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ACCELERATED PUBLICATION

Trehalose-6-phosphate synthase 1 is not the only active TPS in *Arabidopsis thaliana*

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Trehalose metabolism is essential for normal growth and development in higher plants. It is synthesized in a two-step pathway catalysed by TPS (trehalose-6-phosphate synthase) and trehalose phosphatase. *Arabidopsis thaliana* has 11 TPS or TPS-like proteins, which belong to two distinct clades: class I (AtTPS1–AtTPS4) and class II (AtTPS5–AtTPS11). Only AtTPS1 has previously been shown to have TPS activity. *A. thaliana tps1*Δ mutants fail to complete embryogenesis and rescued lines have stunted growth and delayed flowering, indicating that AtTPS1 is important throughout the life cycle. In the present study, we show that expression of AtTPS2 or AtTPS4 enables the yeast *tps1*Δ *tps2*Δ mutant to grow on glucose and accumulate Tre6P (trehalose 6-phosphate) and trehalose. Class II *TPS* genes did not complement the yeast mutant. Thus *A. thaliana* has at least three catalytically active TPS isoforms, suggesting that loss of Tre6P production might not be the only reason for the growth defects of *A. thaliana tps1* mutants.

Key words: *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, trehalose, trehalose phosphatase (TPP), trehalose 6-phosphate, trehalose-6-phosphate synthase (TPS).

INTRODUCTION

Trehalose is a non-reducing disaccharide that functions as a compatible solute, stress protectant, carbon reserve or transport sugar in many bacteria, archaea, fungi and invertebrates. Some vascular plants, such as *Selaginella lepidophylla* and *Myrothamnus flabellifolius,* can accumulate high levels of trehalose, which is considered a prerequisite for surviving extreme drought [1,2]. In *Arabidopsis thaliana* and other drought-intolerant species only trace amounts of trehalose are detected, despite the extensive family of genes potentially encoding enzymes of trehalose biosynthesis [3–5].

Analyses of various mutants and transgenic plants have implicated trehalose metabolism in the regulation of embryogenesis, vegetative growth and flowering [6–12], stomatal conductance [11,13,14] and transitory starch metabolism [15]. Many of the developmental and metabolic effects of perturbing trehalose metabolism are attributable to changes in the level of Tre6P (trehalose 6-phosphate), the intermediate of trehalose biosynthesis. Tre6P is proposed to be a signal of sucrose availability and part of a homoeostatic mechanism for regulating sucrose levels in plant cells [16–18].

Streptophyte plants have multiple *TREHALOSE-6-PHOSPHATE SYNTHASE* (*TPS*) and *TREHALOSE PHOSPHATASE* (*TPP*) genes, with evidence of independent duplications within these families in different plant lineages [4,5,19]. In *A. thaliana*, there are 11 *TPS* genes (*AtTPS1–AtTPS11*) encoding TPS or TPS-like proteins, and ten *TPP* genes (*AtTPPA–AtTPPJ*) encoding catalytically active TPPs [3,20]. The TPS family is split into two distinct clades, class I and class II, and this dichotomy occurred early in the evolution of green plants [3–5,19]. There are four class I *TPS* genes in *A. thaliana*: *AtTPS1–AtTPS4*. These encode proteins with a glycosyltransferase domain that closely resembles the TPS enzymes from *Escherichia coli* (otsA) and yeast (*Sc*Tps1), and a C-terminal domain that has similarity to plant TPP enzymes but lacks some of the active-site residues involved in dephosphorylation of Tre6P [3,4,13,20]. So far, only AtTPS1, and its orthologue from *S. lepidophylla* (SlTPS1), have demonstrated TPS activity in *in vitro* assays of the purified or recombinant protein or by complementation studies in a yeast (*Saccharomyces cerevisiae*) *tps1*Δ mutant. This mutant fails to synthesize Tre6P, thereby deregulating the initial part of glycolysis and causing growth arrest on glucose-containing medium [20–22].

Apart from polyploid and allopolyploid species, *A. thaliana* is quite unusual among angiosperms in having multiple class I *TPS* genes. The *AtTPS2* and *AtTPS3* genes are present in a tandem repeat on chromosome 1, within what is probably a large segmental duplication of the *AtTPS1*-containing region, whereas *AtTPS4* appears to have originated from a more localized duplication event [4]. The AtTPS2–AtTPS4 proteins are smaller than AtTPS1, in part because they lack an autoinhibitory N-terminal region upstream of the glycosyltransferase domain [4,23]. In addition, the putative glycosyltransferase domain of the predicted AtTPS3 protein contains several deletions compared with the other class I proteins, and *AtTPS3* transcripts are expressed at a very low level, if at all, suggesting that *AtTPS3* is a pseudogene [4,13,17]. *AtTPS1* is expressed in meristems of heart-stage embryos and expression is widespread in mature plants, especially in sink organs such as the shoot apical meristem,

Abbreviations: Glc6P, glucose 6-phosphate; HA, haemagglutinin; TPP, trehalose phosphatase; TPS, trehalose-6-phosphate synthase; Tre6P, trehalose 6-phosphate; UDP-Glc, UDP-glucose.

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flower buds and ripening siliques [8]. *AtTPS2* and *AtTPS4* are expressed primarily in developing seeds, although transcripts are detectable in other tissues [13,17,24].

The embryo arrest of the *A. thaliana tps1* mutant and the perturbed phenotypes of the rescued *tps1* mutants showed that a functional AtTPS1 is required for normal growth and development throughout the life cycle of the plant, and that even the closely related class I TPS isoforms, AtTPS2 and AtTPS4, cannot compensate for loss of AtTPS1 function. Unlike *AtTPS1*, the *AtTPS2* and *AtTPS4* genes did not complement the yeast *tps1*Δ mutant for growth on glucose when expressed under the control of a truncated *HXT7* promoter in the pYX212 yeast expression vector [20]. These findings suggested that the AtTPS2 and AtTPS4 proteins do not have TPS activity, and that AtTPS1 is the only catalytically active isoform in *A. thaliana*. This implied that *A. thaliana tps1*-null mutants (e.g. *tps1-1*, *tps1-2*) are unable to synthesize Tre6P, and their defective phenotypes were attributed primarily to loss of this function.

The function of the other class I TPS isoforms remains an open question. Although apparently lacking TPS activity, the AtTPS2 and AtTPS4 proteins retain almost all of the TPS active-site residues found in AtTPS1 and active TPS enzymes from other species [4,20]. To address this apparent paradox, we re-evaluated the class I isoforms by yeast complementation assays using additional yeast mutants. We found that both AtTPS2 and AtTPS4 are able to complement a yeast *tps1*Δ *tps2*Δ strain and that their expression results in high Tre6P levels in yeast cells. The implications of these results for interpretation of the *A. thaliana tps1* mutant phenotype are discussed.

EXPERIMENTAL

Alignment of AtTPS1, AtTPS2 and AtTPS4 and molecular modelling

Protein sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). Alignment was performed using CLC Main Workbench (CLC Bio). Molecular graphics and analysis were performed with the UCSF Chimera package, developed by Resource for Biocomputing, Visualization and Informatics, University of California, San Francisco [25] and based on the crystal structure of otsA in complex with UDP and Glc6P (glucose 6-phosphate) [26] (PDB number 1GZ5).

Plasmid construction

TPS coding sequences (minus the stop codon) were amplified from cDNA derived from *A. thaliana* Col-0 flowers and siliques, and cloned in frame with a C-terminal double HA (haemagglutinin) tag in a yeast multicopy plasmid (pYX212) with a truncated *HXT7* promoter and *URA3* maker. Primers for PCR were as follows: AtTPS1-fwd (5′-ggaattc-ATGCCTGGAAATAAGTACAACTGC-3′, partial digest), AtTPS1-rev (5′-tcccccgggAGGTGAGGAAGTGGTGTCAG-3′), AtTPS2-fwd (5′-cgacgcgtATGGATTATGATGATGCACGTG-3′), AtTPS2-rev (5′-aaggcctCATGCCGATTTGCCCAGTGT-3′), AtTPS4-fwd (5′-catgccATGGCAAGGCCACGACTGCT-3′), AtTPS4-rev (5′-aaggcctAGCATTGTTGTGTGTGCAAAGC-3′). Cloned coding regions were confirmed by sequencing.

Yeast transformation, complementation and growth assays

All experiments were performed in the W303-1A background (Mata leu2-3, 112 ura3-1 trp1-1 his3-11, A5 ade2-1 can1-100 GAL SUC2). Yeast *TPS1*, *A. thaliana TPS1*, Δ*NTPS1*, *TPS2* and *TPS4*, cloned in the pYX212 vector, were introduced into the *S. cerevisiae tps1*Δ deletion strain YSH290 (W303-1A, *tps1*Δ*::TRP1*) and the *tps1*Δ *tps2*Δ deletion strain YSH567 (W303-1A, *tps1*Δ*::TRP1 tps2*Δ*::LEU2*) [27]. A W303-1A wild-type strain carrying the empty pYX212 plasmid was used as a positive control, along with *tps1*Δ and *tps1*Δ *tps2*Δ deletion strains complemented with pYX212/ScTPS1HA2x.

Yeast transformation was performed with the lithium acetate/PEG method without heat shock, as described by Gietz et al. [28], and positive transformants were selected on SD − URA (synthetic dropout medium lacking uracil) galactose plates.

Starter cultures were grown overnight at 30 °C with shaking at 200 rev./min in selective minimal medium containing 2 % (w/v) galactose. For spot assays, six dilution series were made, starting from an OD_{600} of 0.5 and 10 μl of each dilution was spotted on plates containing selective medium supplied either with 2 % galactose or with 2 % glucose. The plates were incubated for 48 h at 30 °C and pictures were taken using a digital camera. For analysis of growth kinetics, cells were diluted to an OD_{600} of 0.05, and growth was monitored by measurement of OD_{600} every 30 min using an Automated Microbiology Growth Analysis System (Bioscreen C) for 3 days at 30 °C with constant shaking at 200 rev./min.

Western blot analysis

Expression of HA-tagged TPS proteins in yeast cells was confirmed by SDS/PAGE of yeast extracts and immunoblotting with horseradish-peroxidase-coupled rat anti-HA High Affinity antibodies (Roche), as described previously by Vandesteene et al. [20].

Metabolite measurements

Tre6P was measured in chloroform/methanol extracts of yeast cells by high-performance anion-exchange chromatography coupled to tandem MS as described by Lunn et al. [16]. Trehalose was measured enzymatically in the same extracts as described in Carillo et al. [29].

RESULTS

Class I TPSs complement yeast *tps1*Δ and *tps1*Δ *tps2*Δ mutants

Crystal structures of the *E. coli* otsA protein with substrates or substrate analogues bound in the active site have revealed which residues are involved in substrate binding and enzymatic catalysis [26,30]. Sequence alignments of otsA with *A. thaliana* TPS proteins showed that almost all of the active-site residues are conserved in AtTPS1 and the other class I TPS proteins [4,20]. Modelling of the putative active sites of the *A. thaliana* class I TPS proteins confirmed that all of the amino acid residues that interact with Glc6P and UDP-Glc (UDP-glucose) are conserved (Figure 1), with just a few exceptions. When UDP-Glc is bound in the active site of the otsA protein, the uracil moiety interacts with Phe^{339}, which is replaced by branched-chain amino acids in the plant proteins: Leu^{446} in AtTPS1, Val^{365} in AtTPS2 and Ile^{357} in AtTPS4. However, the side-chain differences are not expected to have much impact on substrate/product binding because the ligand interacts only with the main-chain hydrogen and carbonyl moieties of Phe^{339} in the otsA protein. Gln^{185}, which interacts with the glucosyl moiety of UDP-Glc, is replaced in all of the *A. thaliana* class I proteins by another positively charged amino acid, histidine. Among the Glc6P-binding residues, Ile^{155} in otsA

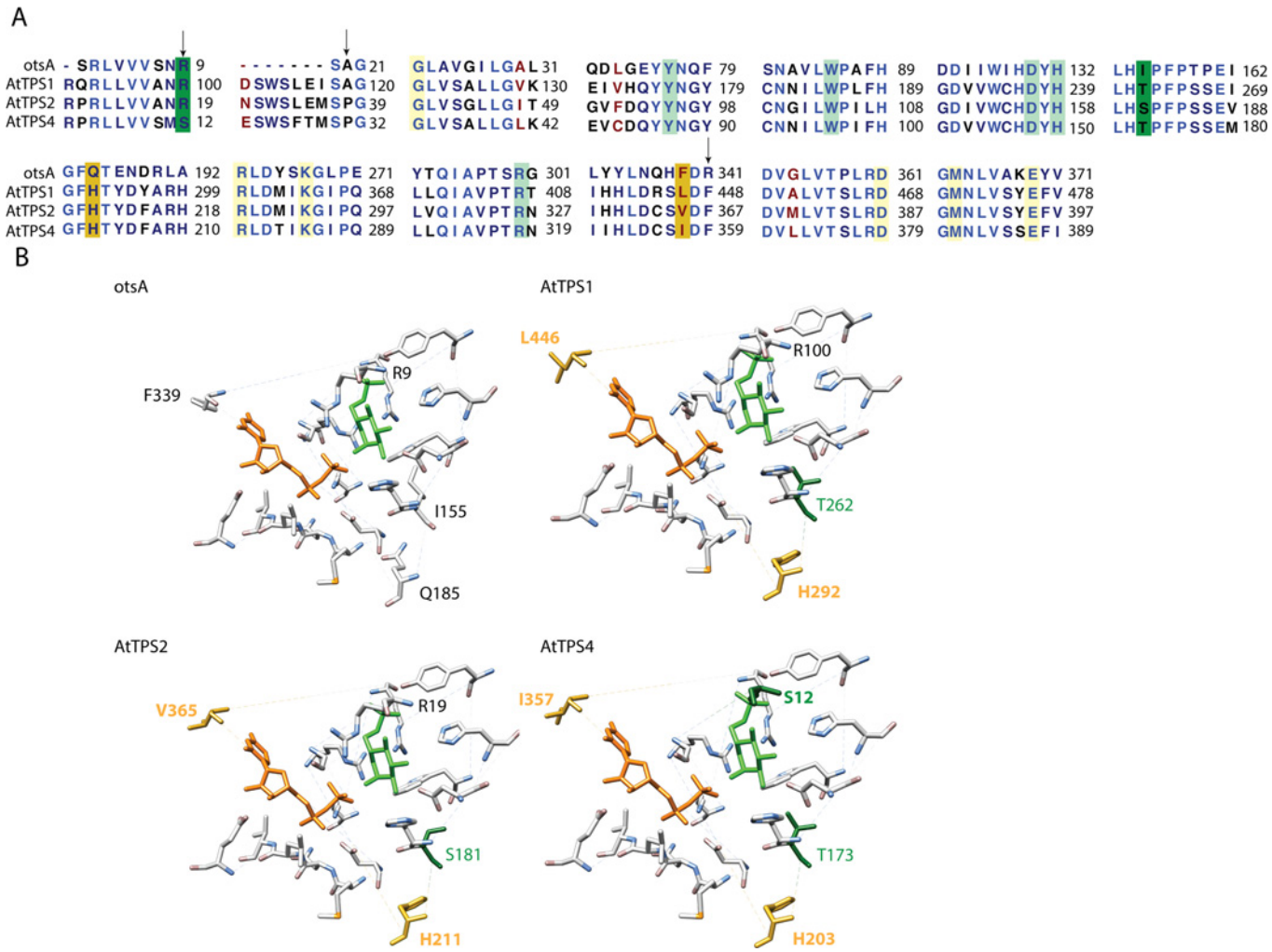

Figure 1 Proposed model of Glc6P and UDP-Glc binding to TPS enzymes

(**A**) Alignment of *E. coli* otsA with AtTPS1, AtTPS2 and AtTPS4. Only relevant sequences are shown. Green boxes show conserved residues important for Glc6P binding. Yellow boxes indicate residues important for UDP-Glc binding. Darker coloured boxes indicate residues that are important for substrate binding, but that differ between the proteins. Arrows indicate residues important for stabilization of the interaction. (**B**) Modelling of the important donor- and acceptor-binding sites as predicted by the crystal structure of otsA with its substrate. UDP-Glc is depicted in orange. Glc6P is depicted in green. Different interactors for UDP-Glc binding are shown in yellow. Different interactors for Glc6P binding are shown in dark green [26,30].

is replaced by hydroxy-gropup-containing amino acids: threonine in AtTPS1 and AtTPS4, and serine in AtTPS2, while Arg^{341} (otsA) is replaced by phenylalanine in all of the plant class I proteins (Figure 1). Ala^{20}, which stabilizes the open conformation of otsA, is replaced by proline in AtTPS2 and AtTPS4. In otsA, Arg^{9} interacts with Glc6P via a solvent-mediated connection between the O3 hydroxy group of the glucosyl moiety and the main-chain amide and the 4-position hydroxy group, and is retained in AtTPS1 and AtTPS2, but replaced by serine in AtTPS4. Thus, with the exception of the A20P substitution, there are few obvious differences between the putative active sites of AtTPS2 and AtTPS4 and the active sites of otsA and AtTPS1.

The enzymatic capacity of the class I proteins was first investigated by complementation assays in yeast mutants. The yeast *tps1*Δ mutant is unable to grow on glucose because of an uncontrolled influx of glucose into glycolysis resulting in a depletion of ATP [23]. There is evidence that both the enzyme itself and the inhibitory effect of Tre6P on hexokinase activity contribute to the deregulated entry of glucose into glycolysis [31,32]. The *tps1*Δ mutant grows normally on galactose because the influx of galactose into glycolysis is not mediated by hexokinase but by galactokinase, the activity of which is not regulated by Tre6P, so the influx of galactose into glycolysis remains normal in the *tps1*Δ mutant. The *tps1*Δ *tps2*Δ double mutant is similarly unable to grow on glucose, but grows on galactose.

The coding sequences of *AtTPS1*, *AtTPS2* and *AtTPS4* were cloned into the pYX212 yeast expression vector under the control of a truncated *HXT7* promoter, which drives constitutive expression. *AtTPS3* was not included in the experiments because previous studies indicate that this likely pseudogene is not expressed and its putative coding region is corrupted [4,20]. No complementation assays were performed in the yeast *tps2*Δ mutant, which has no Tre6P-specific TPP activity, as the TPP-like domains of plant class I TPS proteins lack residues that are essential for Tre6P binding and hydrolysis [4], and previous experiments confirmed that the class I TPS proteins do not complement the yeast *tps2*Δ mutant [5,20,22].

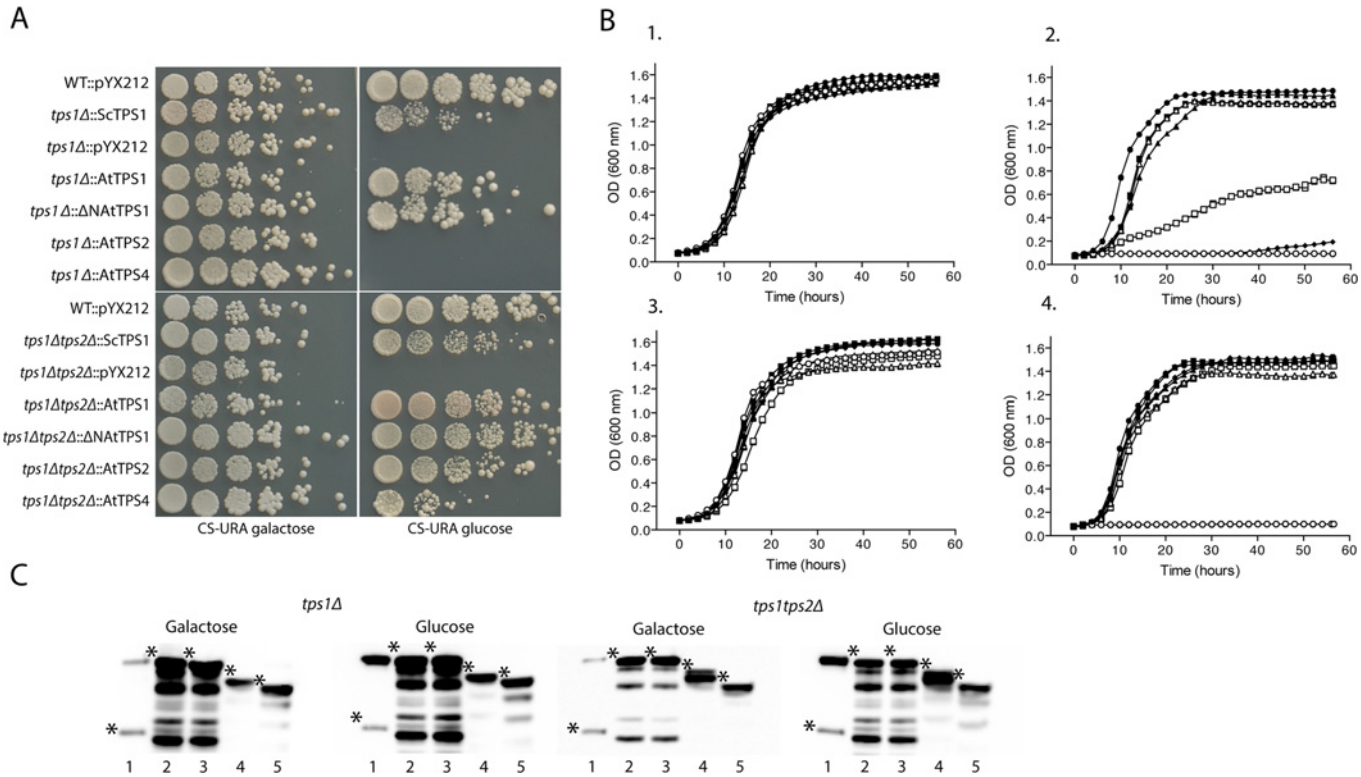

Figure 2 ***A. thaliana*** **has three active TPS isoforms**

(**A**) Complementation assay of AtTPS1, ΔN-AtTPS1, AtTPS2 and AtTPS4 in yeast *tps1*Δ and *tps1*Δ *tps2*Δ strains grown on galactose (left-hand panels) and on glucose (right-hand panels). Control strains are wild-type (WT) with empty pYX212 plasmid and yeast mutants expressing *ScTPS1* or the empty pYX212 plasmid. (**B**) Growth of yeast *tps1*Δ and *tps1*Δ *tps2*Δ strains expressing AtTPS1 (△), ΔN-AtTPS1 (■), AtTPS2 (□) and AtTPS4 (◆) in yeast *tps1*Δ and *tps1*Δ *tps2*Δ growing on galactose (panels 1 and 3) and on glucose (panels 2 and 4). Control strains are wild-type with empty pYX212 plasmid (●) and yeast mutants with *ScTPS1* (▲) and pYX212 (○). (**C**) Western blot analysis confirming the similar expression levels of proteins in yeast *tps1*Δ and *tps1*Δ *tps2*Δ strains grown on galactose (left-hand panels) and supplied with 2 % glucose for 1 h (right-hand panels). Numbers 1–5 indicate ScTPS1, AtTPS1, ΔN-AtTPS1, AtTPS2 and AtTPS4 respectively.

The *A. thaliana* class I proteins were expressed in the *S. cerevisiae tps1*Δ and *tps1*Δ *tps2*Δ mutants. The latter strain was included since previous experiments with *S. lepidophylla TPS1* indicated no restored growth on glucose when expressed in the *tps1*Δ mutant, but a clear complementation of the *tps1*Δ *tps2*Δ mutant. We investigated the ability of the AtTPS1, AtTPS2 and AtTPS4 proteins to suppress the glucose growth defect of the two yeast strains (Figure 2A). In addition, we tested a truncated form of AtTPS1 (ΔN-AtTPS1) that lacks the N-terminal autoinhibitory domain, and thus resembles the structure of the smaller class I isoforms, AtTPS2 and AtTPS4. Each of the plant proteins was expressed with a C-terminal HA tag to allow confirmation of expression by immunoblotting. Serial dilutions of overnight cultures of the different strains were spotted on selective medium containing either 2 % galactose or 2 % glucose. As expected, all strains grew equally well on galactose and both AtTPS1 and ΔN-AtTPS1 complemented the glucose growth defect of the yeast *tps1*Δ strain, but AtTPS2 and AtTPS4 did not [20] (Figure 2A, upper panels). However, in the *tps1*Δ *tps2*Δ background, both AtTPS2 and AtTPS4 did complement the growth defect on glucose, with AtTPS2 being almost as effective at AtTPS1 and ΔN-AtTPS1 (Figure 2A, lower panels). Growth assays in liquid medium confirmed that expression of AtTPS2 or AtTPS4 allowed growth of the *tps1*Δ *tps2*Δ strain on glucose (Figure 2B). Interestingly, growth in glucose-containing liquid medium was also observed for the *tps1*Δ strains expressing AtTPS2 or AtTPS4, although these grew more slowly than the AtTPS1- and ΔN-AtTPS1-expressing strains (Figure 2B). Growth of the AtTPS2- and AtTPS4-expressing *tps1*Δ strains on glucose was confirmed when serial dilutions were spotted on to solid selective medium containing low concentrations (1–10 mM) of glucose (results not shown). None of the *A. thaliana* class II TPS isoforms was able to complement the glucose growth defect of the *tps1*Δ *tps2*Δ mutant when expressed under the control of the *HXT7* promoter, and no Tre6P was detected in class II TPS-expressing strains of the *tps1*Δ *tps2*Δ mutant when grown on galactose (results not shown).

Immunoblotting analysis with anti-HA antibodies was used to compare the expression levels of the various HA-tagged TPS proteins in the yeast cells. Figure 2C shows that the full-length TPS proteins were expressed at very similar levels in both the *tps1*Δ and *tps1*Δ *tps2*Δ mutant backgrounds, irrespective of whether the cells were grown on galactose or glucose. As observed in a previous study [33], several smaller immunoreactive proteins were also seen in the yeast extracts, particularly from cells expressing AtTPS1 and ΔN-AtTPS1 (Figure 2C). It is unclear whether these are truncated forms of the proteins derived from initiation of translation from internal ATG (methionine) codons within the TPS coding sequence, or result from proteolytic cleavage of the full-length proteins. In summary, both AtTPS2 and AtTPS4 are able to fully complement the growth defect of the yeast *tps1*Δ *tps2*Δ mutant on glucose, and also partially

Table 1 Tre6P and trehalose content of wild-type and mutant yeast strains

AtTPS1, ΔN-AtTPS1, AtTPS2 and AtTPS4 from *A. thaliana* were expressed in the yeast (*S. cerevisiae*) *tps1*Δ and *tps1*Δ *tps2*Δ mutants, under the control of a constitutive *HXT7* promoter in yeast expression vector pYX212. Tre6P and trehalose were determined in cells grown in liquid medium containing 2 % (w/v) galactose or 2 % (w/v) glucose. Metabolite contents are compared to wild-type yeast, the parental *tps1*Δ and *tps1*Δ *tps2*Δ mutants, and the mutants complemented with the yeast ScTPS1. Results are single determinations.

		Tre6P (nmol·g^{-1} fresh weight)		Trehalose (μmol·g^{-1} fresh weight)	
Genotype	Construct	2 % Galactose	2 % Glucose	2 % Galactose	2 % Glucose
WT	pYX212	163	20	29.50	24.60
*tps1*Δ	pYX212	3	–*	<0.05	–*
*tps1*Δ	ScTPS1	13	10	3.11	<0.05
*tps1*Δ	AtTPS1	46	5	1.92	<0.05
*tps1*Δ	ΔN-AtTPS1	59	5	2.20	0.05
*tps1*Δ	AtTPS2	1	–*	0.12	–*
*tps1*Δ	AtTPS4	11	–*	<0.05	–*
*tps1*Δ*tps2*Δ	pYX212	5	–*	<0.05	–*
*tps1*Δ*tps2*Δ	ScTPS1	6950	10	1.67	<0.05
*tps1*Δ*tps2*Δ	AtTPS1	224	255	<0.05	0.06
*tps1*Δ*tps2*Δ	ΔN-AtTPS1	523	475	<0.05	0.08
*tps1*Δ*tps2*Δ	AtTPS2	13300	14700	3.44	6.09
*tps1*Δ*tps2*Δ	AtTPS4	942	1030	0.15	1.34

*Cells did not grow.

complement the yeast *tps1*Δ mutant. These results suggested that, in contrast with previous conclusions, both AtTPS2 and AtTPS4 do have TPS activity.

Expression of AtTPS2 and AtTPS4 results in the production of Tre6P

To investigate whether the growth complementation of the yeast mutants by AtTPS2 and AtTPS4 was indeed due to these isoforms having TPS activity, we measured the levels of Tre6P and trehalose in extracts of the various yeast transformants grown with either galactose or glucose. When grown on galactose, *tps1*Δ and *tps1*Δ *tps2*Δ strains carrying the empty pYX212 expression vector appeared to contain trace amounts of Tre6P (3–5 nmol·g^{-1} fresh weight, equivalent to 2–3 % of the level in wild-type cells), but no detectable trehalose (Table 1). Tre6P was measured by high-performance anion-exchange chromatography coupled to tandem MS, with all samples being spiked with a [^{2}H]Tre6P internal standard to allow correction for ion suppression and other matrix effects [16]. However, measurements of the [^{2}H]Tre6P alone (i.e. without yeast extract) showed that it is contaminated by a small amount (<2 %) of unlabelled Tre6P. Although we attempted to normalize and subtract this background for each individual sample, any errors in the correction would be magnified by a factor of 200 during the calculation of the values shown in Table 1. This is because the yeast extracts were diluted 200-fold in order for the extracts with high levels of Tre6P to be within the linear range of the assay. Therefore, as the *tps1*Δ and *tps1*Δ *tps2*Δ mutants have no known source of Tre6P synthesis, we attribute the trace levels of Tre6P seen in the non-complemented mutants to imperfect correction for the background derived from the internal standard.

The AtTPS1- and ΔN-AtTPS1-expressing *tps1*Δ cells had more Tre6P than the non-complemented mutant grown on galactose, although only 28 % and 36 % of the levels in wild-type yeast cells respectively. Tre6P was lower in the AtTPS1 and ΔN-AtTPS1-complemented *tps1*Δ cells when grown on glucose, but still above the background of the *tps1*Δ cells carrying the empty pYX212 vector. In this experiment, there was too little growth of the AtTPS2- and AtTPS4-expressing *tps1*Δ strains on glucose to measure metabolites. However, when grown on galactose, above background levels of Tre6P were detected in the AtTPS4-expressing strain, whereas the AtTPS2-expressing strain contained a small amount of trehalose, presumably derived from synthesis of Tre6P by the AtTPS2 protein.

The *tps1*Δ *tps2*Δ mutant lacks a Tre6P-specific TPP enzyme. Therefore any strains with Tre6P-synthesizing capacity would be expected to contain higher levels of Tre6P than the corresponding strains in the *tps1*Δ background, in which Tre6P can be converted into trehalose. This is indeed what we observed, with all of the complemented strains having higher levels of Tre6P than wild-type yeast cells when grown on galactose (Table 1). With the exception of ScTPS1-complemented cells, the complemented strains also had above wild-type levels of Tre6P when grown on glucose. The AtTPS2- and AtTPS4-expressing strains had particularly high levels of Tre6P, being 81- and 5-fold higher than wild-type on galactose, and 735- and 51-fold higher than wild-type on glucose respectively. Surprisingly, the AtTPS2 and AtTPS4-expressing *tps1*Δ *tps2*Δ strains also contained substantial levels of trehalose, irrespective of the carbon source in the medium (Table 1). Trehalose accumulation was also seen in the ScTPS1-expressing cells, but only when these were grown on galactose.

DISCUSSION

Plants have two distinct clades of *TPS* genes, i.e. class I and class II, but only members of class I have been unequivocally proven to encode catalytically active TPS enzymes [21,23]. *A. thaliana*, in common with other species in the Brassicaceae, has an unusually large family of class I genes for a diploid species, although one of the four genes, *AtTPS3*, is thought to be a pseudogene [4]. The AtTPS1 isoform has been shown to have catalytic activity *in vitro* and is able to complement the growth phenotype of the yeast *tps1*Δ mutant on glucose [21]. In contrast, expression of AtTPS2 or AtTPS4 in the yeast *tps1*Δ mutant failed to restore growth on glucose, leading to the conclusion that these two isoforms did not synthesize Tre6P when expressed in yeast, and that AtTPS1 is the only catalytically active TPS in *A. thaliana* [13]. The apparent inactivity of AtTPS2 and AtTPS4 was rather surprising because TPS active-site residues are highly conserved in both isoforms (Figure 1), with only a single mutation of Ala^{20} to

proline differentiating them from the catalytically active AtTPS1. As discussed below, the conclusion that AtTPS2 and AtTPS4 do not have TPS activity had important implications for interpretation of the embryo lethality and other growth phenotypes of *A. thaliana tps1* mutants. However, as this conclusion was based on a single line of negative evidence, and was difficult to reconcile with the high level of conservation of active-site residues, we felt it was necessary to re-examine the enzymatic capacity of AtTPS2 and AtTPS4 with a more comprehensive yeast complementation analysis.

The *AtTPS2*- and *AtTPS4*-coding regions were expressed not only in the yeast *tps1*Δ mutant background, but also in the *tps1*Δ *tps2*Δ double mutant. We reasoned that the absence of a Tre6P-specific TPP enzyme in the latter might allow accumulation of Tre6P even if the introduced plant proteins had only weak TPS activity, and therefore that the cells might have enough Tre6P to restrict hexokinase activity and so permit growth on glucose. Thus complementation assays in the double mutant are potentially a more sensitive test for TPS activity than in the *tps1*Δ background. Our results showed that AtTPS2 and AtTPS4 do indeed complement the glucose growth phenotype of the *tps1*Δ *tps2*Δ double mutant (Figure 2A), with the complemented strains having similar growth rates to wild-type yeast and the AtTPS1- and ΔN-AtTPS1-complemented mutant strains in glucose-containing medium (Figure 2B). These results indicated that both AtTPS2 and AtTPS4 do have TPS activity, which was confirmed by the detection of high levels of Tre6P in the complemented yeast cells (Table 1). Indeed, the AtTPS2- and AtTPS4-complemented strains had exceptionally high levels of Tre6P compared with the other strains when grown on glucose, suggesting that they might have even higher specific activity than the AtTPS1 and ΔN-AtTPS1 proteins, as each of the *A. thaliana* proteins was expressed at a similar level in the respective yeast strains (Figure 2C). We also tested all of the class II TPS isoforms for complementation of the yeast *tps1*Δ *tps2*Δ mutant, but none of them was able to restore growth on glucose, and strains expressing the class II proteins contained no detectable Tre6P when grown on galactose. This confirms our previous findings that the class II TPS-like proteins do not have TPS activity [34].

The presence of trehalose in some of the complemented strains in the background *tps1*Δ *tps2*Δ was unexpected, because these lack the Tre6P-specific TPP encoded by the *ScTPS2* gene. The concentrations of Tre6P accumulated in the complemented strains were up to 90-fold higher than in wild-type yeast (Table 1), with the AtTPS2-complemented cells having almost 15 mM Tre6P, if an intracellular volume of 1 $ml \cdot g^{-1}$ fresh weight is assumed. Thus, in the presence of millimolar concentrations of Tre6P, even non-specific phosphatases with a low affinity for Tre6P might have significant Tre6P-hydrolysing activity, and account for the presence of trehalose in strains with no Tre6P-specific TPP activity. Two potential candidates are Dog1 and Dog2, which were originally designated as 2-deoxyglucose-6-phosphate phosphatases [35], but were subsequently shown to hydrolyse a broad range of sugar-phosphates [36,37]. *Dog2* expression is repressed by glucose [38–40], so if the Dog2 isoform is primarily responsible for Tre6P hydrolysis in the *tps1*Δ *tps2*Δ background, this would explain the lower levels of trehalose in cells grown with glucose compared with those grown with galactose (Table 1).

Having demonstrated that AtTPS2 and AtTPS4 have TPS activity in the *tps1*Δ *tps2*Δ mutant background, we tested the new expression constructs in the yeast *tps1*Δ mutant to compare with previous studies. In agreement with the findings of Vandesteene et al. [20], we observed no growth of *tps1*Δ (AtTPS2) or *tps1*Δ (AtTPS4) cells when serial dilutions were spotted on selective medium containing 2% glucose and incubated for 48 h (Figure 2A). However, both of these strains did grow in liquid culture medium containing 2% glucose, albeit more slowly than the wild-type and AtTPS1-complemented *tps1*Δ cells (Figure 2B). The reason for the growth difference between solid and liquid cultures is unclear. If regulation of glycolytic fluxes were only partially restored in the AtTPS2- and AtTPS4-expressing strains, it is conceivable that toxic respiratory intermediates accumulated in the cells, but were able to diffuse out of the cells grown in liquid medium allowing the cells to continue growing. Another possibility is that poorly regulated glycolytic fluxes perturbed the phosphate status of the cells, but the liquid-grown cells were better able to cope with this because they had access to a larger supply of P_i from the medium.

When grown in galactose-containing medium, the AtTPS2- and AtTPS4-expressing cells contained detectable amounts of trehalose or Tre6P respectively. Although the levels were very low, they were higher than those in the non-complemented *tps1*Δ (pYX212) mutant (Table 1). Thus, even though the growth phenotype on glucose is only weakly complemented, the metabolite data confirmed that AtTPS2 and AtTPS4 are capable of synthesizing Tre6P in the *tps1*Δ background.

In yeast cells, the two enzymes of trehalose biosynthesis, Tps1 and Tps2 (TPP) associate to form a trehalose-synthesizing complex, which also contains two types of non-catalytic subunit, Tps3 and Tsl1 [41]. The presence of such a complex offers a potential explanation for the marked differences in metabolite levels and degree of complementation in the *tps1*Δ and *tps1*Δ *tps2*Δ backgrounds. If AtTPS2 and AtTPS4 were also able to associate with the yeast Tps2 protein, which is still present in the *tps1*Δ mutant, but not in the double mutant, this might interfere with their activity, limiting their capacity to produce Tre6P. Interference might occur by steric hindrance of their active sites by Tps2, or binding of Tps2 might push them into a less active conformation or one that is susceptible to post-translational modifications (e.g. by yeast protein kinases) that inhibit their activity. In this scenario, the greater ability of the AtTPS1 and ΔN-AtTPS1 isoforms to complement the *tps1*Δ mutant might be explained by a lack of binding to Tps2, or non-interference with their activity if they do bind to Tps2.

Our results, revealing the TPS activity of AtTPS2 and AtTPS4, directly question current interpretations of the *A. thaliana tps1* mutant phenotype, and have wider implications for our understanding of the roles of class I TPS proteins and Tre6P in other plants. When AtTPS1 was thought to be the only catalytically active TPS in *A. thaliana*, loss of Tre6P-synthesizing capacity seemed the most likely explanation for the embryo arrest observed in the *tps1-1* and *tps1-2* null mutants [6], and for the stunted growth and late flowering of the rescued mutants [8,11]. The ability of the *E. coli* otsA protein to partially complement the defective root growth phenotype of the *A. thaliana tps1* mutant appeared to support this conclusion [8]. However, in the light of the discovery that AtTPS2 and AtTPS4 do have TPS activity, and their known expression in developing seeds [20], it seems highly likely that *tps1* embryos retain at least some capacity to produce Tre6P. From this, we can infer that loss of Tre6P-synthesizing capacity might not be the only reason for the arrested development of *tps1* embryos, and that loss of some non-catalytic function of the AtTPS1 protein might also be a contributory factor. In support of this idea, we note that the AtTPS1 protein has been found to associate with a cell-cycle-dependent kinase, CDKA;1, and kinesin KCA1, which are involved in regulation of the cell cycle [33], suggesting that AtTPS1 might be a scaffold or regulatory protein in one or more protein complexes. If AtTPS1 has both catalytic and non-catalytic functions, then its orthologues in other plant species would be expected to have a similar duality. Thus

the implications of our results need to be taken into consideration when investigating the role of AtTPS1 orthologues in other plants, including species outside the Brassicaceae that do not have AtTPS2- or AtTPS4-like isoforms.

As an alternative interpretation, it is possible that the Tre6P-synthesizing capacity of AtTPS1 is required in specific cell types in the embryo where AtTPS2 and AtTPS4 are not expressed, so that loss of AtTPS1 affects embryo development even though some cells (where AtTPS2 and AtTPS4 are expressed) do contain Tre6P. Although current detection methods do not allow us to investigate spatial heterogeneity in the distribution of Tre6P in developing embryos, transcript analysis and promoter–reporter studies lend some weight to this counter argument. *AtTPS1* is expressed in many different cell types, whereas *AtTPS2* is specifically expressed in the chalazal endosperm cyst and possibly in the chalazal proliferative tissue [20]. *AtTPS4* transcripts have been detected in developing embryos, but no expression was detected in embryos of *AtTPS4* promoter–GUS (β-glucuronidase) reporter lines [20]. The chalazal endosperm cyst is a specialized structure of the endosperm involved in seed loading where maternal reserves are transferred to developing endosperm tissue and embryos [42–44], and the chalazal proliferative tissue is maternal tissue that might be involved in translocation of nutrients. Thus AtTPS2, and Tre6P synthesized by this enzyme, might have specific roles in transfer of nutrients between maternal and filial tissues, but be unable to compensate for loss of Tre6P synthesis by AtTPS1 in other cell types. In such a scenario, loss of the Tre6P-synthesizing capacity of AtTPS1 could be the primary or sole reason for the arrested development of *tps1* embryos.

Gene orthologous with *AtTPS2–AtTPS4* are present in syntenic regions of the *Arabidopsis lyrata* and *Brassica rapa* genomes, but have not been found in species outside the Brassicaceae, even in papaya (*Carica papaya*) which belongs to the Caricaceae, a sister family within the order Brassicales [4,18]. It is not immediately obvious why the Brassicaceae have acquired multiple catalytically active TPS isoforms, including truncated forms that lack the autoinhibitory N-terminal domain. Our yeast complementation data suggest that these forms might have higher TPS activity than AtTPS1, raising the possibility that their primary function is to mediate high fluxes of carbon into trehalose rather than generation of small amounts of Tre6P for signalling purposes. Although trehalose levels are usually low in plant tissues [29], and make little contribution to osmoregulation in general, such a role cannot be ruled out in chalazal tissue that has such a specialized function in nutrient transport between maternal and filial tissues.

In summary, our results provide compelling evidence that AtTPS2 and AtTPS4 have TPS activity, and that *A. thaliana* has at least three catalytically active TPS isoforms. This finding opens up the question of the physiological functions of AtTPS1, whose loss in the *tps1* mutant has such a disruptive effect on embryogenesis, vegetative growth and flowering, and why these functions are not substituted by the other catalytically active isoforms. Tissue/cell-specific expression is a plausible, perhaps even likely, explanation for the lack of functional redundancy. However, the possibility that AtTPS1 has non-catalytic functions cannot be excluded, and should be considered when interpreting the phenotypes of *A. thaliana tps1* mutants, and orthologous mutants in other plant species.

AUTHOR CONTRIBUTION

Ines Delorge, Patrick Van Dijck and John Lunn conceived the experiments and wrote the paper. Ines Delorge performed all of the yeast complementation work and all the cloning work. Carlos Figueroa and Regina Feil performed the metabolite measurements.

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