



# Trehalose metabolism in *Arabidopsis*: occurrence of trehalose and molecular cloning and characterization of trehalose-6-phosphate synthase homologues

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## Abstract

Axenically grown *Arabidopsis thaliana* plants were analysed for the occurrence of trehalose. Using gas chromatography–mass spectrometry (GC–MS) analysis, trehalose was unambiguously identified in extracts from *Arabidopsis* inflorescences. In a variety of organisms, the synthesis of trehalose is catalysed by trehalose-6-phosphate synthase (TPS; EC 2.4.1.15) and trehalose-6-phosphate phosphatase (TPP; EC 3.1.3.12). Based on EST (expressed sequence tag) sequences, three full-length *Arabidopsis* cDNAs whose predicted protein sequences show extensive homologies to known TPS and TPP proteins were amplified by RACE–PCR. The expression of the corresponding genes, *AtTPSA*, *AtTPSB* and *AtTPSC*, and of the previously described TPS gene, *AtTPS1*, was analysed by quantitative RT–PCR. All of the genes were expressed in the rosette leaves, stems and flowers of *Arabidopsis* plants and, to a lower extent, in the roots. To study the role of the *Arabidopsis* genes, the *AtTPSA* and *AtTPSC* cDNAs were expressed in *Saccharomyces cerevisiae* mutants deficient in trehalose synthesis. In contrast to *AtTPS1*, expression of *AtTPSA* and *AtTPSC* in the *tps1* mutant lacking TPS activity did not complement

trehalose formation after heat shock or growth on glucose. In addition, no TPP function could be identified for *AtTPSA* and *AtTPSC* in complementation studies with the *S. cerevisiae tps2* mutant lacking TPP activity. The results indicate that while *AtTPS1* is involved in the formation of trehalose in *Arabidopsis*, some of the *Arabidopsis* genes with homologies to known TPS/TPP genes encode proteins lacking catalytic activity in trehalose synthesis.

Key words: *Arabidopsis*, trehalose, trehalose-6-phosphate phosphatase, trehalose-6-phosphate synthase, yeast complementation.

## Introduction

Trehalose ( $\alpha$ -D-glucopyranosyl-1,1- $\alpha$ -D-glucopyranoside) is present in a large variety of microorganisms and invertebrate animals (Elbein, 1974) where it can serve as a reserve carbohydrate and as a stress protectant (Crowe *et al.*, 1984; Wiemken, 1990). The occurrence of trehalose has also been documented for the desiccation-tolerant plants, *Myrothamnus flabellifolia* (Bianchi *et al.*, 1993;

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Abbreviations: GC–FID, gas chromatography–flame ionization detection; GC–MS, gas chromatography–mass spectrometry; EST, expressed sequence tag; HPAEC, high-performance anion exchange chromatography; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase.

Drennan *et al.*, 1993) and *Selaginella lepidophylla* (Adams *et al.*, 1990), whereas reports on the occurrence of trehalose in many other plant species were ambiguous (for review see Müller *et al.*, 1995a).

In yeast, trehalose is synthesized in a two-step process: First trehalose-6-phosphate is formed from UDP-glucose and glucose-6-phosphate in a reaction catalysed by trehalose-6-phosphate synthase (TPS), then trehalose-6-phosphate is hydrolysed to trehalose in a reaction catalysed by trehalose-6-phosphate phosphatase (TPP). The *Saccharomyces cerevisiae* genes for trehalose synthesis, *ScTPS1* (encoding TPS) and *ScTPS2* (encoding TPP), have been cloned (Bell *et al.*, 1992; Vuorio *et al.*, 1993; De Virgilio *et al.*, 1993). Recently, a cDNA from *Selaginella lepidophylla* exhibiting homologies to *ScTPS1* (*SITPS1*; Zentella *et al.*, 1999) and *Arabidopsis* cDNAs exhibiting homologies to *ScTPS1* (*AtTPS1*; Blázquez *et al.*, 1998) and to *ScTPS2* (*AtTPPA* and *AtTPPB*; Vogel *et al.*, 1998) were isolated. Transformation of the respective *S. cerevisiae* mutants with the *Arabidopsis* cDNAs resulted in the complementation of growth and in trehalose formation. These findings prompted speculation that trehalose may be endogenously produced in *Arabidopsis*. Since trehalose induces starch accumulation in the shoots of *Arabidopsis* seedlings and, at the same time, inhibits root elongation (Wingler *et al.*, 2000; Fritzius *et al.*, 2001), it is conceivable that endogenously formed trehalose acts as a signal molecule in the regulation of plant metabolism and development. In addition, it has been suggested that the precursor of trehalose, trehalose-6-phosphate, may play a role in the regulation of carbohydrate metabolism and of sugar sensing in plants (Goddijn and Smeekens, 1998).

Recently, a compound occurring in *Arabidopsis* was tentatively identified as trehalose (Müller *et al.*, 2001). In the present study, sugar extracts from axenically grown plants were analysed by gas chromatography–mass spectrometry (GC–MS) in order to provide unequivocal evidence that trehalose synthesis occurs in *Arabidopsis*. In addition, full-length cDNAs of *Arabidopsis* genes exhibiting homologies to TPS and TPP genes were cloned. In order to analyse their enzymatic function and their involvement in trehalose synthesis, these cDNAs were expressed in the respective *S. cerevisiae* mutants as well as in tobacco protoplasts.

## Materials and methods

### Plant material and growth conditions

*Arabidopsis thaliana* (L.) Heynh. (ecotypes Landsberg *erecta* and Ws-2) seeds were sterilized and germinated on vertically oriented Petri dishes on half-strength MS-medium (Sigma, Buchs, Switzerland) solidified with 1% (w/v) purified agar (Oxoid, Basingstoke, Hampshire, UK). To prevent the breakdown of trehalose by endogenous trehalase activity, 10  $\mu\text{M}$  of

the trehalase inhibitor validamycin A (Asano *et al.*, 1987) was added to the medium where indicated. The plants were grown in a daily cycle of 18 h light (80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 22 °C and 6 h darkness at 18 °C. Two weeks after germination, the plants were transferred into Phytacon plant cell culture vessels (Sigma, Buchs, Switzerland) with lids that allow ventilation. The plants were harvested 4 weeks after germination at between 7 h and 8 h into the photoperiod. Plants were also dark-treated for 24 h or illuminated for 31 h before harvest at midday.

### Identification of trehalose in *Arabidopsis*

For the determination of trehalose in *Arabidopsis* plants (ecotype Ws-2), sugars were extracted, derivatized as described previously (Müller *et al.*, 1995b) and quantified by capillary GC–FID using a Carlo Erba Mega 3500 gas chromatograph equipped with a flame ionization detector (Brechtbühler, Zürich, Switzerland). For the identification of trehalose by GC–MS, complete inflorescences of plants grown axenically on medium containing 10  $\mu\text{M}$  validamycin A were extracted in 80% ethanol at 80 °C and the extracts were freeze-dried. The sugars were then analysed by GC–MS (Fiehn *et al.*, 2000). The peak corresponding to trehalose was compared to a trehalose standard. The total number of peaks in the chromatogram was analysed by the deconvolution of the mass spectra over time using the automated mass spectra deconvolution and identification software AMDIS (Stein, 1999).

### RACE–PCR

Cloning of full-length cDNAs was based on EST sequences that had been found to exhibit homologies to conserved regions of other TPS genes (accession no. T76758, *AtTPSA*; accession no. T76390, *AtTPSB*; accession no. H37578, *AtTPSC*). For RACE–PCR (Frohman *et al.*, 1988), total RNA was extracted from axenically grown *Arabidopsis* plants (ecotype Landsberg *erecta*) and reverse transcribed with EST specific primers (5'–RACE). Primers mapping to internal sequences served to amplify the cDNA ends. Two successive rounds of RACE–PCR were needed to reach the 5' ends. Both strands of the RACE products were sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing kit and an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, USA). The predicted protein sequences were aligned using the PILEUP program from the Wisconsin Package Version 10.0 (GCG, Madison, Wisconsin). The degree of similarity between homologous regions of the proteins was calculated with the BESTFIT program (GCG). The obtained sequence information was used to design oligonucleotides flanking the 5' and the 3' ends of the open reading frames of *AtTPSA*, *AtTPSB* and *AtTPSC*. The corresponding pairs of oligonucleotides were used to amplify the full-length gene sequences using total *Arabidopsis* cDNA as a template. These amplified full-length gene sequences were then ligated into the yeast shuttle vector pFL61 (Minet *et al.*, 1992) or the vector pDH51 and cloned in *E. coli*. As a positive control, the yeast *ScTPS1* gene was amplified and cloned in a similar way. The pFL61–*AtTPS1* construct was provided by M Blázquez (La Jolla, USA).

### Yeast transformation

The *S. cerevisiae tps1* (YSH 6.106.-1A) and *tps2* (YSH 6.106.-8C) deletion mutants (Reinders *et al.*, 1997) were transformed with the pFL61-based constructs as described earlier (Vogel *et al.*, 1998). To select for transformants, the cells were

first grown on minimal media supplemented with 2% galactose and 1% raffinose (Sgal/raf) at 28 °C. For complementation tests, transformants of the *tps1* deletion mutant were then replica plated onto minimal plates with 2% glucose as a carbon source. Transformants of the *tps2* deletion mutant were replica plated onto Sgal/raf medium and incubated at 38.6 °C.

#### Determination of trehalose in transformed yeast cells

For measuring trehalose formation during heat shock, the *S. cerevisiae* cells were grown in Sgal/raf liquid medium to a density of  $5 \times 10^6$  to  $2 \times 10^7$  cells ml<sup>-1</sup> and transferred to 40 °C for 60 min. The cells were collected by filtration, resuspended in H<sub>2</sub>O and boiled for 10 min. Sugars in the extracts were determined by HPAEC–PED analysis using an anion-exchange column (CarboPac PA-10, Dionex, Olten, Switzerland) and a pulsed amperometric detector (Dionex) as described previously (De Virgilio *et al.*, 1993).

#### Measurement of TPS activity

TPS activity was assayed with permeabilized *S. cerevisiae* cells. The cells were grown to a density of  $5 \times 10^6$  to  $2 \times 10^7$  cells ml<sup>-1</sup>, harvested, suspended in 200 mM Tricine (pH 7.0) containing 0.05% Triton X-100 and frozen in liquid nitrogen. After thawing for 3 min at 30 °C, the cells were washed four times with 200 mM K-phosphate buffer (pH 7.0) or 200 mM MES-KOH (pH 7.1). 60 µl of the resuspended cells (equivalent to 0.84 mg protein) was incubated at 35 °C with 5 mM UDP-[U-<sup>14</sup>C]glucose (5.4 MBq mmol<sup>-1</sup>), 10 mM glucose-6-phosphate, 12.5 mM MgCl<sub>2</sub> and 2 mM DTT in a total volume of 240 µl. The reaction was also measured with ADP-glucose or UDP-galactose instead of UDP-glucose and with the addition of fructose-6-phosphate. After 20, 40 and 60 min, aliquots of 60 µl were boiled for 10 min. The reaction products were quantified by HPAEC–PED analysis as described above and by online-detection of radioactive compounds with a radio-chromatography detector (FLO-ONE\Beta Series A-500; Radiomatic, Meriden, Connecticut, USA). For the calculation of TPS activities, the rates of trehalose and trehalose-6-phosphate formation were added. Protein concentrations were quantified with a modified Lowry assay (Peterson, 1977).

#### Transient expression in tobacco protoplasts and assay of TPS activity

Tobacco (*Nicotiana plumbaginifolia*) protoplasts were isolated and aliquots of 10<sup>6</sup> cells were transformed with 10 µg of the pDH51-based constructs as described previously (Goodall *et al.*, 1990). After the final wash, the protoplasts were resuspended and lysed in 50 mM K-phosphate buffer (pH 7.0). An equivalent of 600 000 protoplasts was assayed for trehalose-6-phosphate synthase activity by incubation at 30 °C with 1 mM UDP-[U-<sup>14</sup>C]glucose (27 MBq mmol<sup>-1</sup>), 2 mM glucose-6-phosphate, 12.5 mM MgCl<sub>2</sub>, 1 µM validoxylamine, and 1 mM DTT in a total volume of 240 µl. After 5, 15, 30, and 60 min, aliquots of 60 µl were boiled for 10 min and centrifuged at 20 000 *g* for 10 min. The radioactive reaction products in the supernatant were analysed after separation by TLC followed by phosphor-imager analysis or by HPAEC-separation with online-detection of radioactive compounds using a radio-chromatography detector (FLO-ONE\Beta Series A-500; Radiomatic, Meriden, Connecticut, USA).

#### Quantitative RT–PCR

Total RNA was extracted from axenically grown *Arabidopsis* plants (ecotype Ws-2) using the Qiagen Rneasy kit (Qiagen, Basel, Switzerland). The RNA was treated with DNase using the MessageClean kit (GenHunter, Nashville, TN) and reverse-transcribed using a reverse-transcription kit (Boehringer, Mannheim, Germany) with oligo-dT and random primers in the reaction. Quantitative PCR was carried out with an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Rotkreuz, Switzerland) using the SYBR Green PCR reagents (Applied Biosystems) according to the manufacturer's protocol. The genes tested, primers used and sizes of the amplified fragments were: *AtTPS1* (accession no. Y08568) 5'-TTGAGGTCCCCG-AAGTCAAAC-3' and 5'-TGCGGCAACAATTTTCATG-3', 255 bp; *AtTPSA* 5'-TGGATGTTCCCCTTCGCTT-3' and 5'-TTCTCATGCCGTAGCTGTTTCTC-3', 123 bp; *AtTPSB* 5'-TCCGTGTTAATCCGTGGAACA-3' and 5'-TCTTTG-ACGCCCTTTGAAG-3', 175 bp; *AtTPSC* 5'-CGAGGAG-GATCAATGAGCGTT-3' and 5'-TTGTGCGAGGCGATG-AATC-3', 222 bp; *AtH3G* (accession no. X60429) 5'-GAT-TTGGCTTTCCAGAGCCA-3' and 5'-CGAGCGAGCTG-AATGTCTTTG-3', 143 bp; *AtACT2* (accession no. U41998) 5'-GCAAGTCATCACGATTGGTGC-3' and 5'-GAACC-ACCGATCCAGACACTGT-3', 297 bp; *AtCAB1* (accession no. X56062) 5'-GCTGTTGGCGTTTGTAGGATTC-3' and 5'-CAATGTTGTTGTGCCATGGATC-3', 107 bp. Before their use in quantitative PCR, all primer pairs were shown specifically to produce amplification products of the expected size. The PCR conditions were initial denaturation at 95 °C for 10 min, followed by cycles of 15 s at 95 °C, 30 s at 55 °C, and 60 s at 60 °C. Monitoring of the logarithmic increase in the amount of the PCR products showed that the amplification reactions were equally efficient for all samples. The relative amount of cDNA was calculated from the cycle number at which the amplified target reached a fixed threshold. All samples were analysed in duplicate. To account for possible contamination with genomic DNA, negative controls (no reverse transcriptase in the cDNA synthesis reaction) were included in the PCR analysis. For all samples, the amount of contaminating DNA was below 2%.

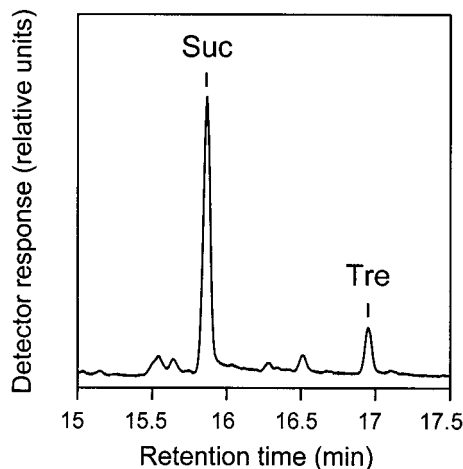
## Results

### Identification of trehalose in *Arabidopsis*

When extracts from axenically grown *Arabidopsis* plants were analysed for sugars by GC–FID, a peak with the same retention time as trehalose was regularly detected. The amount of this compound increased when the plants were grown in the presence of validamycin A, which has been shown to inhibit the activity of trehalase in *Arabidopsis* (Müller *et al.*, 2001). For example, assuming the detected compound was trehalose, its content rose from 0.14 mg g<sup>-1</sup> DW in the flowers of plants grown in the absence of validamycin A to 0.66 mg g<sup>-1</sup> DW in the presence of validamycin A (for a more detailed analysis of trehalose contents in *Arabidopsis* see Müller *et al.*, 2001).

To confirm the identity of this compound as trehalose, sugars were analysed by GC–MS. For this analysis, whole inflorescences (including the flowers, stems and cauline leaves) of plants grown axenically in the presence of

validamycin A were extracted. In three independent experiments, trehalose contents in the inflorescences as measured by GC-FID (Fig. 1) ranged between 0.11 and 0.28 mg g<sup>-1</sup> DW (0.20 ± 0.05 mg g<sup>-1</sup> DW; mean ± SE). No trehalose was found in concentrated growth medium,

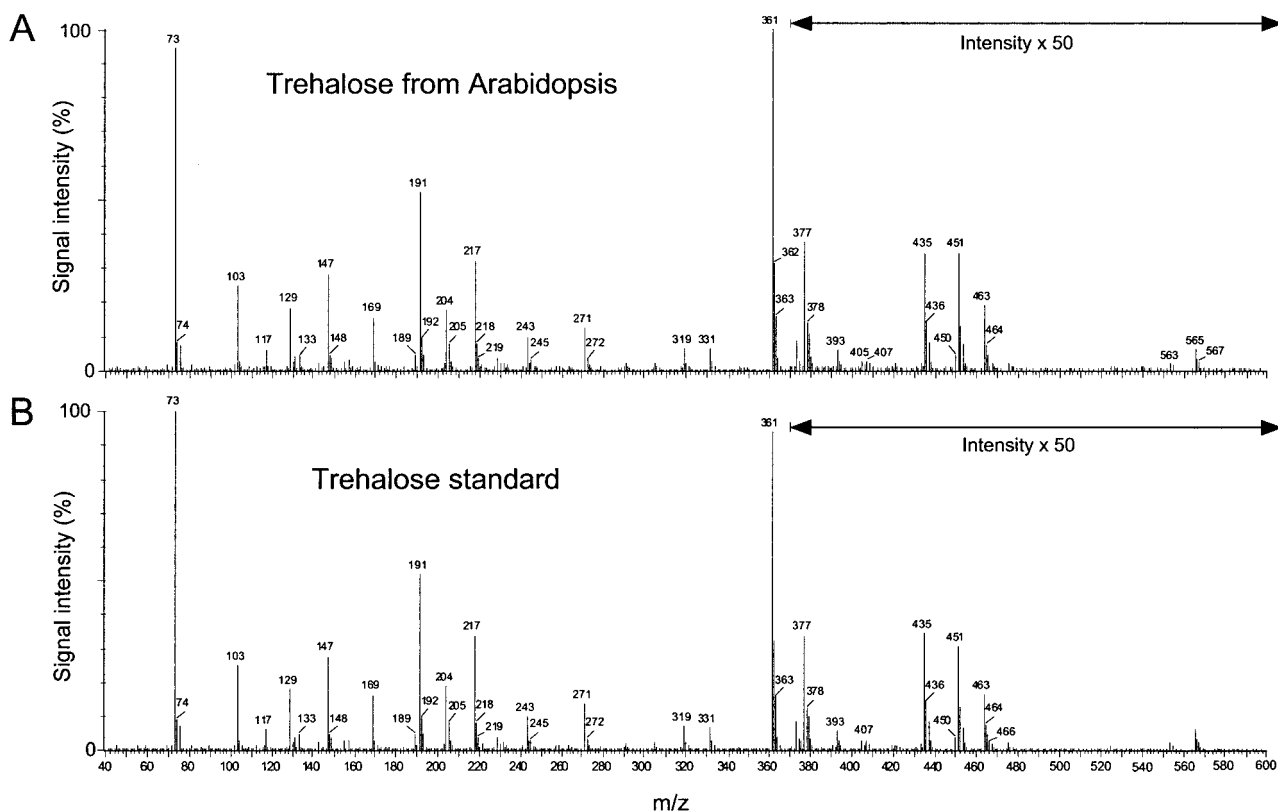


**Fig. 1.** Detection of trehalose by GC-FID in inflorescences of *Arabidopsis* plants grown axenically in the presence of 10 μM validamycin A. The trehalose peak (Tre) is shown in relation to the sucrose peak (Suc). The content of trehalose was 0.28 mg g<sup>-1</sup> DW compared to 1.84 mg sucrose g<sup>-1</sup> DW.

showing that the trehalose peak was not due to a contamination of any of the reagents used in the analysis. When the polar fraction of extracts from the experiment yielding the highest trehalose contents was analysed by GC-MS, more than 700 components were found after mass spectra deconvolution. The retention time of one of the major peaks corresponded precisely with the retention time of trehalose in an external reference chromatogram. Despite the extreme complexity of the plant chromatogram, this peak was unambiguously identified as trehalose by comparison with the trehalose mass spectrum (Fig. 2). High m/z fragments are highly characteristic for trehalose, since most of these fragments are not present in other disaccharides.

### Cloning and sequencing of TPS homologues from *Arabidopsis*

Full-length *Arabidopsis* cDNAs were amplified by the RACE method (Frohmann *et al.*, 1988) based on the sequence of previously published *Arabidopsis* ESTs with homologies to microbial TPS genes. Three different full-length sequences were obtained and the respective genes were named *AtTPSA*, *AtTPSB* and *AtTPSC*. In the meantime, all three sequences were found on BACs from *Arabidopsis* chromosomes, confirming that they



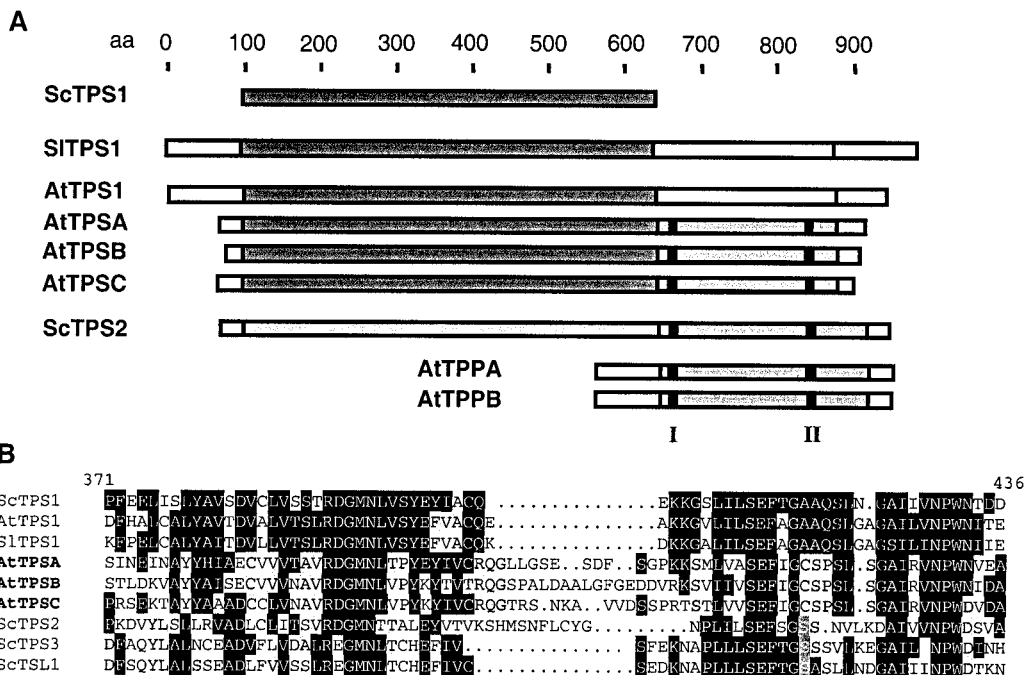
**Fig. 2.** Mass spectra of the trehalose peak identified by GC-MS in inflorescences of *Arabidopsis* plants grown axenically in the presence of 10 μM validamycin (A) and of a trehalose standard (B). The intensity for the mass/charge ratio range from 370 to 600 was magnified by a factor of 50.

originated from *Arabidopsis*. *AtTPSA* is located on chromosome 1 (accession no. AC068143), *AtTPSB* is located on chromosome 2 (accession no. AC005724), and *AtTPSC* is located on chromosome 1 (accession no. AC003671). Full-length sequences of all three genes were translated and aligned with other plant and microbial TPS and TPP proteins. The proteins encoded by *AtTPSA*, *AtTPSB* and *AtTPSC* show homologies to TPS and TPP proteins (Fig. 3; Table 1). In contrast to the *E. coli* EcOTSA and the *S. cerevisiae* ScTPS1, the plant proteins have an additional C-terminal part homologous to the *S. cerevisiae* TPP, ScTPS2 (Fig. 3A). This phosphatase-like part is also present in the *Selaginella*

*lepidophylla* SITPS1 (Zentella *et al.*, 1999). In contrast to other TPS proteins, *AtTPSA*, *AtTPSB* and *AtTPSC* possess a variable insert of 12, 15 or 13 amino acids, respectively, between amino acid position 405 and 406 of ScTPS1 (Fig. 3B).

*Expression analysis of AtTPSA, AtTPSB and AtTPSC*

Probably due to the low expression of *AtTPSA*, *AtTPSB* and *AtTPSC*, no signal was obtained by Northern blot analysis. Therefore, the expression of these genes was analysed by quantitative RT-PCR (Table 2). *AtTPSA*, *AtTPSB*, *AtTPSC*, and *AtTPS1* were expressed in all



**Fig. 3.** Comparison of *AtTPSA*, *AtTPSB*, and *AtTPSC* with known TPS proteins. (A) Block diagram of the proteins. I and II indicate conserved motifs in the phosphatase part. (B) Alignment of a part of the predicted protein sequence of *AtTPSA*, *AtTPSB* and *AtTPSC* with other TPS proteins. The proteins are: ScTPS1: *S. cerevisiae* TPS; AtTPS1: *Arabidopsis* TPS1; AtTPSA: *Arabidopsis* TPSA; AtTPSB: *Arabidopsis* TPSB; AtTPSC: *Arabidopsis* TPSC; SITPS1: *Selaginella lepidophylla* TPS1; AtTPPA: *Arabidopsis* TPPA; AtTPPB: *Arabidopsis* TPPB; ScTPS2: *S. cerevisiae* TPP; ScTPS3: 115 kDa regulatory subunit of TPS complex of *S. cerevisiae*; ScTSL1: 123 kDa regulatory subunit of TPS complex of *S. cerevisiae*.

**Table 1.** Percentage similarity between homologous regions of TPS and TPP proteins

Values in parentheses are from sequences for which only short regions (about 100 amino acids long) of homology were detected; n.o. indicates that no significant overlap was detected. The sequences are: ScTPS1: *S. cerevisiae* TPS; AtTPS1: *Arabidopsis* TPS1; AtTPSA: *Arabidopsis* TPSA; AtTPSB: *Arabidopsis* TPSB; AtTPSC: *Arabidopsis* TPSC; SITPS1: *Selaginella lepidophylla* TPS1; AtTPPA: *Arabidopsis* TPPA; AtTPPB: *Arabidopsis* TPPB; ScTPS2: *S. cerevisiae* TPP.

	ScTPS1	AtTPS1	AtTPSA	AtTPSB	AtTPSC	SITPS1	AtTPPA	AtTPPB	ScTPS2
ScTPS1	100								
AtTPS1	59	100							
AtTPSA	53	49	100						
AtTPSB	51	48	68	100					
AtTPSC	52	46	70	69	100				
SITPS1	62	73	50	46	47	100			
AtTPPA	n.o.	n.o.	39	38	41	(32)	100		
AtTPPB	n.o.	n.o.	39	38	37	(35)	61	100	
ScTPS2	49	44	48	46	45	44	40	39	100

**Table 2.** Expression of *AtTPS1*, *AtTPSA*, *AtTPSB*, *AtTPSC*, *AtCAB1* (photosystem I chlorophyll a/b binding protein gene), *AtACT2* (actin 2 gene) and *AtH3G* (histone 3 gene) in rosette leaves, roots, stems, and flowers of *Arabidopsis* plants

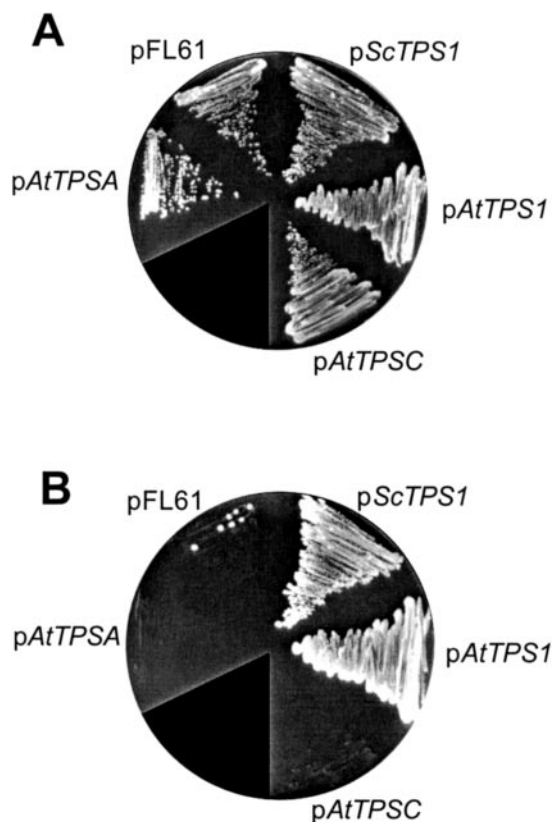
Rosettes of plants that were kept in the dark for 24 h (dark) or illuminated for 31 h (light) were also analysed. The expression was determined using a quantitative PCR system (see Materials and methods).

	Relative gene expression (%)				Light	Dark
	Leaf	Root	Stem	Flower		
<i>AtTPS1</i>	100	5	76	32	91	3
<i>AtTPSA</i>	100	10	73	66	116	56
<i>AtTPSB</i>	100	8	129	27	92	355
<i>AtTPSC</i>	100	8	134	29	121	282
<i>AtCAB1</i>	100	2	76	26	120	16
<i>AtACT2</i>	100	34	115	78	63	137
<i>AtH3G</i>	100	50	120	188	84	66

organs tested (rosette leaves, roots, stems, and flowers). Expression levels of all four TPS genes were similar in the rosette leaves and the stems. In the flowers, expression varied considerably between different experiments (data not shown), probably due to different developmental stages harvested. In the roots, the expression of all TPS genes was lower than in the shoot organs. The expression of *AtTPS1* was consistently lower in rosettes of dark-incubated plants than in rosettes of illuminated plants. Expression of the chlorophyll *a/b*-binding protein gene, *AtCAB1*, of the histone gene, *AtH3G*, and of the actin gene, *AtACT2*, was used as control. As expected, the expression levels of *AtACT2* and *AtH3G* were similar in all organs (between 34% and 188%), while the expression of *AtCAB1* was highest in the leaves and stems, lower in the flowers and very low in the roots, and strongly reduced in by dark incubation.

#### Expression in the *S. cerevisiae tps1* deletion mutant

The function of the *Arabidopsis* genes was analysed by complementation studies with *S. cerevisiae* mutants. For this purpose, the RACE-PCR products were ligated into the yeast shuttle vector pFL61 behind a strong phosphoglycerate kinase promoter (Minet *et al.*, 1992) and cloned in *E. coli*. Whereas this step was repeatedly successful for *AtTPSA* and *AtTPSC*, no transformants were obtained for constructs containing *AtTPSB*, indicating that *AtTPSB* might be toxic in *E. coli*. pFL61-based constructs containing *AtTPSA*, *AtTPSC*, *AtTPS1* or *ScTPS1* were transformed into the *S. cerevisiae tps1* mutant. Due to the lack of TPS activity, this mutant is unable to grow on glucose, although it does grow on galactose. After transformation with the *S. cerevisiae ScTPS1* or the *Arabidopsis AtTPS1* cDNAs, growth on glucose was restored (Fig. 4). In contrast, growth on glucose was not complemented by expression of *AtTPSA*



**Fig. 4.** Complementation of growth of the *S. cerevisiae tps1* deletion mutant (YSH 6.106.-1A) by transformation with pFL61 based constructs. *S. cerevisiae* cells were grown on minimal media containing 2% galactose and 1% raffinose (A) or 2% glucose (B).

**Table 3.** In vivo formation of trehalose after 60 min heat shock at 40 °C and in vitro activity of TPS in the *S. cerevisiae tps1* deletion mutant (YSH 6.106.-1A) transformed with different pFL61 based constructs

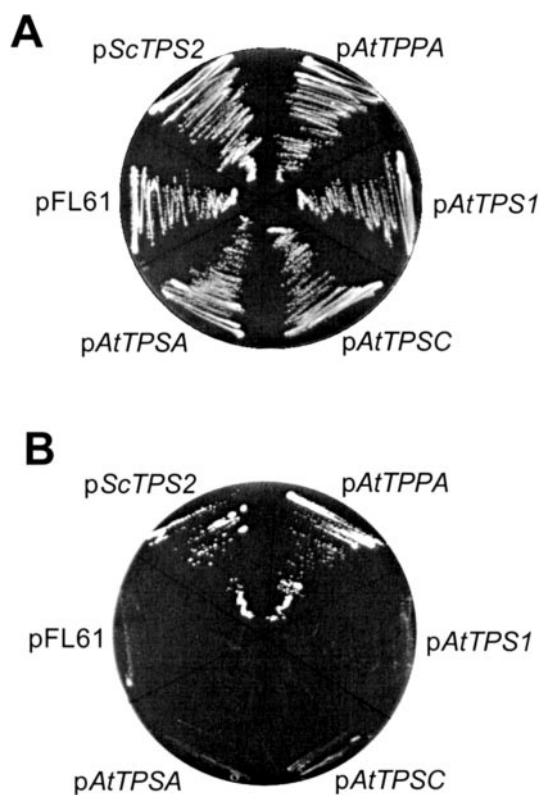
Construct	Formation of trehalose (nmol 10 <sup>-6</sup> cells)	Trehalose-6-phosphate synthase (nkat g <sup>-1</sup> protein)
pFL61	<0.05	<5
pScTPS1	2.30	370
pAtTPS1	0.94	<5
pAtTPSA	<0.05	<5
pAtTPSC	<0.05	<5

or *AtTPSC*. In addition, whereas expression of *ScTPS1* and *AtTPS1* restored the ability to accumulate trehalose after heat shock (Table 3), no trehalose was produced in heat-shocked cells transformed with the *AtTPSA* or *AtTPSC* cDNAs. *In vitro* activities of TPS were assayed with permeabilized *S. cerevisiae* cells. While *ScTPS1* restored TPS activity (Table 3), TPS activities were below the detection limit in cells transformed with any of the *Arabidopsis* cDNAs (*AtTPS1*, *AtTPSA* or *AtTPSC*). Furthermore, no activity was detected when ADP-glucose or UDP-galactose (instead of UDP-glucose)

or fructose-6-phosphate was added, when MES-KOH instead of K-phosphate was used as a buffer system or when the activity was assayed with protein extracts instead of permeabilized cells. When GST-fusions of AtTPSA and AtTPSC were expressed in the *tps1* mutant, fusion proteins of the correct size were detected (data not shown), indicating that the *Arabidopsis* proteins were expressed and not degraded in the yeast cells.

#### Expression in the *S. cerevisiae tps2* deletion mutant

Since the C-terminal parts of AtTPSA and AtTPSC show homologies to TPP proteins (Table 1; Fig. 3), AtTPSA and AtTPSC were also expressed in the *S. cerevisiae tps2* mutant, which cannot grow at high temperatures. As expected, expression of the *S. cerevisiae* TPP, ScTPS2, and of the *Arabidopsis* TPP, AtTPPA, complemented growth at 38.6 °C (Fig. 5). Growth at this temperature was, however, not restored after transformation with the *AtTPSA*, *AtTPSC* or *AtTPS1* cDNAs. In addition, trehalose formation after heat shock was only restored by transformation with *ScTPS2* and *AtTPPA*, but not by transformation with the *AtTPSA*, *AtTPSC* or *AtTPS1* cDNAs (data not shown).



**Fig. 5.** Complementation of growth of the *S. cerevisiae tps2* deletion mutant (YSH 6.106.-8C) by transformation with pFL61 based constructs. *S. cerevisiae* cells were grown on minimal media containing 2% galactose and 1% raffinose at 28 °C (A) or at 38.6 °C (B).

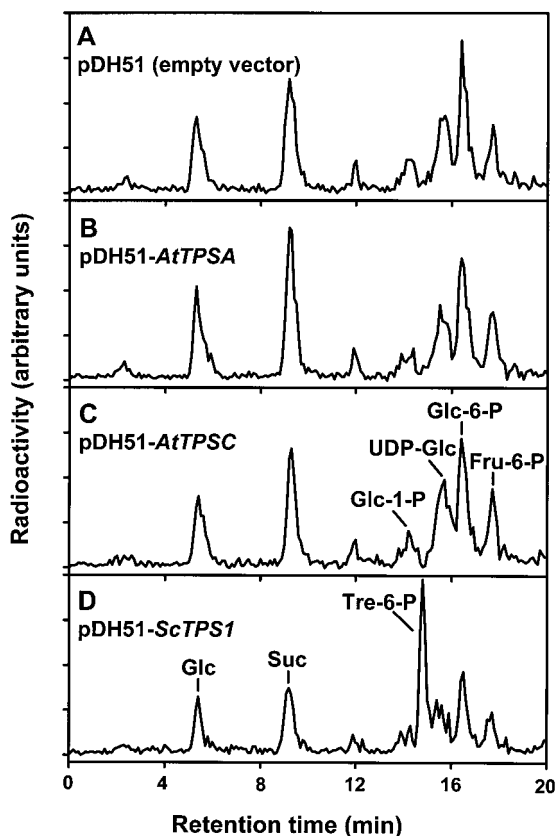
#### Expression in tobacco protoplasts

To exclude the possibility that the lack of TPS activity of AtTPSA and AtTPSC was due to unsuitable conditions in the yeast cells, their function was also studied in tobacco protoplasts. Whereas transformation with the positive control, pDH51-*ScTPS1*, resulted in the formation of trehalose-6-phosphate, no formation of trehalose-6-phosphate was found in protoplasts transformed with pDH51-*AtTPSA* or pDH51-*AtTPSC* (Fig. 6). No conversion of trehalose-6-phosphate into trehalose could be detected in the protoplasts transformed with pDH51-*ScTPS1*.

## Discussion

#### Occurrence of trehalose in Arabidopsis

Until recently, most higher plants, such as *Arabidopsis*, were not considered to form trehalose (Müller *et al.*,



**Fig. 6.** Assay of trehalose-6-phosphate formation in tobacco protoplasts transformed with pDH61-based constructs. Formation of trehalose-6-phosphate (retention time 14.8 min) from UDP-[U-<sup>14</sup>C]glucose and glucose-6-phosphate was analysed by HPAEC-separation followed by detection with a radio-chromatography detector. (A) Empty vector control; (B) vector expressing *AtTPSA*; (C) vector expressing *AtTPSC*; (D) vector expressing *ScTPS1*. Fru-6-P, fructose-6-phosphate; Glc, glucose; Glc-1-P, glucose-1-phosphate; Glc-6-P, glucose-6-phosphate; Suc, sucrose; Tre-6-P, trehalose-6-phosphate; UDP-Glc, UDP-glucose.

1995a). However, the discovery of an *Arabidopsis* TPS gene, *AtTPS1* (Blázquez *et al.*, 1998), and of two TPP genes, *AtTPPA* and *AtTPPB* (Vogel *et al.*, 1998), suggested that *Arabidopsis* has the potential for trehalose synthesis.

Here, it is shown that trehalose, the product of the TPS and TPP reactions, does occur in *Arabidopsis* (Figs 1, 2). This finding confirms results of other studies in which chromatographic techniques were used for measuring trehalose in plants: for example, trehalose was found in tobacco plants grown hydroponically in the presence of validamycin A (Goddijn *et al.*, 1997), and in a salt-stressed rice plant (Garcia *et al.*, 1997). In *Arabidopsis*, a compound that increased in the presence of validamycin A was tentatively identified as trehalose (Müller *et al.*, 2001). To provide unambiguous evidence that trehalose occurs in plants it was, however, necessary to identify trehalose using GC-MS or NMR analysis. Recently, trehalose was identified by GC-MS analysis in soil-grown potato tubers (Roessner *et al.*, 2000). In the present study, axenically grown *Arabidopsis* plants were used to determine trehalose by GC-MS analysis in order to rule out that microorganisms were the source of trehalose. Unless axenically grown *Arabidopsis* plants contain seed-borne microbial endophytes, an involvement of microorganisms in the formation of the trehalose found in this study can be excluded. One of the reasons why the occurrence of trehalose in *Arabidopsis* has not been described earlier, is probably the cleavage of trehalose by the endogenous trehalase activity of *Arabidopsis* (Müller *et al.*, 2001). When trehalase activity was inhibited by validamycin A, the content of trehalose rose to easily detectable amounts.

The detection of trehalose in higher plants, such as *Arabidopsis*, raises the question about its function (Müller *et al.*, 1999). In view of its low abundance, it probably does not play a role as a stress protectant. However, there are indications that trehalose and/or trehalose-6-phosphate act as signal molecules in the regulation of plant metabolism and development. The results obtained by the expression of microbial TPS proteins in plants, by inhibition of trehalase activity by validamycin A and by external supply of trehalose support this hypothesis (Wagner *et al.*, 1986; Müller *et al.*, 1998, 2000; Pilon-Smits *et al.*, 1998; Romero *et al.*, 1997; Wingler *et al.*, 2000; Fritzius *et al.*, 2001).

#### *Involvement of the individual TPS homologues in trehalose synthesis*

Having established that trehalose synthesis occurs in *Arabidopsis*, it was interesting to investigate where the *Arabidopsis* TPS and TPP homologues are expressed and which of them catalyse reactions of trehalose synthesis. Since *AtTPSA*, *AtTPSB*, *AtTPSC*, and *AtTPS1* are

expressed in all organs of *Arabidopsis* plants (Table 2), they could be simultaneously involved in trehalose synthesis. To analyse their function, the open reading frames of *AtTPSA* and *AtTPSC* were amplified by PCR and cloned. Complementation studies with yeast mutants (Figs 4, 5; Table 3) confirm that *AtTPS1* (Blázquez *et al.*, 1998) encodes a functional TPS, while no enzymatic function could be ascribed to *AtTPSA* and *AtTPSC*.

After transformation of *S. cerevisiae* with the *AtTPS1* cDNA, trehalose formation in the heat-shocked *tps1* mutant was detected by HPAEC-PED analysis. Previously, trehalose formation in this system had only been demonstrated using a coupled enzymatic assay (Blázquez *et al.*, 1998), which is not necessarily specific. Although expression of *AtTPS1* restored trehalose formation *in vivo*, it did not result in measurable *in vitro* activity of TPS. Much lower *in vitro* activities compared with the *in vivo* rates of trehalose plus trehalose-6-phosphate formation have also been reported for ScTPS1 in *S. cerevisiae* mutants with deletions of other components of the TPS complex (ScTPS2, ScTPS3 or ScTSL1; Reinders *et al.*, 1997). This indicates that the TPS activity is underestimated in the *in vitro* assay, when no TPS complex can be formed. For *AtTPS1* this might be due to a lack of interaction with the *S. cerevisiae* proteins.

In contrast to *AtTPS1*, expression of *AtTPSA* and *AtTPSC* did not restore trehalose formation and did not complement growth of the *S. cerevisiae tps1* mutant on glucose. Fusion proteins of *AtTPSA* or *AtTPSC* with GST were also expressed in *S. cerevisiae*. In this case, fusion proteins of the expected size were readily detected (data not shown), indicating that *AtTPSA* or *AtTPSC* were correctly expressed at the protein level. Moreover, the lack of trehalose formation was probably not due to an incompatibility of *AtTPSA* and *AtTPSC* function with specific conditions in the *S. cerevisiae* cells, since expression of *AtTPSA* and *AtTPSC* in tobacco protoplasts did not result in trehalose or trehalose-6-phosphate synthesis either (Fig. 6). ScTPS1, in contrast, was also active in the tobacco protoplasts, showing that tobacco protoplasts are a suitable system for studying the function of yeast enzymes. Taken together, the results suggest that *AtTPSA* and *AtTPSC* probably do not encode functional TPS enzymes.

As the C-terminal parts of *AtTPSA* and *AtTPSC* show homologies to functional TPP proteins, it was also attempted to complement the *S. cerevisiae tps2* mutant lacking TPP. However, neither growth at 38.6 °C nor trehalose formation could be restored. Similarly, the *Selaginella lepidophylla* SITPS1 probably does not exhibit TPP activity, even though it contains a C-terminal domain homologous to microbial TPP proteins (Zentella *et al.*, 1999).

The results presented here suggest that trehalose is indeed formed in *Arabidopsis* plants and that the first

step of trehalose synthesis is catalysed by AtTPS1. The function of AtTPSA and AtTPSC, on the other hand, remains unclear. Since AtTPSA and AtTPSC also show homologies to ScTPS3 and ScTSL1, two regulatory proteins of the *S. cerevisiae* TPS complex (Vuorio *et al.*, 1993; Reinders *et al.*, 1997), they might present regulatory proteins of a putative plant TPS complex. The cloning of the full-length cDNAs makes it possible to manipulate the expression of *AtTPSA* and *AtTPSC* in *Arabidopsis* in order to analyse their function in *planta*.

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