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

Nelson Avonce, Barbara Leyman, José O. Mascorro-Gallardo², Patrick Van Dijck, Johan M. Thevelein, and Gabriel Iturriaga*²

Instituto de Biotecnología, Universidad Nacional Autónoma de México, Col. Chamilpa, Cuernavaca 62210, Mexico (N.A); Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Col. Chamilpa, Cuernavaca 62210, Mexico (G.I.); and Laboratorium voor Moleculaire Celbiologie, Katholieke Universiteit Leuven and Vlaams Interuniversitair Instituut voor Biotechnologie (VIB), B–3001 Leuven-Heverlee, Flanders, Belgium (B.L., P.V.D., J.M.T.)

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 SITPS1 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

---

¹ This work was supported by the European Union International Cooperation (grant no. CA4-CT-2000-30041 to G.I.), the Flanders Interuniversity Institute for Biotechnology (VIB), and the Fund for Scientific Research-Flanders and the Fund of the Katholieke Universiteit Leuven (Concerted Research Actions). B.L. is a postdoctoral fellow of the Fund for Scientific Research (Flanders, FWO). N.A. was supported by a CONACyT Ph.D. fellowship, Mexico.

² Present address: Departamento de Fitotécnia, Universidad Autónoma Chapingo, Carretera México Texcoco Km 38.5, Chapingo, Edo.Mex. CP 56230, Mexico.

* Corresponding author; e-mail iturri@cbi.uaem.mx; fax 52-777-3297030.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.052084.
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
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 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.

### Table 1. 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.

<table>
<thead>
<tr>
<th>Transgenic Lines</th>
<th>Trehalose</th>
<th>T6P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS</td>
<td>MS + Validamycin A</td>
</tr>
<tr>
<td>1.1</td>
<td>9.9 ± 3.8</td>
<td>19.26 ± 2.1</td>
</tr>
<tr>
<td>2.4</td>
<td>6.5 ± 4.0</td>
<td>30.21 ± 2.7</td>
</tr>
<tr>
<td>3.2</td>
<td>16.4 ± 1.3</td>
<td>24.12 ± 1.6</td>
</tr>
<tr>
<td>3.4</td>
<td>16.8 ± 1.5</td>
<td>20.47 ± 1.8</td>
</tr>
<tr>
<td>4.4</td>
<td>26.1 ± 2.0</td>
<td>32.24 ± 3.8</td>
</tr>
<tr>
<td>5.4</td>
<td>15.0 ± 1.0</td>
<td>21.89 ± 2.1</td>
</tr>
<tr>
<td>6.2</td>
<td>23.1 ± 2.1</td>
<td>30.21 ± 3.6</td>
</tr>
<tr>
<td>7.5</td>
<td>21.9 ± 1.3</td>
<td>35.88 ± 3.2</td>
</tr>
<tr>
<td>10.6</td>
<td>19.2 ± 2.1</td>
<td>17.84 ± 3.5</td>
</tr>
<tr>
<td>12.3</td>
<td>25.3 ± 1.5</td>
<td>35.88 ± 3.0</td>
</tr>
<tr>
<td>Wild type</td>
<td>10.1 ± 1.3</td>
<td>22.22 ± 2.4</td>
</tr>
</tbody>
</table>

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)
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 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).

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.
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.

### 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

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

<table>
<thead>
<tr>
<th></th>
<th>MS</th>
<th>MS + 6% Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>25.79 ± 2</td>
<td>41.98 ± 5</td>
</tr>
<tr>
<td>35S::AtTPS1</td>
<td>23.68 ± 1</td>
<td>26.00 ± 3</td>
</tr>
</tbody>
</table>

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.
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 35S::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 semi quantitative 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-
plings, \textit{AtTPS1} expression is silent in the presence of Glc (Fig. 6). This result suggests a Glc control of \textit{AtTPS1} in the absence of \textit{HXK1}. The expression of \textit{HXK1} and \textit{HXK2} was also analyzed in these \textit{HXK1}-sense and \textit{HXK1}-antisense lines. As expected, \textit{HXK1} was absent in antisense plants and up-modulated in sense transgenics. In the case of \textit{HXK2}, no differences in gene expression levels were observed in the \textit{HXK1}-sense and \textit{HXK1}-antisense plants after comparison with the wild-type, except that \textit{HXK2} expression decreased in the presence of Glc (Fig. 6). These results suggest that \textit{AtTPS1} might participate in the \textit{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 \textit{TPS1} genes has been found in a wide variety of non-stress tolerant plant species (Goddijn and van Dun, 1999). Although Arabidopsis has 11 \textit{TPS} genes (Leyman et al., 2001), 3 of them closely related to \textit{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 \textit{AtTPS1} gene to test for possible effects on stress tolerance and development. We showed that although Arabidopsis overexpressing \textit{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 \textit{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 \textit{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 \textit{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 \textit{AtTPS1}. The overexpression of a chimeric fusion of \textit{E. coli} TPS and TTP 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 in high Glc or ABA concentrations. It has been shown that T6P has a regulatory role in the glucose transport (Eastmond et al., 2002; Schluepmann et al., 2003). These results suggest 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 Diijk 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 gln2 and gln6 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 HXK1. 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; Leon 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. AITPS1 is probably part of the HXK1-dependent Glc-signaling pathway and could be modulating the ABI4 gene expression in concert with Glc. Thus, AITPS1 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 AITPS1 gene. HXK1-sense or HXK1-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- to 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, 0.05% MES (w/v), and 0.8% (w/v) phytagar. 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), seedlings were grown on 1:1:1 vermiculite:perlite:peat moss into 20-cm pots containing solid medium (Invitrogen, Carlsbad, CA) supplemented with 1% (w/v) Suc, B5 vitamins, 0.05% MES (w/v), and 0.8% (w/v) phytagar. To break dormancy, seeds were incubated at 4°C for 4 d with cool-white illumination (20 μE m⁻² s⁻¹).

Gene Constructs and Plant Transformation

For overexpression of AITPS1 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 AAtHPS1 (5’-CATGCGATCGGGCTTAAGGTGAGGAAGTGGTGTCAG-3’) and AAtHPS3 (5’-ATATCTTTCGGCGCGCTTAAAGGTGAGAATTGGTGTCAG-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 NcoI/NorI DNA fragment was ligated into the pSAL6 vector and checked for complementation and trehalose synthesis capacity in the yeast (Saccharomyces cerevisiae) byΔΔ mutant (Van Dijk et al., 2002). Insert was excised from pSAL6 clone and ligated to its 5’-leader deduced from the reported sequence (Blázquez et al., 1998), which contains a NcoI site of pBluescript SK⁺ to release the insert and cloned in pBin19 (Stratagene, La Jolla, CA) before Agrobacterium tumefaciens (Bevan, 1984) was transformed into the yeast and in transgenic plants. After DNA digestion with SuperScript II reverse transcriptase (Invitrogen) and oligo(dT), the resulting DNA synthesis was checked using oligonucleotides NAA (5’-CTAGCCCGGGGGC-CAGGTGAGTAATTTAGTTTTGGTGTGAGCGTC-3’) and AAtHPS3 (5’-ATAGTTTTCCGGCGCTTAAAGGTGAGGAATTGGTGTCAG-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 NcoI/NorI DNA fragment was ligated into the pSAL6 vector and checked for complementation and trehalose synthesis capacity in the yeast (Saccharomyces cerevisiae) byΔΔ mutant (Van Dijk et al., 2002). Insert was excised from pSAL6 clone and ligated to its 5’-leader deduced from the reported sequence (Blázquez et al., 1998), which contains a NcoI site of pBluescript SK⁺ 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/AITPS1-NOS. The construct was introduced by electroporation in Agrobacterium tumefaciens C58C1 strain containing the pGV2296 plasmid. The resulting bacteria were used to transform Arabidopsis by in planta vacuum infiltration (Buchhold 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 T₂ 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: AITPS1, 5’-GGACTATGAGAATGGG-3’ and 5’-TAAAGAGGGCAAGACCTTCACACTTC-3’; and 5’-AGATGACAGACGATGACAG-3’, 934 bp, U28214; HXX2, 5’-GAAAGTGCTGAAGAAGCTGC-3’ and 5’-CAGACTCATCTCTCTCGACATGCAGCTG-3’, 502 bp, U28215; and AITP1, 5’-TCCGAGAGGCAAGTACATG-3’ and 5’-CCCTTCTTAAATGTCGTGCTG-3’, 478 bp, BT00357. Each RT-PCR product 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 UBC4 detector was used as a housekeeping reference. The following primers (melting temperature 59°C) and probes (melting temperature 69°C) were used: AITPS1, 5’-GCGTAGATGAGAACAAAGACGCTAGAGT-3’ and 5’-TGCACTGATCTACCATGAG-3’ probes; CAB2, 5’-GCCAAAGGGGCGCCATCAG-3’ and 5’-TCGGTAGATCGCCAGAGA-3’; AP3, 5’-TACGACGGCTATCGGATGAGAAGCC-3’ and 5’-CAGTTGCTGCTCGAGAACAA-3’; LAC, 5’-CTTCTGAGATCGCCAGAGA-3’; probe; AP1, 5’-TACGACGGCTATCGGATGAGAAGCC-3’ and 5’-CAGTTGCTGCTCGAGAACAA-3’ probes; CAB2, 5’-GCCAAAGGGGCGCCATCAG-3’ and 5’-TCGGTAGATCGCCAGAGA-3’; probe; AP3, 5’-TACGACGGCTATCGGATGAGAAGCC-3’ and 5’-CAGTTGCTGCTCGAGAACAA-3’; probe; AP1, 5’-TACGACGGCTATCGGATGAGAAGCC-3’ and 5’-CAGTTGCTGCTCGAGAACAA-3’ probes; CAB2, 5’-GCCAAAGGGGCGCCATCAG-3’ and 5’-TCGGTAGATCGCCAGAGA-3’; probe; AP3, 5’-TACGACGGCTATCGGATGAGAAGCC-3’ and 5’-CAGTTGCTGCTCGAGAACAA-3’; probe; AP1, 5’-TACGACGGCTATCGGATGAGAAGCC-3’ and 5’-CAGTTGCTGCTCGAGAACAA-3’; probe.

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 PBS buffer (135 mM NaCl, 5 mM KCl, 120 mM Na₂HPO₄, 10 mM KH₂PO₄, 0.05% Tween 20) containing 5% fat-free milk powder, incubated overnight in 4°C with AITPS1 rabbit antiserum diluted 1:1,000, washed two times with PBS at 25°C, and incubated with a secondary antibody conjugated to alkaline phosphatase diluted 15,000 (Zymed, San Francisco, CA). Immune complexes were detected by 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 10,000g), the samples were filtered through a 1-mL column containing Dowex ion-exchange resin (11:1 [v/v]) 50WXH 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 Carboxp PA-100 column (N Oxford, Sunnyvale, CA) eluted with 90 mM NaOH. Trehalose, Glc, Fructose, and Suc standards were used to determine the concentrations. To confirm the identity of trehalose in plant extracts, samples were incubated with TH (Hummel stir) and analyzed as described before.


Downloaded from on July 20, 2017 - Published by www.plantphysiol.org

Copyright © 2004 American Society of Plant Biologists. All rights reserved.
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 Schluemmann et al. (2003). After centrifugation (10 min at 10,000g), the samples were vacuum desiccated. The dry samples were extracted with 1.2 mL of boiling 0.1 M NaOH and centrifuged as described before. The ABA assay was neutralized with 1 M NaOH and ABA (1993) concentration.


Bell W, Klaassen P , Ohnacker M, Boller T, Herweijer M, Schoppink P , Van

concentrations.

The dry samples were extracted with 1.2 mL of boiling 0.1M NaOH for 1 h and centrifuged. supernatant was neutralized with 1.2 mL of 200 mM triethanolamine, pH 7.6. The sample pH was adjusted to 4 with 10 mM H2SO4 and analyzed at 65°C by HPLC on an Aminex HP-87H column (Bio-Rad, Richmond, CA) that was run isocratic with 5 mM H2SO4 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, the supernatant was neutralized with 1 M NaOH and ABA quantified by ELISA with a Phytodetect-ABA kit (Agdia, Elkhart, IN).

ACKNOWLEDGMENTS

We are indebted to Drs. J.-C. Jang for his kind gift of transgenic Arabidopsis seeds expressing the sense or antisense HXK gene and Patricia León for Col-0 and abi-t mutant. We also thank Drs. Rudy Varga and Lorena Pedrada for their assistance with trehalose and T6P determinations. Juan Estevez for his technical advice on Arabidopsis transformation and ABA determination, Katrien Royackers for AtTPS1 antiserum, and Paul Gaytán and Eugenio López for oligonucleotide synthesis.

Received August 19, 2004; returned for revision August 31, 2004; accepted September 3, 2004.

LITERATURE CITED


Nucleic Acids Res 12: 8711–8721


