Arabidopsis Trehalose-6-Phosphate Synthase 1 Is Essential for Normal Vegetative Growth and Transition to Flowering[1]

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In resurrection plants and yeast, trehalose has a function in stress protection, but the absence of measurable amounts of trehalose in other plants precludes such a function. The identification of a trehalose biosynthetic pathway in angiosperms raises questions on the function of trehalose metabolism in nonresurrection plants. We previously identified a mutant in the Arabidopsis trehalose biosynthesis gene *AtTPS1*. Plants homozygous for the *tps1* mutation do not develop mature seeds (Eastmond et al., 2002). *AtTPS1* expression analysis and the spatial and temporal activity of its promoter suggest that this gene is active outside the seed-filling stage of development as well. A generally low expression is observed in all organs analyzed, peaking in metabolic sinks such as flower buds, ripening siliques, and young rosette leaves. The arrested *tps1/tps1* embryonic state could be rescued using a dexamethasone-inducible *AtTPS1* expression system enabling generation of homozygous mutant plants. When depleted in *AtTPS1* expression, such mutant plants show reduced root growth, which is correlated with a reduced root meristematic region. Moreover, *tps1/tps1* plants are retarded in growth and remain generative during their lifetime. Absence of Trehalose-6-Phosphate Synthase 1 in Arabidopsis plants precludes transition to flowering.

Trehalose (α-D-glucopyranosyl-[1,1] -α-D-glucopyranoside) accumulation has been observed in a variety of species, most typically in anhydrobionts, which are able to survive complete dehydration. However, trehalose was not detected in plant species, with the exception of stress-induced trehalose accumulation in resurrection plants such as *Selaginella lepidophylla* (Crowe et al., 1992). Recent isolation of trehalose biosynthetic genes from Arabidopsis (Blazquez et al., 1998; Vogel et al., 1998; Müller et al., 2001) has led to the identification of a trehalose biosynthesis pathway in many, if not all, angiosperms. The first trehalose biosynthetic step is catalyzed by trehalose-6-P synthase (TPS), which converts Glc-6-P and UDP-Glc into trehalose-6-P (T-6-P). The second step is catalyzed by T-6-P phosphatase (TPP), which hydrolyzes T-6-P and releases trehalose. This biosynthesis pathway is similar in plants and yeast (*Saccharomyces cerevisiae*), where this metabolism was first described (Cabib and Leloir, 1958). Trehalose is readily hydrolyzed into two Glc units by trehalase (TRE), which is present in all organs of Arabidopsis and *Glycine max* (Müller et al., 2001).

The nonreducing disaccharide trehalose is highly resistant to nonenzymatic hydrolysis (Paiva and Panek, 1996) and is known to stabilize proteins during dehydration (Crowe et al., 1992). However, trehalose levels in nonresurrecting plants are barely detectable and insufficient for this function. It was proposed that trehalose metabolism may play a regulatory role in these plant species (Goddijn and Smeekens, 1998). The ability to utilize available sugars depends on trehalose biosynthesis and is most likely linked to the T-6-P intermediate (Eastmond et al., 2002; Schluepmann et al., 2003). Different TPS and TPP overexpressing transgenic plants displaying increased resistance to abiotic stress have been described (Holmström et al., 1996; Romero et al., 1997; Pilon-Smits et al., 1998; Goddijn and Van Dun, 1999; Paul et al., 2001; Garg et al., 2002; Jang et al., 2003; Schluepmann et al., 2003), but resistance phenotype in these plants did not correlate with trehalose accumulation (Jang et al., 2003). Observed effects on growth phenotypes in these transgenic lines were not attributed to increased trehalose levels. Tobacco (*Nicotiana tabacum*) overexpressing TPS from yeast (*ScTPS1*) or *Escherichia coli* (*OTSA*) display retarded growth and lancet-shaped dark green leaves (Goddijn and Van Dun, 1999). Furthermore, photosynthesis per unit leaf area is increased in *OTSA*-overexpressing tobacco plants, whereas it is reduced in *OTSB* (*E. coli* TPP) overexpressors (Paul and Pellny, 2003). Similarly, transgenic Arabidopsis plants expressing *OTSA* display opposing phenotypes to plants expressing *OTSB* and *E. coli* T-6-P hydrolase (*TREC*) (Schluepmann et al., 2003). *TREC* directly cleaves T-6-P into Glc and Glc-6-P without a trehalose intermediate. In *OTSA*-overexpressing plants, T-6-P levels are increased, whereas in *OTSB*- and *TREC*-expressing plants, T-6-P is reduced. Hence, studies with transgenic plants overexpressing microbial enzymes suggest that T-6-P may be a key regulator of metabolism.

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The function of plant trehalose biosynthetic enzymes was investigated by identification of mutations in the Arabidopsis trehalose biosynthetic *TPS1* gene, which was reported to complement yeast mutants defective in the homologous gene functions (Blazquez et al., 1998; Vogel et al., 1998; Müller et al., 2001). Reverse genetics identified transposon insertions in the *AtTPS1* gene. The *tps1-1* allele is located in the second exon and *tps1-2* in the first exon, as described previously (Eastmond et al., 2002); a third allele obtained from the SAIL (Syngenta Arabidopsis Insertion Library) collection has a T-DNA insertion in the last exon of *AtTPS1*. All insertions display an embryonic growth arrest in the homozygous state. These embryo lethal phenotypes of homozygous plants carrying the *tps1* mutation demonstrated that the trehalose biosynthetic pathway is essential for Arabidopsis embryo maturation (Eastmond et al., 2002).

We investigated the function of *AtTPS1* at other stages of plant development. We analyzed *AtTPS1* expression during development of wild-type plants and examined the effect of TPS1 deletion throughout Arabidopsis development. *AtTPS1* gene expression is increasing during wild-type embryo development in concordance with its essential function during Arabidopsis embryo maturation. The presence of *AtTPS1* mRNA is not restricted to the seed-filling stage but is observed in seedlings, roots, leaves, stems, flowers, and siliques. Expression is peaking in metabolic sink organs, including flower buds, ripening siliques, and young rosette leaves. TPS1 may therefore be important beyond the seed-filling process as well.

We used a dexamethasone (DEX)-inducible *AtTPS1* system to study the *tps1/tps1* phenotype throughout the plant life cycle. Such a system is essential to generate viable mutant seeds. Homozygous *tps1* seedlings display delayed growth and short-root phenotypes compared to wild-type and heterozygous seedlings. *tps1* mutant plants transgenic for the inducible *AtTPS1* construct remain in the vegetative growth phase in the absence of transgene induction. DEX application induces the switch to flowering; however, the mutant plants flower several weeks later than wild type and develop many flowering stems. Comparable phenotypes were observed in mutant plants complemented with a bacterial TPS1 enzyme (OTSA). Hence, besides its necessity for seed maturation, *AtTPS1* function is also important for vegetative growth and transition to flowering.

RESULTS

Cloning and Functional Analysis of the *AtTPS1* Promoter

The function of plant trehalose metabolism in angiosperms was studied using mutants in the first step of Arabidopsis trehalose biosynthesis, identified using a reverse genetic approach (Eastmond et al., 2002). Homozygous *tps1* plants arrest during the seed-filling stage of embryonic development, whereas heterozygous plants appear like wild type. Application of trehalose, T-6-P, or Suc does not allow rescue of *tps1/tps1* embryos, which precludes study of the vegetative mutant phenotype. A 3.1-kb *AtTPS1* promoter fragment and a 5.9-kb *AtTPS1* gene fragment were isolated from an Arabidopsis genomic library. These fragments were combined in a wild-type complementation construct (*AtTPS1*::*AtTPS1*), which was then transformed to *tps1-2* heterozygous mutant plants. Siliques of several T1 plants showed a mutant embryo frequency of one-fifteenth, in accordance with complementation by a single transgene locus. Complemented offspring seeds and plants were indistinguishable from wild type. Thus, expression of the wild-type *AtTPS1* sequence fully rescues the *tps1/tps1* embryonic defects, indicating that the 3.1-kb promoter is able to drive functional *AtTPS1* gene expression during the essential stages of Arabidopsis embryo maturation.

AtTPS1 Is Expressed throughout the Plant

The 3.1-kb *AtTPS1* promoter region was fused to the β-glucuronidase reporter gene (*GUS*) and 27 independent transgenic lines were generated, of which 11 were studied in more detail. In the *AtTPS1*::*GUS*-expressing transgenic lines, *GUS* activity was detected in developing seeds, where it appeared already in the shoot apical meristem of heart-stage embryos (Fig. 1A). Staining is also observed in the funiculus attachment region. The early embryonic expression might explain the growth retardation in *tps1* mutant embryos prior to the developmental arrest (Eastmond et al., 2002). From torpedo stage onward, expression expands throughout the hypocotyl; in mature embryos, faint expression is also observed in cotyledon tips (Fig. 1A). In other plant organs, *AtTPS1*-specific *GUS* expression could not be detected. Sensitive quantitative PCR (Q-PCR) analysis was used with an *AtTPS1*-specific Taqman probe. All gene-specific expression levels were normalized for *AtACTIN2* expression. Q-PCR confirms the strong increase of *AtTPS1* expression during embryo maturation and its absence in *tps1/tps1* embryos (Fig. 1B). In wild-type Arabidopsis seedlings and plant organs isolated from plants at principal growth stage 8, flowering plants with siliques ripening (Boyes et al., 2001), *AtTPS1* expression is observed in all tissues analyzed. It peaks in metabolic sink organs, including flower buds, ripening siliques, and young rosette leaves (Fig. 1C). *AtTPS1* mRNA level in roots is about one-third the level in the arial part of seedlings grown on half-strength Murashige and Skoog medium (MS; Fig. 1D). Presence of 1% Suc (29 mM) in the medium enhances seedling growth and increases the expression level slightly, as does sorbitol.

In Arabidopsis, 11 *TPS* gene homologs are found, divided into classes I and II (Leyman et al., 2001). *AtTPS1* belongs to class I. As an example of a class II gene, we studied the expression of *TPS5*. The expression level of *TPS5* is low in all organs analyzed, when

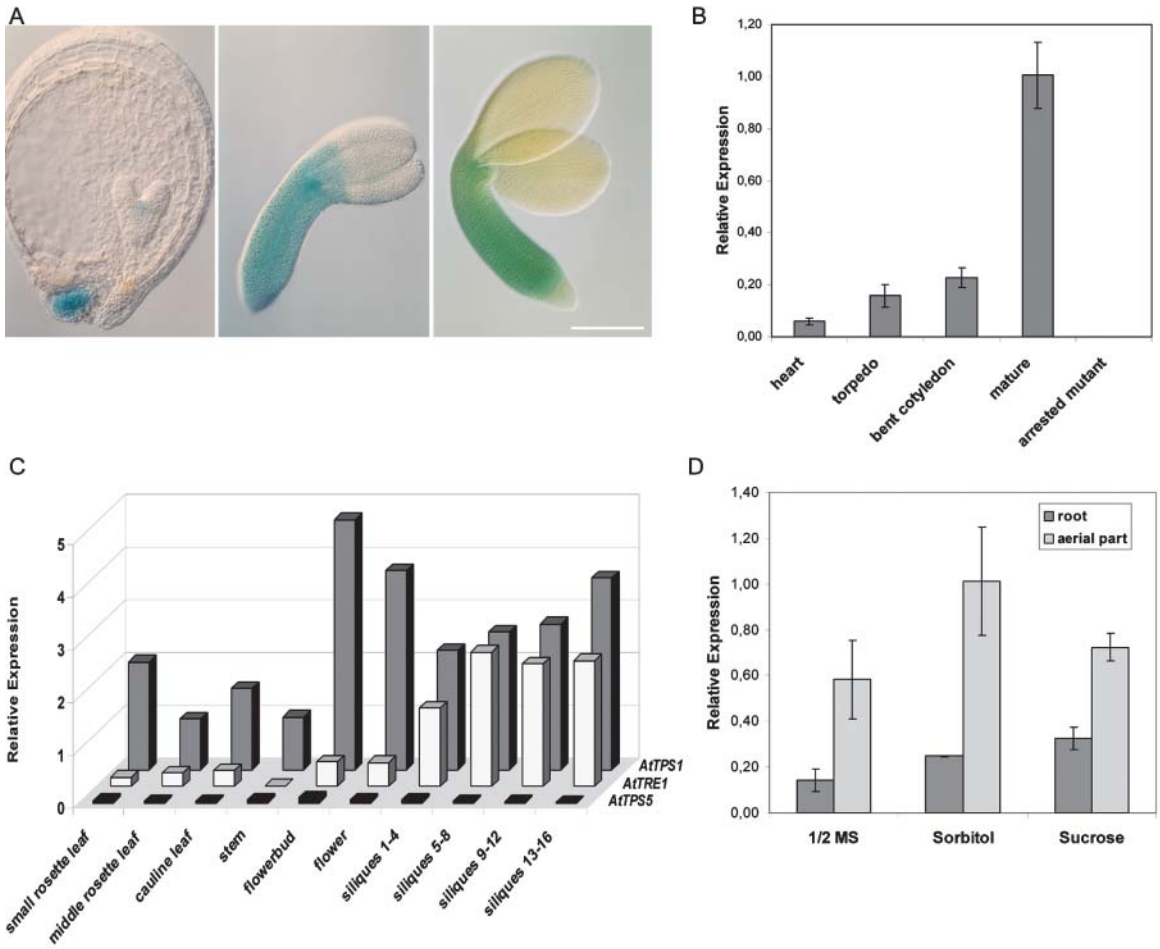

Figure 1. Histochemical localization of *GUS* activity in *AtTPS1*::*GUS* transgenic plants during Arabidopsis seed development. From left to right in A, heart, torpedo, and mature stage. Bar = 1 mm. B, Relative *AtTPS1* expression analyzed by Q-PCR in developing embryos; C, in distinct organs during plant growth of Arabidopsis; and D, in root and arial tissues of 8-d-old seedlings grown on half-strength MS, sorbitol, or Suc. Relative expression of *AtTPS5* and *AtTRE1* is displayed in C as a fraction of the *AtTPS1* level in stem. Silique numbering was based upon appearance of the first silique below the lowest flower, with further numbering downward ($n = 3$).

compared to the *AtTPS1* expression level (Fig. 1C). These data further support the specificity of the primer-probe sets used. Similar to *AtTPS1*, TRE (*AtTRE1*) is expressed ubiquitously in Arabidopsis, and the TRE1 mRNA levels increase in ripening siliques. However, in young leaves and flowers, it does not correlate with increased TPS1 levels (Fig. 1C).

The requirement of a functional *AtTPS1* during Arabidopsis embryo maturation is reflected in *AtTPS1* gene expression, which increases during wild-type seed development. *AtTPS1* is also expressed throughout the plant life cycle, peaking in metabolic sink organs. TPS1 might therefore play a role beyond the seed-filling process as well.

Conditional Rescue of the *tps1* Mutation

Inducible *AtTPS1* expression would enable rescue of the *tps1* embryonic defect and thus allow study of the *AtTPS1* role throughout plant development. For this, the DEX-inducible system (Aoyama and Chua, 1997) was modified by replacing the 35S cauliflower mosaic virus promoter with the *UBIQUITIN10* promoter of Arabidopsis (Sun and Callis, 1997) and inserting the *AtTPS1* gene behind the upstream activation sequence to facilitate *AtTPS1* expression during all stages of embryo development upon DEX application. Heterozygous mutant *tps1-2* plants were transformed with this inducible *TPS1* (*ind-TPS1*) construct. In total, 37 independent inducible lines were obtained, which were selected on phosphinotricin for the transposon insertion in *tps1-2*, on hygromycin for the inducible *AtTPS1* transgene construct, and on DEX for generation of viable seeds. Fourteen T1 lines showed a mutant seed frequency of one-fifteenth instead of one-third, indicative of complementation by a single transgene locus. Viable seeds were harvested from these lines and all flower stems were removed, after which the T1 plants were repotted in fresh soil and grown without DEX induction. The newly formed siliques contained

mutant embryos arrested in the torpedo stage of development corresponding to the *tps1/tps1* embryonic arrested state, suggesting that the system is inducible, as expected. Four independent inducible mutant lines containing a single transgene insertion were selected to study in more detail. Selection was based on antibiotic resistance segregation and Southern-blot analysis (data not shown). Q-PCR analysis confirmed the absence of *AtTPS1* expression in these lines under noninducing conditions and the presence of *AtTPS1* mRNA following DEX treatment (Fig. 2). Control plants expressing inducible *GUS* and heterozygous *TPS1/tps1* plants expressing the *ind-TPS1* construct are indistinguishable from wild type both in the absence and presence of DEX, showing that DEX treatment is not affecting the plant phenotype nor causing an overexpression phenotype (data not shown). Thus, the DEX-inducible *AtTPS1* system can rescue the arrested embryonic phenotype and is switchable, allowing study of the mutant phenotype beyond the seed maturation stage.

Deleting *AtTPS1* Perturbs Root and Shoot Growth

Rescued homozygous mutant seeds germinate in the absence of further DEX induction of the *AtTPS1* gene. Mutant *tps1* seedlings display a short root (Fig. 3A, MS) compared to wild type. DEX induction of *AtTPS1* partially restores root length, whereas it does not affect wild type (Fig. 3A, MS + DEX). Exogenous sugar application of 1% (29 mM) Suc or sorbitol (osmotic control) does not stimulate root growth (Fig. 3, Suc or Sorbitol + DEX). Confocal microscopy shows that the cell patterning in *tps1/tps1* roots is not affected (Fig. 3D). By contrast, the length of the meristematic region is much reduced in *tps1/tps1* roots versus wild type. This is indicated by the presence of enlarged cells, which are visible immediately above the short meristematic region in the mutant picture. Conversely, the photograph of wild-type roots at this stage display only the meristematic region consisting of small cells (Fig. 3D, Col-0). Partial complementation is obtained in *tps1/tps1* plants expressing *E. coli* TPS (*OTSA*, driven by the 3.1-kb *AtTPS1* promoter), resulting in an intermediate root-length phenotype (*TPS1*::*OTSA)* (Fig. 3, B and D). Mutant plants expressing the wild-type *TPS1* gene (*AtTPS1*::*AtTPS1*) display normal roots (Fig. 3, C and D).

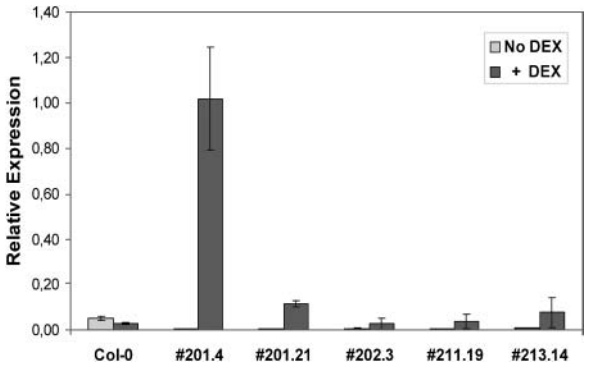

Figure 2. Relative *AtTPS1* expression analyzed by Q-PCR in wild-type (Col-0) and *ind-TPS1* transgenic *tps1/tps1* plants. Plants were grown for 1 month on soil, where DEX was added during watering of the plants ($n = 3$).

Following seedling establishment, the mutant plants are retarded in growth in the absence of DEX induction. Plants develop small, rounded rosette leaves, often with brownish leaf margins (Fig. 4A, left). The rosette diameter is largely reduced compared to wild type. Under inductive conditions, the rosette diameter is much increased; however, leaf and petiole elongation is still somewhat behind wild type grown under similar conditions (Fig. 4, A, right, and B). Without induction, the leaf initiation rate in *tps1* mutant plants is comparable to wild type. This is reflected by a similar number of rosette leaves compared to wild type at the stage where wild type has developed its first floral bud (Fig. 4C, No DEX). Mutant plants grown in the presence of DEX application have a significantly increased leaf number at the time of the first floral bud appearance (Fig. 4C, +DEX).

AtTPS1 Is Essential for Floral Transition

Floral transition in *tps1* mutant plants is dependent on DEX application (Fig. 5A). In the absence of DEX, the vegetative state is maintained until the plants die (>6 months after germination). All four *AtTPS1*-inducible mutant lines tested flower exclusively upon induction. In the presence of DEX, transition from vegetative to generative phase is delayed (Fig. 4C). Continuously induced mutant plants flower several weeks later than similarly treated wild-type plants. Such induced mutant plants showed a reduced apical dominance, resulting in the production of many flowering stems with small siliques and, regularly, aerial rosette formation (Fig. 5A, +DEX). Therefore, it is difficult to generate sufficient quantities of viable *tps1/tps1* offspring seed. Growth under continuous light, short-day, or long-day conditions with or without application of 1% Suc does not affect flowering time; neither does vernalization of the seeds for 6 weeks prior to germination. This late-flowering phenotype of *tps1* mutant plants might be a result of altered expression of inflorescence initiator genes. However, the expression of flowering-time genes *CO*, *SOC1*, *FLC*, *FT*, and *LFY* is not altered in the induced or non-induced mutant plants compared to wild type (Q-PCR analysis on 12-d-old seedlings grown in long-day conditions on half-strength MS or half-strength MS with 10 μM DEX; data not shown).

The *tps1/tps1* lines carrying the *AtTPS1*::*OTSA* transgene showed a range of phenotypes from wild type to much delayed in flowering time (Fig. 5B). Mutant plants expressing *AtTPS1*::*AtTPS1* are fully complemented for seed maturation, root length, growth, and flowering time (Figs. 3C and 5C). Hence, besides its

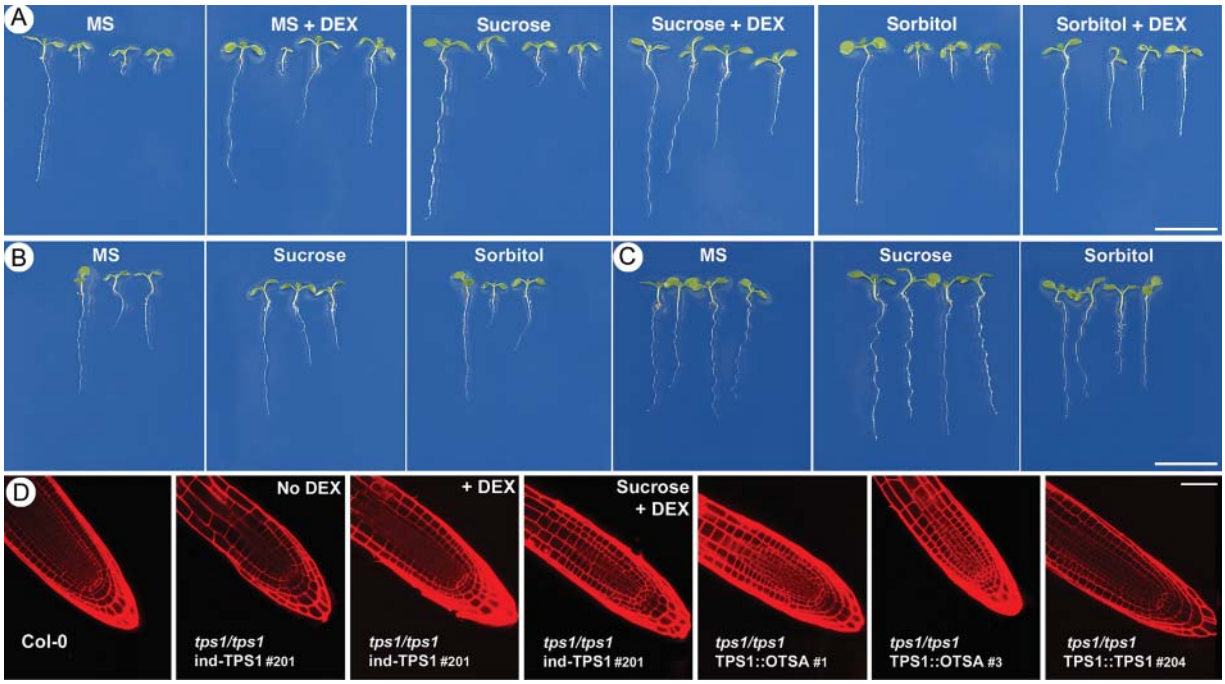

Figure 3. Root phenotype of 8-d-old seedlings grown on half-strength MS or half-strength MS containing 29 mM (1%) Suc or 29 mM sorbitol. A, Transgenic *tps1/tps1* inducible *AtTPS1* seedlings grown in the presence or absence of 10 μM DEX as indicated. In each section from left to right: Col-0 and *ind-TPS1* lines 201, 211, and 213. B, Col-0 and transgenic *tps1/tps1 AtTPS1*::*OTSA* lines 1 and 3 in each section from left to right, respectively. C, Col-0 versus *tps1/tps1* seedlings complemented with *AtTPS1*::*AtTPS1*. From left to right: Col-0 and *TPS1*::*TPS1* lines 201, 205, and 204. Bar = 1 cm. D, Detail of root tips by confocal microscopy following propidium iodine staining. Root meristematic region in wild type is represented by files with small cells. In the *tps1* mutant, this region is very short; cells immediately elongate and become large mature root cells. Bar = 0.1 mm.

essential role in seed maturation, *AtTPS1* activity is also required for normal growth and transition from the vegetative to the generative state in Arabidopsis.

DISCUSSION

TPS Gene Expression in Arabidopsis

The function of trehalose metabolism in nonresurrection plants is enigmatic. The work of Eastmond et al. (2002) and Schluepmann et al. (2003) afforded a glance at a possible signaling function. *AtTPS1* is essential for Arabidopsis seed maturation (Eastmond et al., 2002), since *tps1/tps1* embryos are developmentally arrested at the torpedo stage. Viable *tps1/tps1* seeds can be obtained by introduction of *E. coli OTSA* under control of the *AtTPS1* promoter, indicating that T-6-P is necessary for complementation (Schluepmann et al., 2003). The OTSA protein is a biosynthetically active enzyme that consists of a catalytic domain only (Goddijn and Van Dun, 1999). *AtTPS1* contains an additional domain next to the catalytic domain. This C-terminal domain of *AtTPS1* may harbor as-of-yet unknown functions. Absence of this domain in OTSA precludes such additional functions, which may explain partial complementation of the *tps1* mutant phenotype by OTSA. Here, we further investigated the role of *AtTPS1* throughout the Arabidopsis life cycle.

In the Arabidopsis genome, a total of 11 TPS homologs are present, which can be divided into two classes based upon their sequence homology (Leyman et al., 2001). Within each class, the DNA sequences are highly conserved, which requires use of gene-specific probes for quantitative measurements of gene expression levels. We developed gene-specific Taqman probes to determine the expression of *AtTPS1* (class I) and *AtTPS5* (class II) representatives by Q-PCR (see "Materials and Methods").

The requirement for a functional TPS1 during Arabidopsis embryo maturation is reflected by *AtTPS1* gene expression. Furthermore, *AtTPS1* is expressed at generally low levels in all Arabidopsis organs tested, in spite of the presence of 10 additional TPS homologs. The question then arises whether these homologs are all biosynthetically active in distinct expression domains or whether they are part of a multimeric trehalose-synthesizing protein complex. Only for TPS7 and TPS8 was biosynthetic activity tested by complementation in the yeast *tps1*Δ mutant. Whereas *AtTPS1* complements such a yeast mutant, complementation was not detected for *AtTPS7* or *AtTPS8* (Vogel et al., 2001). However, this does not exclude in planta TPS biosynthetic activity mediated by these proteins.

AtTPS1 expression in Arabidopsis is highest in metabolically active organs such as young rosette

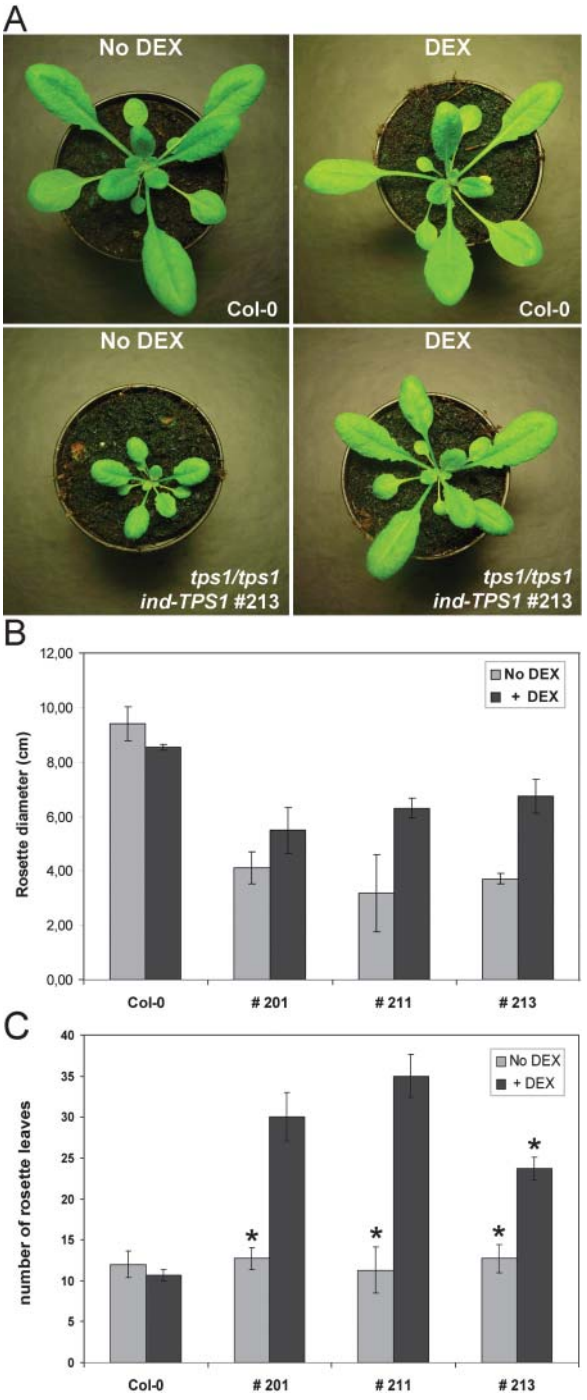

Figure 4. A, Wild type (top) and *tps1/tps1 ind-TPS1* transgenic plants (bottom) grown for 1 month on soil in long-day light regime. Left, No DEX induction; right, plants grown with DEX treatment. B, Rosette diameters (cm) of 1-month-old soil-grown plants (as depicted in A) with and without DEX application. Wild type (Col-0) versus *tps1/tps1* transgenic for *ind-TPS1* lines 201, 211, and 213 ($n = 3$). C, Number of rosette leaves of soil-grown plants at the time when in wild type the first floral bud appeared. The same plant lines were used as described in B. No DEX, Growth without DEX. +DEX, Number of rosette leaves of plants grown in presence of DEX, at appearance of the first floral bud. Asterisk indicates plants without floral bud development.

leaves, flower buds, ripening siliques, and maturing embryos (Fig. 1, B and C). In wild-type embryos, Suc levels are highest at the seed-filling stage when growth is arrested in the *tps1* mutant (Eastmond et al., 2002), suggesting an involvement in metabolism for *AtTPS1* or T-6-P. Carbon status itself only slightly affects *AtTPS1* expression levels in seedling tissues (Fig. 1D). Feeding experiments with 8-d-old seedlings supplied with Suc for 0.5 h up to 24 h similarly did not significantly affect *TPS1* expression (data not shown). The large induction of *AtTRE1* expression after fertilization in ripening siliques (Fig. 1C) coincides with *TPS1* induction in these organs. Conversely, *AtTRE1* is

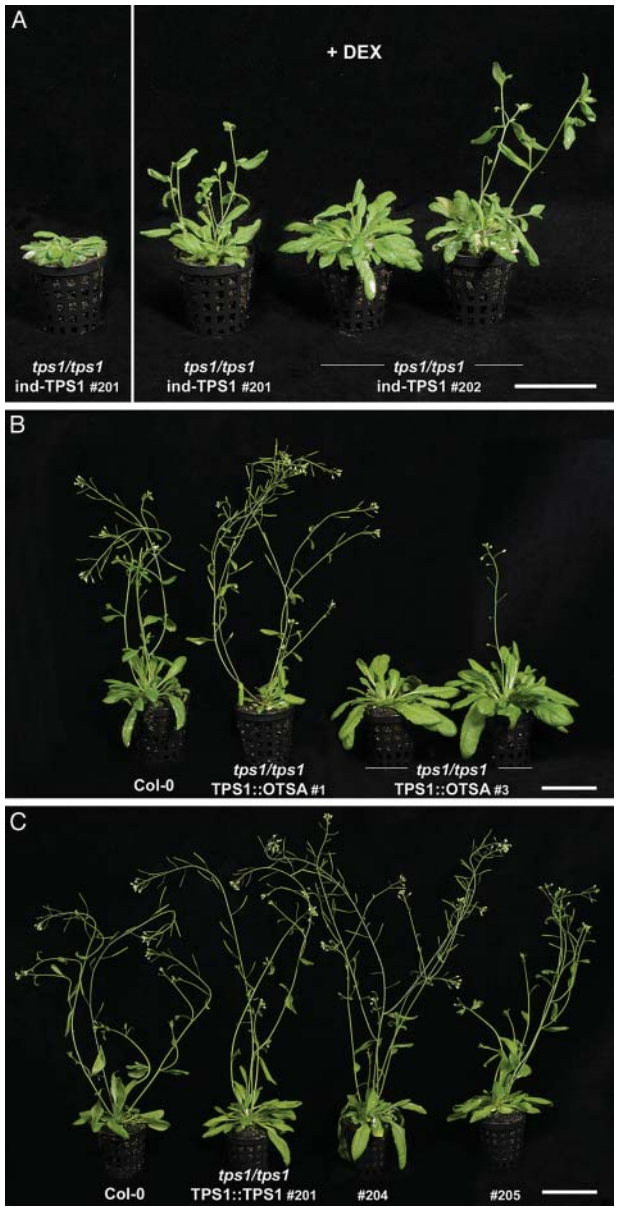

Figure 5. Flowering phenotypes. A, *tps1/tps1* plants expressing inducible *AtTPS1*, grown without (left) and with (right) DEX induction. B, Col-0 versus *tps1/tps1* carrying the *AtTPS1*::*OTSA* transgene. C, Col-0 versus *tps1/tps1* complemented with *AtTPS1*::*AtTPS1*. Bar = 5 cm.

not increased in floral organs and young rosette leaves, as is *AtTPS1*. Thus, increasing *AtTPS1* expression does not seem to be linked to *AtTRE1* induction.

AtTPS5 mRNA levels remain relatively low in the plant organs analyzed (Fig. 1C). The public Arabidopsis microarray database (available at http://www.arabidopsis.org/links/microarrays.jsp) shows that *TPS1* expression is generally low and stable. *TPS5*, *7*, *8*, *10*, and, especially, *TPS11* are in general significantly expressed and respond to diverse treatments or conditions. These data suggest that the diverse *TPS* homologs from classes I and II might have different spatial and temporal regulations. The ubiquitous presence of *AtTPS1* mRNA in Arabidopsis organs and the pronounced phenotypes observed in *tps1* mutant plants suggests an important role for the TPS1 protein.

AtTPS1 Enzyme Activity Is Required for Growth

The effect of the *tps1* mutation beyond seed development was studied using a DEX-inducible *AtTPS1* system to generate viable *tps1/tps1* seeds. Rescued *tps1/tps1* seeds are able to germinate and grow in the absence of *AtTPS1* induction. Therefore, TPS1 does not seem to be required for seed germination. However, we cannot exclude residual TPS1 protein in the germinating mutant seed, which might remain from the rescued maturing seeds. Established *tps1/tps1* seedlings display a short root containing a reduced meristematic region (Fig. 3). In wild-type seedlings, *AtTPS1* is expressed at low levels in roots (Fig. 1D), consistent with microarray data from root cell files, where *AtTPS1* is expressed exclusively in root stele tissues (Birnbaum et al., 2003). The *tps1/tps1* root has a normal structure (Fig. 3D), thus AtTPS1 is probably not involved in developmental patterning.

Arabidopsis Floral Transition Requires AtTPS1 Function

Vegetative *tps1* mutant plants are retarded in growth, with small rosette leaves. In the absence of functional TPS1, no transition from vegetative to generative development occurs. *AtTPS1* induction in *ind-TPS1* mutant lines can overcome this developmental arrest, but induced plants have a reduced apical dominance, and flowering is delayed compared to wild type. Some *tps1* mutant lines carrying the *AtTPS1*::*OTSA* transgene show delayed flowering also, whereas others are similar to wild type. Apparently, *E. coli OTSA* expression under the control of the *AtTPS1* promoter is capable of complementing seed maturation and floral transition phenotypes, whereas root growth is only partially complemented. Possibly, normal root growth requires a higher level of TPS1 activity than seed maturation and floral transition. Alternatively, *OTSA* may lack additional functions present in *AtTPS1*, such as phosphorylation by protein kinases, 14-3-3 protein binding (Moorhead et al., 1999), or allosteric control sites. In yeast, *E. coli OTSA* is able to restore T-6-P levels in *tps1*Δ *S. cerevisiae*, but this does not lead to a full restoration of growth on Glc, suggesting a role for the TPS1 protein in the control of Glc influx into glycolysis (Bonini et al., 2000, 2003). The *UBIQUITIN10* promoter from Arabidopsis drives *AtTPS1* expression in the *TPS1*-inducible mutant lines. Possibly, this promoter is insufficiently active in important cell types, which might explain the observed phenotypes under inductive conditions. It is unlikely that ectopic *AtTPS1* expression causes the observed phenotypes since strong ectopic TPS enzyme activity in *35S*::*OTSA* transgenic plants does not result in short-root and late-flowering phenotypes (Schluepmann et al., 2003).

Factors known to be involved in floral transition are light quality, photoperiod or GA, and signaling pathways requiring vernalization (Simpson and Dean, 2002). Several of these stimuli were applied in an attempt to overcome the late-flowering phenotype. Growth on 1% Suc, which is the optimal concentration to stimulate flowering (Ohto et al., 2001), or elevated light conditions to enhance carbon availability in these plants do not shorten the late flowering. Also, seed vernalization for 6 weeks was ineffective in enhancing flowering time. Furthermore, no significant change in expression of flowering-time genes (*CO*, *SOC1*, *FLC*, *FT*, and *LFY*) was detected in noninduced versus induced mutant lines or versus wild-type plants. This suggests that the *tps1* mutation acts downstream or parallel to these floral integrators.

Taken together, the *AtTPS1* expression data and the phenotypes observed for the *tps1* mutant suggest an essential role for *AtTPS1* throughout the Arabidopsis life cycle. In *tps1* mutants, the root meristematic region is dramatically decreased, leaf growth is reduced, and floral transition is absent. Clearly, other TPS homologs are unable to complement *AtTPS1* function. Biosynthesis of T-6-P by the *AtTPS1*::*OTSA* construct can overcome many, but not all, of the *tps1* mutant phenotypes. Previously it was shown that T-6-P, the product of TPS activity, is required for plant growth (Schluepmann et al., 2003). T-6-P levels control utilization of available carbon in a way that is not yet understood. TPS1 likely regulates the metabolic status of the plant through T-6-P biosynthesis. Therefore, the observed phenotypes in the *tps1* mutant might have a metabolic basis. Suc addition does not rescue *tps1* mutant phenotypes because T-6-P is required for carbon to be utilized for normal growth. Identification of targets of T-6-P and methodology for determining cellular T-6-P levels would be most helpful in further understanding the role of trehalose metabolism in plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis Columbia (Col-0) and *tps1-2* were obtained from En/Spm transposon insertion collection (Tissier et al., 1999) as described by Eastmond et al. (2002). After seed stratification for 3 d at 4°C, plant material was

transferred to 16-h-light/8-h-dark (long-day) growth conditions at 22°C and 200 μmol m^{-2} s^{-1} irradiance. In vitro growth and selection were performed on half-strength MS (Murashige and Skoog, 1962) solidified with 0.8% agarose. For resistance marker selection, 20 mg L^{-1} hygromycin, 10 mg L^{-1} phosphinotricin (PPT), or 50 mg L^{-1} kanamycin were added to the medium. For Basta (PPT) selection on soil, plants were sprayed twice with 75 mg L^{-1} PPT, 0.01% Silwet L77 solution at about a 3-d interval. For DEX treatment, 10 μM were supplied in the in vitro growth medium or in water for watering soil grown plants.

Constructs and Plant Transformation

AtTPS1 cDNA (accession no. Y08568) was cloned by PCR (Titan One Tube RT-PCR System; Roche, Basel), which was used to screen a LambdaGem-11 phage library (Promega, Madison, WI) of Arabidopsis genomic DNA (Col-0). A 3,110-bp (5′ untranslated region ATG; total fragment was 5,007 bp) promoter containing *Xho*I-fragment and a 5,886-bp (ATG-3′ untranslated region; total fragment was 6,716 bp) *TPS1* gene-containing fragment were cloned into pBluescript II KS+ (Stratagene, La Jolla, CA).

The *AtTPS1*::*AtTPS1* complementation construct was obtained by fusing the complete *TPS1* promoter fragment from pBlue into pBin19 (*Kpn*I/*Bst*EII). In this binary vector (*Spe*I/blunted *Xba*I), the *TPS1* gene was introduced (*Spe*I/*Sma*I).

For the *AtTPS1*::*OTSA* construct, a 3,067-bp *AtTPS1* promoter fragment (*Eco*RI/blunted *Bst*EII) was cloned into *Eco*RI/blunted Sse8387I from pMog 1423 (provided by Zeneca, Leiden, The Netherlands) to drive *Escherichia coli OTSA* gene expression.

The *ind-TPS1* construct was created in pTA7002 vector (Aoyama and Chua, 1997), where the 35S cauliflower mosaic virus promoter was replaced by 1,020-bp *AtUBQ10* promoter (accession no. L05361) from p3325 (Sun and Callis, 1997). Into PmeI (blunted and T-overhang created by Taq-polymerase)/Sse8387I of pTA7002 vector, the *UBQ10* promoter fragment (*Bam*HI, blunted with added A-overhang/Sse8387I) was ligated, thereby creating pTA-UBQ10::GVG. Next, a 5,934-bp genomic *AtTPS1* (*Bst*EII, blunted/*Xba*I) fragment was inserted into vector sites *Xho*I (blunted)/*Spe*I. As an inducible control vector (*ind-GUS*), the *GUSA* reporter gene (1,812 bp, *Sal*I/*Nhe*I fragment from pCambia1381) was inserted into *Xho*I/*Spe*I of pTA-UBQ10::GVG.

In pCambia1303, the *Nco*I/*Bst*EII *GUSA* fragment was replaced by *GUSA-GFP* (green fluorescent protein) fragment from pCambia1381, creating pCambia1383. The *TPS1* promoter was PCR amplified (using reverse primer and tpsNcoI5′u primer 5′-ttccagccatgggctcacacca-3′), from which a 3.1-kb *Eco*RI/*Nco*I fragment was inserted into the corresponding vector sites giving the *AtTPS1*::*GUS* reporter construct.

The floral dip method (Clough and Bent, 1998) was used for transformation of plant material with *Agrobacterium tumefaciens* strain GVG2260.

DNA Extraction, RNA Extraction, and PCR Analysis

Genomic DNA was isolated using the cetyl-trimethyl-ammonium bromide method (Doyle and Doyle, 1990) or the quick-prep method (Cheung et al., 1993). For genotyping the *OTSA* complementing lines, PCR detection was performed using primers (tps4d, 5′-tgtgagcgtatgcctggaaataag-3′; tps471u, 5′-agcccatcctatccatctg-3′; and EnRB544, 5′-ggcttgtgtggaacttactatg-3′, located in the transposon right border) discriminating between wild-type *TPS1* (557-bp product) and transposon-inserted *tps1-2* (1,060-bp product).

Qiaex RNeasy plant columns (Qiagen USA, Valencia, CA) were used to isolate RNA for Q-PCR analysis. Small-scale RNA isolation from embryos was performed with the PureScript plant RNA extraction kit (Gentra, Minneapolis) as described previously (Eastmond et al., 2002). For Q-PCR analysis, total RNA was DNAseI (DNA-Free; Ambion, Austin, TX) treated to remove genomic DNA. Absence of DNA was analyzed by performing a PCR reaction (40 cycles, similar to the real-time PCR program) on the DNAseI-treated RNA using Taq-DNA polymerase. Sixty units of M-MLV Reverse Transcriptase (Promega, Madison, WI) were used according to the manufacturer's protocol on 1 μg of total RNA to synthesize cDNA with 0.5 μg of odT16V (custom oligo from Invitrogen, Carlsbad, CA) and 0.5 μg random hexamer (Invitrogen). After cDNA synthesis, the sample volume was increased to 200 μL with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), from which 5 μL were used as template per Q-PCR reaction. The ABI-prism 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) was used for the real-time PCR measurements. Per reaction, 12.5 μL of Taqman Universal PCR Master Mix (PE-Applied Biosystems), 250 nM gene-specific Taqman probe, and 900 nM of each gene-specific primer (see below) were supplied in a 25-μL reaction volume. Q-PCR was performed according to the standard Taqman Universal PCR Master Mix protocol (PE-Applied Biosystems). Relative quantitation of gene expression is based on the comparative C_T method (User Bulletin No. 2: ABI PRISM 7700 Sequence Detection System, 1997) using *AtACTIN2* as a calibrator reference. Relative expressions are represented as a fraction from the highest level of each gene, unless otherwise mentioned.

Gene-Specific Primer-Probe Pairs and Their Efficiencies

AtTPS1 (At1G78580) specific: AtTPS1-probe (5′-FAM-atctccttggctcacctgacgacgtc-TAMRA-3′); AtTPS1-F (5′-tgggtcgtactcgcaccaa-3′) and AtTPS1-R (5′-tttgcttccttgagaagctcg-3′); Efficiency: 1.90. *AtACT2* (At3g18780) specific: AtACT2-probe (5′-FAM-aagtcttgttccagccctcgtttgtgc-TAMRA-3′); AtACT2-F (5′-gctgagagattcagatgccca-3′) and AtACT2-R (5′-atggaagctgctggaatccac-3′); Efficiency: 1.97. *AtTPS5* (At4g17770) specific: AtTPS5-probe (5′-FAM-tcccaagaatatcgtgtacctcgtcagtgg-TAMRA-3′); AtTPS5-F (5′-ccgcgaaacaatcgaaatct-3′) and AtTPS5-R (5′-ttcccagtctgttccatcatttg-3′); Efficiency: 1.93. *AtTRE1* (At4G24040) specific: AtTRE1-probe (5′-FAM-ttcgtctcagatccctccggcttcc-TAMRA-3′); AtTRE1-F (5′-gctgcaccacgaaccagtaga-3′) and AtTRE1-R (5′-ttcttcgttctccacgttgga-3′); Efficiency: 1.98. Flowering-time gene pairs are described by El-Din El-Assal et al. (2003). Fluorogenic probes were obtained from Sigma-genosys (Cambridge, UK), and all custom primers were from Invitrogen (Paisley, UK).

Microscopy and β-Glucuronidase Assay

Siliques were opened and embryos excised using precision forceps. For Nomarski images, embryos were taken in chloralhydrate [chloralhydrate: water:glycerol = 8:3:1 (v/v)], placed between an object and a covering slide, after which photos were directly made using Nomarski optics on a Zeiss photomicroscope (Jena, Germany). In case of *GUS* localization, the excised seeds and embryos were incubated in X-Gluc buffer {5× GUS-buffer, 100 mL: 250 mg X-Gluc, 0.1 g Triton X-100, 82.3 mg K-ferricyanide [$K_3Fe(CN)_6$, M = 329.9 g mol^{-1}], 105.6 mg K-ferrocyanide [$K_4Fe(CN)_6$-$3H_2O$, M = 422.4 g mol^{-1}] in 50 mM phosphate buffer} at 37°C for 10 h before clearing in chloralhydrate and Nomarski imaging. For confocal laser scanning microscopy (Leica, Wetzlar, Germany), seedlings were stained with propidium iodine (1 μg mL^{-1}).

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