Bonini et al., 2003).

The *SlTPS1* gene from *S. lepidophylla* encodes a functional enzyme as shown by complementation of the yeast *tps1Δ* mutant, which restored its ability to grow on Glc and to accumulate trehalose (Zentella et al., 1999). In higher plants, trehalose rarely occurs, although an Arabidopsis (*Arabidopsis thaliana*) *AtTPS1*

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homolog was also able to support limited trehalose synthesis upon expression in yeast *tps1Δ* mutant (Blázquez et al., 1998). The capacity to synthesize trehalose by higher plants was first revealed by using the TH inhibitor validamycin A, leading to trehalose accumulation in tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) plants, albeit at low levels (Goddijn et al., 1997). N-terminal deletion of the *S. lepidophylla* and Arabidopsis *TPS1* gene product results in a dramatic increase in TPS activity (Van Dijck et al., 2002). This indicates a high potential trehalose synthesis capacity in plants in spite of the near universal absence of trehalose. In the past few years, it has been found that most plants, including non-stress tolerant species, encode *TPS1* transcripts, after analysis of many expressed sequence tag collections. Thus, besides its role in trehalose synthesis, *TPS1* has other possible roles in plants. It has been reported that an Arabidopsis transposon-insertion mutant in the *AtTPS1* gene was deficient in embryo maturation and growth, and the role of this gene is essential for vegetative growth and flowering (Eastmond et al., 2002; van Dijken et al., 2004).

Overexpression of *TPS1* genes in plants has been attempted to improve stress tolerance. So far, this strategy has been reported only with bacterial and yeast *TPS1* genes. When the *E. coli TPS1* (*otsA*) gene was overexpressed in tobacco and potato, trehalose accumulated at low levels in tobacco and was undetectable in potato (Goddijn et al., 1997). These tobacco plants, however, also showed a stress tolerance phenotype as well as striking morphological changes (Goddijn et al., 1997; Pilon-Smits et al., 1998). Expression in rice (*Oryza sativa*) of a bifunctional gene fusion of *otsA* and *otsB* (encoding TPP) driven by a stress-regulated promoter confers resistance to abiotic stress without causing morphological changes (Garg et al., 2002). On the other hand, overexpression of the yeast *TPS1* gene in tobacco led to moderate trehalose accumulation and dehydration tolerance (Holmström et al., 1996). Here, we report that the overexpression of Arabidopsis *AtTPS1* gene in Arabidopsis conferred drought tolerance without causing morphological changes and that seedlings displayed Glc- and abscisic acid (ABA)-insensitive phenotypes. Expression analysis of several genes involved in Glc sensing and ABA signaling displayed an altered gene expression pattern.

RESULTS

Overexpression of *AtTPS1* Confers Dehydration Tolerance

Sixteen independent Arabidopsis transgenic lines (*35S::AtTPS1*) were obtained after transformation with *Agrobacterium tumefaciens* harboring the p35S-AtTPS1-NOS plasmid. Homozygous plants were selected from each of these transgenic lines containing a single gene insertion (*1.1*, *2.4*, *3.2*, *3.4*, *4.4*, *5.4*, *6.2*, *7.5*, *10.6*, and line *12.3*), after genetic analysis using kanamycin to score a 3:1 segregation ratio. To assay for gene expression of *AtTPS1* in transgenic plants, we used reverse transcription (RT)-PCR since it has been reported that this gene is expressed at very low levels in wild-type Arabidopsis (Blázquez et al., 1998). All 10 transgenic lines overexpressed *AtTPS1* at moderately higher levels than the wild type (Fig. 1). The adenine ribosyl phosphotransferase 1 gene (*APT1*) was used as a constitutive control (Fig. 1). To analyze the expression of AtTPS1 protein, western blotting was conducted using 6-d-old seedlings. AtTPS1 protein was almost undetectable in wild-type plants and the line *1.1*, whereas it could be seen in all other transgenic

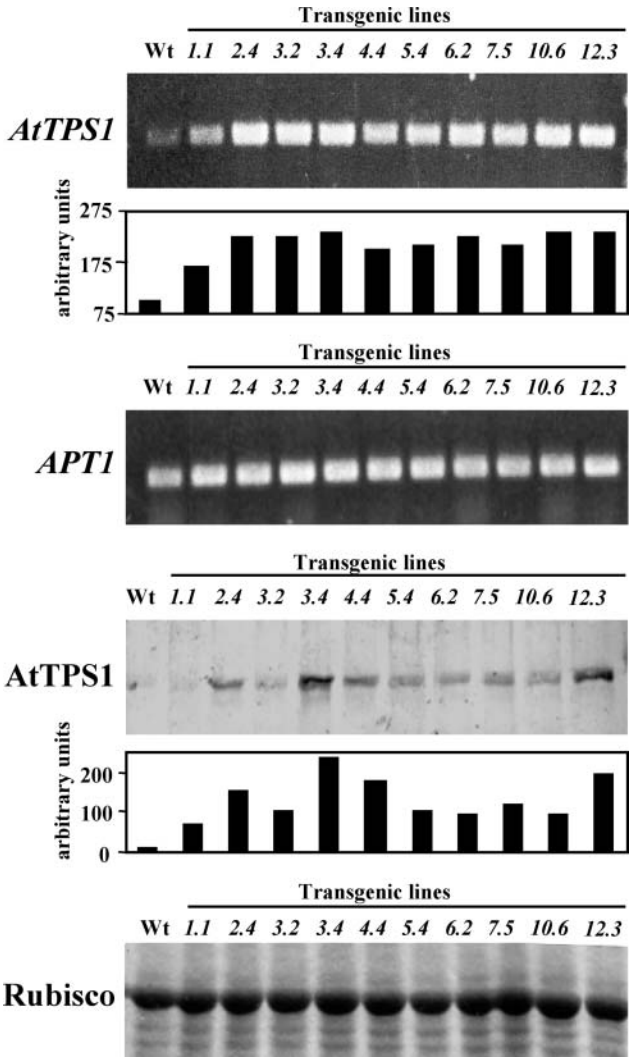

Figure 1. Semiquantitative RT-PCR analysis of *AtTPS1* expression. *AtTPS1* transcript accumulation in wild-type and 10 independent transgenic lines. *APT1* was used as a constitutive control. Western-blot analysis of AtTPS1 protein accumulation in wild-type and transgenic lines. SDS-PAGE showing Rubisco protein as a constitutive control. Graphics represent the quantification of the shown bands normalized according to the corresponding controls (*APT1* and Rubisco).

lines but especially at higher levels in lines *3.4* and *12.3*, corresponding to approximately 10 times higher than wild type (Fig. 1). Staining of Rubisco in an SDS-PAGE gel showed that equal amounts of protein were used for the western blot (Fig. 1).

The trehalose concentration was determined in *35S*::*AtTPS1* lines and wild-type plants since endogenous TPP or unspecific phosphatases can convert T6P to trehalose. A small increase of trehalose in the different transgenic lines was found, with the highest levels in lines *4.4* and *12.3*, being around 2.5 times more than in wild-type plants (Table I). The use of the TH inhibitor validamycin A caused higher trehalose accumulation in all transgenic and wild-type plants, with the line *12.3* displaying again the highest levels. T6P, the product of AtTPS1 enzyme activity, was also measured in the different transgenic lines and wild-type plants. As shown in Table I, there is an increase in T6P concentration in plants overexpressing the *AtTPS1* gene, which correlates with the corresponding trehalose levels for each independent line. For the transgenic lines *4.4* and *12.3*, there is a 4-fold increase in T6P in comparison to wild-type plants.

A detailed analysis of possible changes in organ shape or size and plant growth habit was monitored in the 10 homozygous *35S*::*AtTPS1* lines during their whole life cycle. No morphological changes in individual organs or at the whole-plant level were observed in any of the lines overexpressing *AtTPS1*, except for a delayed flowering time (1–2 weeks) in all of them. To assess the role of the *AtTPS1* gene in stress tolerance, drought tolerance tests with adult plants grown in soil were performed. Ten individuals from each of the 10 selected *35S*::*AtTPS1* lines and wild-type plants were grown for 4 weeks under fully watered conditions, followed by 2 weeks of water deprivation. Most transgenic lines (*3.2*, *3.4*, *4.4*, *5.4*, *6.2*, *7.5*, *10.6*, and *12.3*) recovered from water deprivation after rewatering for 1 d. In Figure 2, the line *12.3* is shown recovering its full shape after rewatering (Fig. 2, A and B), whereas wild-type plants did not survive the same dehydration treatment (Fig. 2, C and D). After rewatering, transgenic plants continued their normal growth and set viable seeds. The relative water content (RWC) of the plants and soil gravimetric water content (SGWC) were determined during the experimental time. There was a higher RWC in the transgenic line *12.3* compared to the line *5.4* and wild-type plants up to 12 d after water deprivation, and thereafter the RWC of all plants declined sharply, reaching around 10% after 14 d of dehydration (Fig. 2E). The SGWC also dropped to less than 3% after 14 d without watering (Fig. 2E, inset). These results strongly suggest that overexpression of the *AtTPS1* gene in Arabidopsis confers drought tolerance.

Glc- and ABA-Insensitive Phenotypes in *35S*::*AtTPS1* Plants

Sugars regulate gene expression in many organisms (Rolland et al., 2001). In plants, genes involved in photosynthesis and mobilization of stored reserves are repressed upon increase in sugar concentrations, whereas genes required for catabolism of carbon metabolites are induced (Pego et al., 2000). Sugar-mediated regulation of gene expression in Arabidopsis has been shown to be dependent on the hexokinase (HXK1) protein, representing a primary sensor of this pathway (Jang et al., 1997; Moore et al., 2003). The antisense expression or gene knock out of *HXK1* in Arabidopsis leads to a sugar-insensitive phenotype when plants are germinated on 6% Glc, whereas overexpression of *HXK1* promotes a Glc-hypersensitive response. In yeast, it has been suggested that the TPS1 protein might be involved in sugar sensing through interaction with hexokinase (Thevelein and Hohmann, 1995). Therefore, we decided to test whether the overexpression of *AtTPS1* in Arabidopsis would lead to a sugar-response phenotype. Different transgenic lines expressing *AtTPS1* at low (lines *3.2*, *3.4*, and *5.4*) or at relatively high (lines *4.4* and *12.3*) levels and wild-type seeds were germinated on Murashige and Skoog (MS)

Table I. *Trehalose and T6P concentration in 7-d-old plantlets*

Trehalose and T6P content was determined for wild type and 10 independent *35S*::*AtTPS1* transgenic lines grown on MS and MS supplemented with 1 mM validamycin A. Concentration is expressed in μg/g of fresh weight.

Transgenic Lines	Trehalose		T6P
	MS	MS + Validamycin A	MS
1.1	9.9 ± 3.8	19.26 ± 2.1	0.75 ± 0.26
2.4	6.5 ± 4.0	30.21 ± 2.7	0.69 ± 0.17
3.2	16.4 ± 1.3	24.12 ± 1.6	1.67 ± 0,57
3.4	16.8 ± 1.5	20.47 ± 1.8	1.75 ± 0.46
4.4	26.1 ± 2.0	32.24 ± 3.8	3.12 ± 0.92
5.4	15.0 ± 1.0	21.89 ± 2.1	1.50 ± 0.38
6.2	23.1 ± 2.1	30.21 ± 3.6	2.51 ± 0.72
7.5	21.9 ± 1.3	35.88 ± 3.2	2.33 ± 0.76
10.6	19.2 ± 2.1	17.84 ± 3.5	2.28 ± 0.83
12.3	25.3 ± 1.5	35.88 ± 3.0	3.06 ± 0.88
Wild type	10.1 ± 1.3	22.22 ± 2.4	0.74 ± 0.25

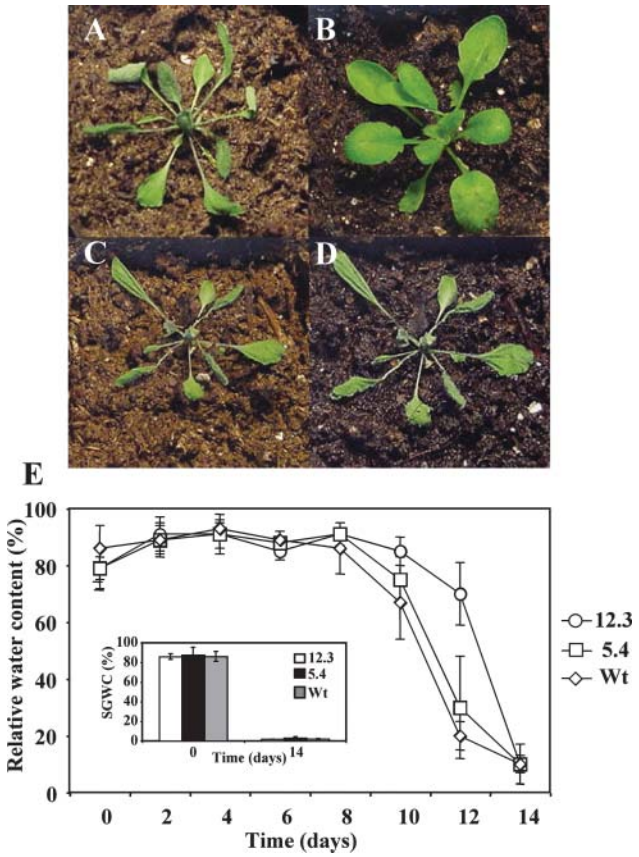

Figure 2. Stress-tolerance analysis of *AtTPS1*-overexpressing plants. Four-week-old *12.3* transgenic line (A and B) and wild-type plants (C and D) were water deprived until desiccation (A and C) and then rehydrated for 24 h (B and D). Kinetics of RWC of 10 individuals of *12.3* (○), *5.4* (□), and wild-type (◇) plants (E). The SGWC of 5 individuals of transgenic lines *12.3* (white bars), *5.4* (black bars), and wild type (gray bars) was determined at the beginning and end of the experiment (E, inset).

media containing 6% Glc. Possible phenotypic changes were scored daily up to 7 d after germination, when the most obvious changes were visible. Transgenic lines expressing higher levels of *AtTPS1* germinated well and developed at a normal rate with green well-expanded leaves (Fig. 3B), in contrast with wild-type seedlings that developed at a smaller size, showing absence of greening, well-expanded leaves, and root elongation (Fig. 3A). The other transgenic lines with a lower *AtTPS1* expression level showed an intermediate phenotype with some growth retardation and smaller leaves, but they grew better than wild-type plants. These results show that overexpression of *AtTPS1* in Arabidopsis confers a Glc-insensitive phenotype. Transgenic *35S*::*AtTPS1* lines *5.4* and *12.3* were chosen for further experiments as representative lines.

To further characterize the effect of Glc on plant development, the germination rate was determined. The *abi4* mutant was used as a positive control since it is Glc insensitive (Arenas-Huertero et al., 2000). In MS media, the *abi4* mutant and the line *12.3* germinated at a higher rate than the line *5.4* and wild-type plants; a clear difference was observed 2 d after sowing (Fig. 3C). In the presence of 6% Glc, the *abi4* mutant germinated at a higher rate compared with the rest of the lines, followed closely by the line *12.3*, which reached a similar germination percentage 4 d after sowing. By contrast, the line *5.4* and wild-type seedlings germinated 15% and 30% less, respectively, than *abi4* mutant and line *12.3* (Fig. 3D). These results suggest that *AtTPS1* promotes germination in contrast with *ABI4* gene, which is known to inhibit this process (Arenas-Huertero et al., 2000).

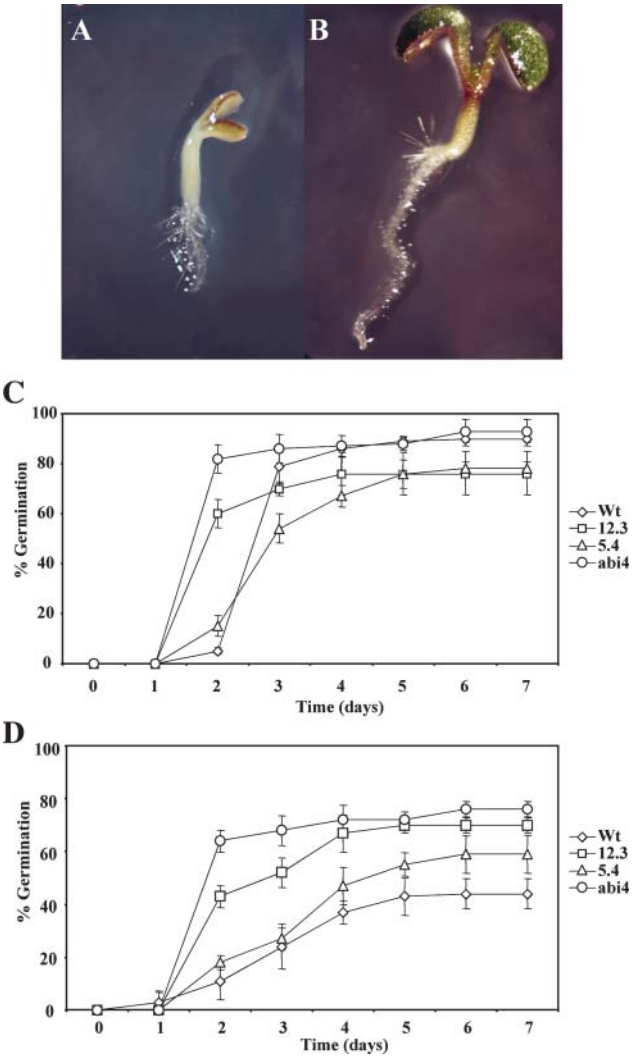

Figure 3. Glc sensitivity of *35S*::*AtTPS1* plants. Phenotype of 7-d-old wild-type (A) and *12.3* transgenic-line (B) seedlings growing on MS supplemented with 6% Glc. Germination kinetics of transgenic lines overexpressing *AtTPS1* in comparison to wild type and *abi4* mutant growing on MS media alone (C) or on MS supplemented with 6% Glc (D). Germination was defined as complete protrusion of the radicule. The data are the mean of 3 independent experiments evaluating 100 seeds per data point. Error bar represents SD. □, Line *12.3*; △, line *5.4*; ◇, wild-type seedlings; and ○, *abi4* mutant.

Several reports have shown a cross-talk between Glc sensing and ABA signaling in germination and seedling growth (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). To ascertain whether Arabidopsis seeds overexpressing the *AtTPS1* gene display an ABA-insensitive response, their germination was assayed in the presence of different concentrations of ABA. Seeds of the line *12.3* and *abi4* mutant were able to germinate in the presence of higher ABA concentrations than wild-type and *5.4* line seedlings, with the largest difference observed at 2.5 μM ABA (Fig. 4A). At this ABA concentration, a germination kinetics experiment was conducted, which showed about 20% higher germination for the *abi4* and line *12.3* than the line *5.4* and wild-type seedlings (Fig. 4B). The ABA-insensitive phenotype of seedlings overexpressing *AtTPS1* (Fig. 4D) can be clearly distinguished from the sensitive phenotype of wild-type plants (Fig. 4C). These results raised the question whether the phenotypic differences on Glc of transgenic plants overexpressing the *AtTPS1* gene could be due to an altered ABA content. To test this possibility, the endogenous levels of ABA were measured in 7-d-old line *12.3* and wild-type seedlings (Table II). The ABA content of the wild type and transgenic line *12.3* was not significantly different when seeds were germinated in MS media. However, when external Glc was added, the ABA concentration in wild-type plants increased, as has been reported before (Arenas-Huertero et al., 2000), whereas in *12.3* plants the ABA levels remained constant. This result strongly suggests an interaction between *AtTPS1* gene and ABA metabolism.

Table II. *Determination of endogenous ABA concentration in the transgenic 12.3 line*

The ABA concentration was determined in 7-d-old plantlets grown on MS or MS supplemented with 6% Glc. ABA is expressed in ng ABA/g of fresh weight. The data are the mean of three biologically independent experiments.

	MS	MS + 6% Glc
Wild type	25.79 ± 2	41.98 ± 5
35S::AtTPS1	23.68 ± 1	26.00 ± 3

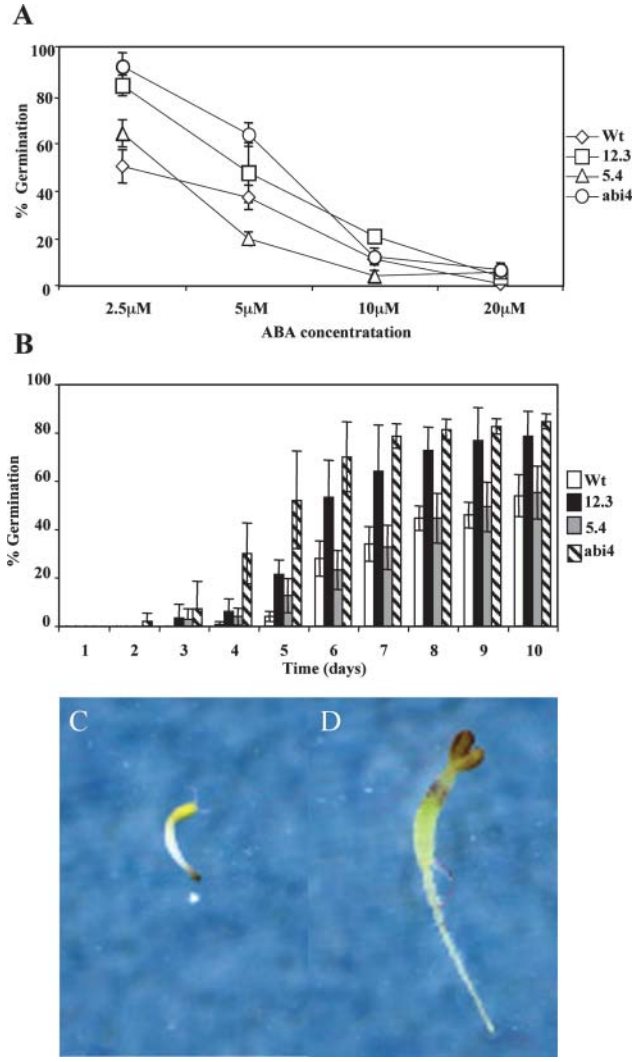

Figure 4. ABA sensitivity of *35S::AtTPS1* plants. A, Germination dose response after 10 d on MS media supplemented with 2.5, 5, 10, or 20 μM ABA. Representative lines *12.3* (□) and *5.4* (△) are shown in comparison to wild-type seedlings (◇), and *abi4* mutant (○). B, Germination kinetics of plants growing on MS media containing 2.5 μM ABA. Lines *12.3* (black bars) and *5.4* (gray bars) are shown in comparison to wild-type seedlings (white bars) and *abi4* mutant (dashed bars). Germination was defined as complete protrusion of the radicule. The data are the mean of 3 independent experiments evaluating 100 seeds per data point. Error bar represents SD. Phenotype of 7-d-old wild-type (C) and *12.3* transgenic-line seedlings (D) growing on MS supplemented with 2.5 μM ABA.

Altered Transcription of Glc- and ABA-Regulated Genes in *35S::AtTPS1* Plants

Glc and ABA regulate the transcription of genes involved in a wide variety of cellular processes, such as embryo maturation, stress adaptation, ABA-response, Glc metabolism, and photosynthesis (Koch, 1996; Merlot and Giraudat, 1997; Sheen et al., 1999; Finkelstein et al., 2002; León and Sheen, 2003). In this study, it has been shown that *AtTPS1* participates in the Glc and ABA-signaling pathways controlling germination and vegetative development. Therefore, it was decided to analyze the expression pattern of genes regulated by ABA or Glc in *35S::AtTPS1* plants. For this purpose, transgenic lines *12.3*, *5.4*, and *4.4* and wild-type 7-d-old seedlings, grown for 7 d in MS media, were treated with 7% Glc or mannitol for 6 h, and RNA was extracted for analysis by quantitative RT-PCR. We monitored the relative expression levels of signaling components of sugar (*HXK1*) and ABA signal transduction (*ABI4*) and of specific genes that are markers for photosynthesis (*CAB1*) and starch production (*APL3*). In a first experiment, we measured the expression level of *AtTPS1* itself, which increased

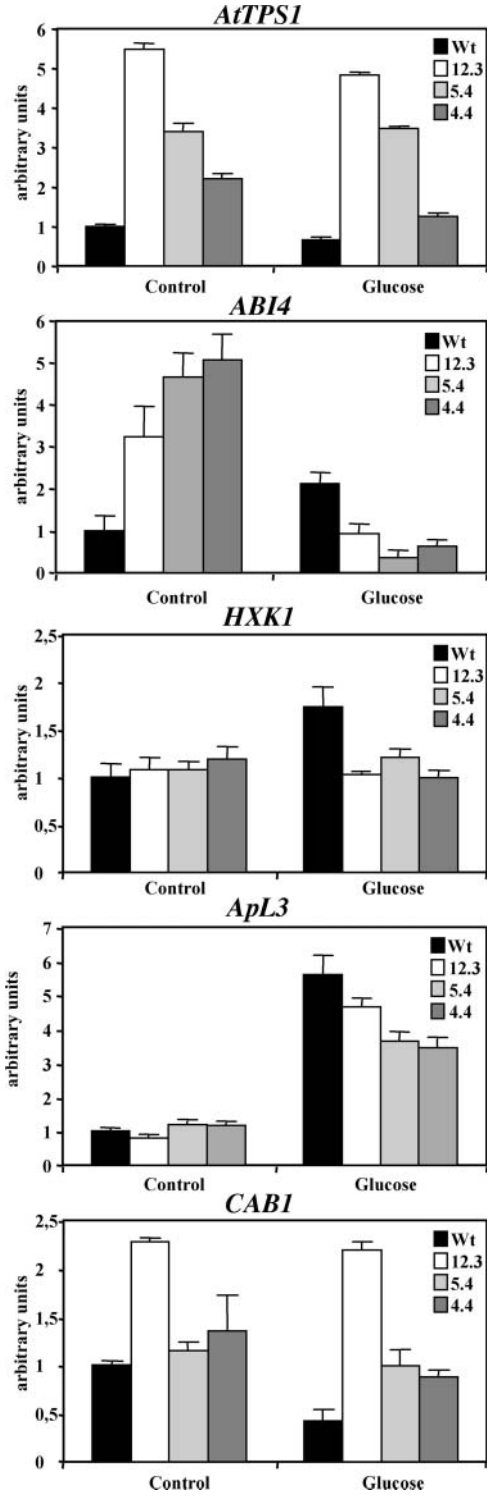

Figure 5. Quantitative PCR analysis of selected genes expressed in *35S::AtTPS1* plants. Relative expression levels measured by QPCR in response to 6 h of treatment with 7% Glc or mannitol (Control) of transgenic lines *12.3*, *5.4*, and *4.4* and wild-type seedlings. The data were obtained from three biologically independent experiments. Detector probes: *AtTPS1*, *ABI4*, *HXK1*, *ApL3*, and *CAB1*.

3- to 5-fold in the transgenic *35S::AtTPS1* lines and slightly decreased in all the different lines when plants were incubated with Glc (Fig. 5). In response to high levels of external Glc, development of the photosynthetic apparatus was inhibited in wild-type plants as shown before (Fig. 3A), which coincided with a reduction in *CAB1* expression (Fig. 5). However, overexpression of *AtTPS1* reduced the sensitivity of Arabidopsis significantly to external Glc. In line with this observation, the expression of *CAB1* remained as high as when Glc was replaced by the nonsignaling osmotic control mannitol (Fig. 5). A higher expression of *CAB1* is consistent with previous observations that plants overexpressing *E. coli TPS1* have dark-green leaves and higher photosynthesis rates (Paul et al., 2001). Starch synthesis and *ApL3* transcript expression are induced when high concentrations of trehalose are fed to Arabidopsis wild-type plants (Wingler et al., 2000). In the *35S::AtTPS1* lines, the expression of *ApL3* is comparable to wild-type seedlings without Glc in the medium (Fig. 5). This could be a consequence of the small increase in trehalose content in those transgenic plants. In wild-type plants, *ApL3* is highly induced by Glc; however, the *35S::AtTPS1* seedlings showed a reduced induction in *ApL3* transcript levels compared to wild type (Fig. 5). Therefore, the lower response of *ApL3* to Glc in plants overexpressing *AtTPS1* is in agreement with a Glc-insensitive phenotype.

HXK1 encodes a central sugar-sensing and -signaling protein, and *ABI4* is indispensable for ABA signaling (Moore et al., 2003). *HXK1* is not significantly induced upon overexpression of *AtTPS1* in the absence of Glc. The induction of *HXK1* by Glc is about 2-fold, whereas in *AtTPS1* overexpressors, *HXK1* levels do not respond to Glc (Fig. 5). Additionally, the overexpression of *AtTPS1* provoked a 3- to 5-fold increase in *ABI4* steady-state mRNA levels (Fig. 5). In a similar manner to the effect on *HXK1*, *ABI4* expression in *35::AtTPS1* plants dropped significantly when Glc was present (Fig. 5). *ABI4* was clearly repressed by the synergistic effect of Glc and *AtTPS1*, which could explain the Glc insensitivity of those plants.

Involvement of *AtTPS1* in Glc-Signaling Pathway

To substantiate the involvement of *AtTPS1* in Glc signaling, a gene expression analysis by semiquantitative RT-PCR was conducted in Arabidopsis ecotype Landsberg *erecta* in which *HXK1* expression was reduced (*HXK1*-antisense), resulting in a Glc-insensitive phenotype, and in plants overexpressing *HXK1* (*HXK1*-sense), which have Glc hypersensitivity (Jang et al., 1997). The *APT1* gene was used as a constitutive control (Fig. 6). Expression of *AtTPS1* was absent in *HXK1*-antisense plants, suggesting the dependence of *AtTPS1* expression on the presence of *HXK1* (Fig. 6). By contrast, there is a dramatic increase in *AtTPS1* transcript levels in *HXK1*-antisense plants grown in 6% Glc, whereas in wild-type and *HXK1*-sense seed-

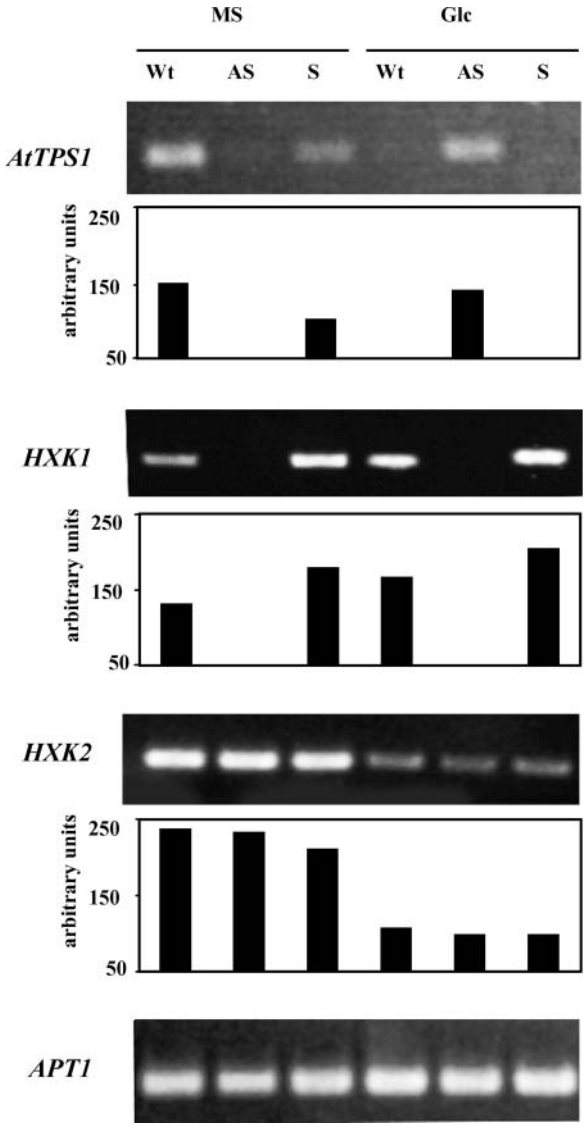

Figure 6. *AtTPS1* expression analysis in *HXK1*-sense or -antisense plants. Semiquantitative RT-PCR analysis in 7-d-old wild-type (Wt), antisense (AS) or sense (S) *HXK1* transgenic lines grown on MS alone or supplemented with 6% Glc. Graphics represent the quantification of the shown bands normalized according to *APT1* expression.

lings, *AtTPS1* expression is silent in the presence of Glc (Fig. 6). This result suggests a Glc control of *AtTPS1* in the absence of *HXK1*. The expression of *HXK1* and *HXK2* was also analyzed in these *HXK1*-sense and *HXK1*-antisense lines. As expected, *HXK1* was absent in antisense plants and up-modulated in sense transgenics. In the case of *HXK2*, no differences in gene expression levels were observed in the *HXK1*-sense and *HXK1*-antisense plants after comparison with the wild-type, except that *HXK2* expression decreased in the presence of Glc (Fig. 6). These results suggest that AtTPS1 might participate in the HXK-dependent signaling pathway.

DISCUSSION

In plants, light and sugars have a profound influence on growth and development. Light is essential for the synthesis of carbon skeletons in photosynthesis and is also a signal for photomorphogenesis (Pego et al., 2000). For many years, sugars have been regarded as the cell fuel and location of carbon storage. Sugars also control growth via metabolic effects on enzyme activity and sink-source allocation (Koch, 1996). In bacteria, yeast, and animals, sugar signaling has been shown to control gene expression in evolutionary conserved pathways involving secondary messengers, protein kinases, and phosphatases and in particular hexokinases (Hunter, 2000; Rolland et al., 2001). In recent years, it has been established that sugars, such as Glc, signal plant development and growth during germination by modulating the overall carbon status in a complex and still unexplained cross-talk with plant hormones such as ethylene and ABA (Rolland et al., 2002). There is strong evidence that hexokinase (HXK1) is a hexose sensor that leads to gene activation or repression by an unknown pathway (Jang et al., 1997; Moore et al., 2003). In addition, HXK-independent sugar signaling has also been described (Sheen et al., 1999).

Initially regarded as a rare sugar involved in desiccation tolerance, trehalose seems to occur in most plants, although at almost undetectable levels. The presence of *TPS1* genes has been found in a wide variety of non-stress tolerant plant species (Goddijn and van Dun, 1999). Although Arabidopsis has 11 *TPS* genes (Leyman et al., 2001), 3 of them closely related to *AtTPS1*, which encodes an active enzyme involved in trehalose biosynthesis (Blázquez et al., 1998), it does not accumulate significant amounts of this disaccharide (Vogel et al., 2001).

In this study, we overexpressed in Arabidopsis the *AtTPS1* gene to test for possible effects on stress tolerance and development. We showed that although Arabidopsis overexpressing *AtTPS1* accumulated trehalose only at low levels, transgenic plants have acquired desiccation tolerance. This is similar to previously reported work in tobacco where bacterial or yeast *TPS1* genes were successfully used to engineer stress tolerance in spite of the absence of significant levels of trehalose accumulation (Holmström et al., 1996; Pilon-Smits et al., 1998). In addition, these previous studies showed that transgenic plants overexpressing bacterial or yeast *TPS1* exhibited unexpected phenotypical changes, such as dark-green and lanceolate leaves, and a growth retardation habit that we did not observe in Arabidopsis expressing *AtTPS1*, except for a delayed flowering time. The low concentration of trehalose accumulated in transgenic plants, as documented in this and other reports, is unlikely to be sufficient for its action as an osmoprotectant (Gaff, 1996). In fact, we found that T6P accumulates at a higher concentration than trehalose in plants overexpressing *AtTPS1*. The overexpression of a chimeric fusion of *E. coli* TPS and TPP encoding genes in rice led

to trehalose accumulation and abiotic stress-tolerant plants, but no morphological abnormalities were observed probably due to the absence of T6P accumulation (Garg et al., 2002). Therefore, all these results could be explained as a consequence of an alternative role of T6P as a signal molecule involved in control of carbon metabolism in connection with growth and development. A *tps1* mutant in Arabidopsis has been characterized as embryo lethal, strongly suggesting a role for T6P in plant development (Eastmond et al., 2002). It has been shown that T6P has a regulatory role in carbohydrate utilization during plant growth and development (Eastmond et al., 2002; Schluepmann et al., 2003, 2004; van Dijken et al., 2004). Therefore, we tested whether the overexpression of *AtTPS1* in Arabidopsis would lead to a sugar-response phenotype. Our results showed that Arabidopsis plants overexpressing *AtTPS1* display Glc- and ABA-insensitive phenotypes allowing them to normally grow and develop in high Glc or ABA concentrations. Similar phenotypes are displayed by the allelic *gin6/sis5/sun6* mutants, all impaired in the *ABI4* transcriptional activator gene, which has a central role in mediating Glc sensitivity by ABA (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). Thus, besides being involved in the synthesis of trehalose, the AtTPS1 enzyme also has a role in sugar and ABA signaling during vegetative development. We also showed that *AtTPS1* promotes germination in contrast with the *ABI4* gene, which is known to inhibit this process (Arenas-Huertero et al., 2000). An important point is that ABA levels remained almost unchanged when plants overexpressing *AtTPS1* were grown in the presence of Glc, in contrast with the wild type and *gin6* mutant, which show a substantial increase in ABA content under this condition (Arenas-Huertero et al., 2000; León and Sheen, 2003).

Recently, the overexpression of trehalose metabolism genes from *E. coli* in Arabidopsis has been reported (Schluepmann et al., 2003). Plants expressing the *otsA* (encoding TPS) gene accumulate T6P, whereas those expressing *otsB* (encoding TPP) contain almost undetectable levels of T6P. Seedlings expressing *otsA* grew better than *otsB* or *treF* (encoding TH) expressors in 100 mM Glc, Fru, or Suc. However, when germination was assayed in the presence of 7% (389 mM) Glc, seedlings of wild-type or *otsA* (TPS)-expressing plants were similarly sensitive to high sugar concentration, with no greening or cotyledonary leaf expansion. Growth of *otsB* (TPP)-expressing plants was completely arrested at an earlier developmental stage (Schluepmann et al., 2003). These results contrast with those obtained in this work, where overexpression of *AtTPS1* in Arabidopsis confers insensitivity to 6% Glc. The T6P levels in Arabidopsis determined in this study were comparable to those reported in plants overexpressing the *E. coli otsA* gene (Schluepmann et al., 2003). A likely explanation for this discrepancy is that the N-terminal and/or C-terminal protein extensions of AtTPS1, which are absent in the corresponding proteins from *E. coli* and yeast (Blázquez et al., 1998; Goddijn and van Dun, 1999; Zentella et al., 1999), play a role in the sugar-signaling response. In this respect, it is interesting to mention that we have shown previously that deletion of the N-terminal part of AtTPS1 results in a dramatic increase in intrinsic TPS catalytic activity (Van Dijck et al., 2002). Additionally, when this N-terminal deleted AtTPS1 was expressed in Arabidopsis, the plants were Glc sensitive, strongly suggesting that the N-terminal region of *AtTPS1* somehow takes part in the Glc-signaling process (N. Avonce and G. Iturriaga, unpublished data).

A relevant aspect of this study is that *35S::AtTPS1* plants displaying a Glc-insensitive phenotype showed a down-regulation of *ABI4*, *HXK1*, and *ApL3* genes when seedlings were germinated in Glc. This is consistent with the phenotype of *gin2* and *gin6* mutants abrogated in *HXK1* or *ABI4* genes, respectively, also displaying Glc insensitivity (Arenas-Huertero et al., 2000; Moore et al., 2003). However, the expression of *AtTPS1* in the *abi4* mutant was at similar levels to wild-type plants, suggesting that *AtTPS1* is probably not regulated by *ABI4* (N. Avonce and G. Iturriaga, unpublished data). The contribution of other genes not analyzed in this study to explain the phenotypes of *35S::AtTPS1* plants cannot be excluded.

The analysis of *AtTPS1* in *HXK1*-antisense or *HXK1*-sense transgenic lines is indicative of its possible involvement in the HXK-dependent Glc-signaling pathway. Absence of *AtTPS1* expression was observed in *HXK1*-antisense plants germinated in media without Glc, indicating the dependence of *AtTPS1* expression on the presence of *HXKI*. These results suggest that *AtTPS1* participates downstream of HXK1. Glc controls ABA-signaling genes like the ABI4 transcription factor known to act in a signaling network downstream of HXK1 Glc sensor (Arenas-Huertero et al., 2000; León and Sheen, 2003). We have shown that *AtTPS1* up-regulates *ABI4* or down-regulates it if Glc is present. Therefore, we suggest that *AtTPS1* is regulating ABI4 transcription factor in a signaling cascade to control germination, shoot development, and cotyledon greening and expansion. It remains to be shown whether the gene regulation effect caused by *AtTPS1* is at transcriptional level alone and/or whether mRNA stability, changes of protein levels, or protein modifications could also be important.

CONCLUSION

This work reveals that plants overexpressing *AtTPS1* show Glc- and ABA-insensitive phenotypes and that these are due at least in part to an altered regulation of genes involved in Glc and ABA signaling during seedling vegetative growth. It is likely that in the signaling process T6P and possibly the AtTPS1 protein are involved. In addition, since trehalose does not accumulate at significant levels in plants overexpressing *AtTPS1*, their increased drought tolerance

seems a consequence of the same signaling process. *AtTPS1* is probably part of the HXK1-dependent Glc-signaling pathway and could be modulating the *ABI4* gene expression in concert with Glc. Thus, *AtTPS1* has a pivotal role in gene regulation to integrate environmental and metabolic cues during vegetative development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Col-0 ecotype was used for overexpressing the *AtTPS1* gene. *HXKI*-sense or *HXKI*-antisense plants (Jang et al., 1997) are in the Landsberg *erecta* ecotype. Plants were routinely grown on Metro-Mix 200 (Grace Sierra, Milpitas, CA) soil at 24/20°C with 16-h-light/8-h-dark cycle. Surface-sterilized seeds were germinated on MS media: 1× MS basal salt mixture medium (Invitrogen, Carlsbad, CA) supplemented with 1% (w/v) Suc, B5 vitamins, 0.05% MES (w/v), and 0.8% (w/v) phytoagar. To break dormancy, seeds were incubated at 4°C for 4 d with cool-white illumination (20 μE m^{-2} s^{-1}). Arabidopsis seeds were germinated in different ABA concentrations of (±) cis-trans isomer (Sigma, St. Louis) or in 6% (w/v) Glc. For dehydration tolerance tests (Gaxiola et al., 2001), 4-week-old Arabidopsis plants grown on 1:1:1 vermiculite:perlite:peat moss were used. Watering was held for 2 weeks, and then plants were rewatered and let 1 d to recover before being photographed. For quantitative PCR (QPCR) experiments, wild-type Arabidopsis seeds and seeds overexpressing *35S::AtTPS1* were germinated on top of Whatman 3MM filter paper (Clifton, NJ) on MS medium supplemented with 1% Suc. Ten days after sowing, the seedlings were moved with the filter paper to a new MS plate containing 7% Glc, 7% mannitol, or 1% Suc, and 6 h later seedlings were collected for RNA extraction.

Gene Constructs and Plant Transformation

For overexpression of *AtTPS1* in transgenic plants, total RNA was extracted from 2-week-old Arabidopsis plants using TRIZOL reagent (Invitrogen) according to manufacturer's instructions. Five micrograms of total RNA was reverse transcribed with Superscript II (Invitrogen) using oligo(dT) primer and amplified by PCR using Expand High Fidelity PCR system (Roche, Indianapolis) with AthTPS5′ (5′-CATGCCATGGCTGGAAATAAGTACAACTGTC-3′) and AthTPS3′ (5′-ATAGTTTTGCGGCCGCTTAAGGTGAGGAAGTGGTGT-CAG-3′) primers derived from the reported sequence (Blázquez et al., 1998). The PCR program consisted of 40 cycles of amplification (94°C, 1 min; 50°C, 1 min; 72°C, 2 min). The resulting 2.8-kb *Nco*I/*Not*I DNA fragment was ligated into the pSAL6 vector and checked for complementation and trehalose synthesis capacity in the yeast (*Saccharomyces cerevisiae*) *tps1Δ* mutant (Van Dijck et al., 2002). Insert was excised from pSAL6 clone and ligated to its 57-bp 5′-leader deduced from the reported sequence (Blázquez et al., 1998), which was synthesized using oligonucleotides NA4 (5′-CTAGAGCGGCCGC-CAGTGTGAGTAATTTAGTTTTGGTTCTGTTTTGGTGTGAGCGTC-3′) containing a *Xba*I site and NA5 (5′-CATGGACGCTCACACCAAAACAGAAC-CAAAACTAAATTACTCACACTGGCGGCCGCT-3′) with a *Nco*I site, and cloned in *Xba*I and *Not*I sites of pBluescript SK^- (Stratagene, La Jolla, CA) before determining its DNA sequence. This plasmid containing *AtTPS1* (pSKAtTPS1) was digested with *Xba*I and *Kpn*I to release the insert and cloned in pBin19 vector (Bevan, 1984) containing the 0.8-kb 35S-promoter and 0.3-kb NOS polyadenylation site, resulting in plasmid pBin35S/AtTPS1/NOS. The construct was introduced by electroporation in *Agrobacterium tumefaciens* C58C1 strain containing the pGV2260 plasmid. The resulting bacteria were used to transform Arabidopsis by in planta vacuum infiltration (Bechtold et al., 1993). Transgenic seedlings were selected on MS media containing 50 μg mL^{-1} kanamycin (Sigma). One-week-old seedlings were transferred to pots under the indicated conditions until plants formed seeds. Homozygous lines from the T_3 generation were used in this work.

RT-PCR Analysis

RT-PCR experiments were performed using 5 μg of total RNA extracted as described before from Arabidopsis tissues and used for first-strand cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen) and oligo(dT). PCR was conducted at linearity phase of the exponential reaction for each gene after comparison of the PCR products at different cycles. The gene-specific primers derived from database entries to amplify the corresponding gene fragment from transgenic lines or wild-type Arabidopsis plants, the corresponding size products and the GenBank accession numbers were as follows: *AtTPS1*, 5′-GCAGATATCGAATTCGGG-3′ and 5′-TTAAGGTGAG-GAAGTGGTGTCAG-3′, 662 bp, Y08568; *HXK1*, 5′-CAGACTTCTCTGTCCT-CTGG-3′ and 5′-AGGTAGAGAGAGTGAGAAGC-3′, 934 bp, U28214; *HXK2*, 5′-GAAGATGGCTGAAGAAGCTGC-3′ and 5′-CAGAGTCATCTTCAAGT-TCG-3′, 502 bp, U28215; and *APT1*, 5′-TCCCAGAATCGCTAAGATTGCC-3′ and 5′-CCTTTCCCTTAAGCTCTG-3′, 478 bp, BT000370. Each RT-PCR result was confirmed in at least three independent experiments. RT-PCR products were resolved in 1× Tris-acetate EDTA, 1% agarose gels stained with ethidium bromide. The bands shown represent the negative of the fluorescent images, and a densitometric quantification using Quantity One software (Bio-Rad, Hercules, CA) of the RT-PCR reaction was performed and normalized for each gene band using the *APT1* gene as a control.

QPCR

Total RNA was extracted from Arabidopsis seedlings, and after DNAse I treatment (Roche), cDNA was synthesized using AMV reverse transcriptase system (Promega, Madison, WI). QPCR was carried out using an ABI-Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA) as described by the manufacturer. The *UBQ4* detector was used as a housekeeping reference. The following primers (melting temperature 59°C) and probes (melting temperature 69°C) were used: *AtTPS1*, 5′-GAGCTTAGAGAGAA-GAGGAAGAGCAA-3′ and 5′-TTCTAAACGCAAGTCATTCTCAGAGT-3′, 5′-CGTAATCCTAATGACGTTGC-3′ probe; *CAB1*, 5′-GCCAAAGGGCCCAT-CAG-3′ and 5′-TCGGTGATTCGCCAGAGAA-3′, 5′-TGACCGTGTCAAG-TACT-3′ probe; *APL3*, 5′-TCAGCACCATGTCGATAGTAAAGC-3′ and 5′-CAGTTGGTTTCTCAGAGAAATGGA-3′, 5′-TCTTTCGTGCGCGCCG-3′ probe; *HXK1*, 5′-GGTAGCAAACTCAAGATGCTTATCAG-3′ and 5′-CCC-TAGGTCCAATGCATAAAAGAG-3′, 5′-CGTTGATAATCTTCCTTCCG-3′ probe; *ABI4*, 5′-CGGTGGGTTCGAGTCTATCAA-3′ and 5′-ACCCATAGAA-CATACCGGATCAA-3′, 5′-CATCCACCGCCGTTG-3′ probe; *UBQ4*, 5′-GCT-TCTGAGCTTTTGTGATGTGAT-3′ and 5′-GAAACCAAACCAGGTGAA-GATCTC-3′, 5′-TGTTCGAGTCTCATGCAC-3′ probe. Probes were labeled with FAM (6-carboxyfluorescein) reporter fluorochrome at the 5′ end and with TAMRA (6-carboxy-tetramethyl-rodamine) quencher fluorochrome at the 3′ end.

Western Blot

Protein extracts were prepared by homogenization of 100 mg of plant tissue in a buffer containing 50 mM Tris-HCl, pH 7.0. Protein concentration was assayed using the Bradford method (Bio-Rad, Hercules, CA). Ten micrograms of protein per lane were separated in a 7.5% SDS-PAGE and transferred onto nitrocellulose Hybond-C membrane (Amersham, Buckinghamshire, UK). Membranes were blocked with PBST buffer (135 mM NaCl, 5 mM KCl, 120 mM Na_2HPO_4, 10 mM KH_2PO_4, 0.05% Tween 20) containing 5% fat-free milk powder, incubated overnight at 4°C with AtTPS1 rabbit antiserum diluted 1:1,000, washed two times with PBST at 25°C, and incubated with a secondary antibody conjugated to alkaline phosphatase diluted 1:5,000 (Zymed, San Francisco, CA). Immune complexes were detected by a color assay using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphatase (Sigma).

Trehalose Determination

Plant extracts were prepared by adding 1 mL of milliQ water to 100 mg (fresh weight) of frozen plant tissue and boiled for 10 min. After centrifugation (10 min at 10,000*g*), the samples were filtered through a 1-mL column containing Dowex ion-exchange resins (1:1 [v/v]) 50WX8 100-200 and 1X8 100-200 (Dow Chemical, Midland, MI). The eluate pH was adjusted to 7.0 with 1 M NaOH and analyzed by HPLC using a Carbopac PA-100 column (Dionex, Sunnyvale, CA) eluted with 90 mM NaOH. Trehalose, Glc, Fru, and Suc standards were used to determine the concentrations. To confirm the identity of trehalose in plant extracts, samples were incubated with TH (*Humicola grisea*) and analyzed as described before.

Trehalose-6-P Determination

T6P was extracted from 100 mg of frozen plant tissue by adding 500 μL of 80% ethanol and boiled for 20 min as described by Schluepmann et al. (2003). After centrifugation (10 min at 10,000*g*), the samples were vacuum desiccated. The dry samples were extracted with 1.2 mL of boiling 0.1 M NaOH for 1 h and centrifuged as described before. The obtained supernatant was neutralized with 1.2 mL of 200 mM triethanolamine, pH 7.6. The sample pH was adjusted to 4 with 10 mM H_2SO_4 and analyzed at 65°C by HPLC on an Aminex HP-87H column (Bio-Rad, Richmond, CA) that was run isocratic with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. A T6P (Sigma) standard was used to determine the concentrations.

ABA Determination

To determine the ABA content in wild-type and transgenic plants, 7-d-old seedlings were homogenized in ABA extraction buffer (10 mM HCl, 1% polyvinylpyrrolidone in methanol) and incubated overnight at 4°C. After centrifugation, supernatant was neutralized with 1 M NaOH and ABA quantified by ELISA with a Phytodetek-ABA kit (Agdia, Elkhart, IN).

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