Truncation of *Arabidopsis thaliana* and *Selaginella lepidophylla* trehalose-6-phosphate synthase unlocks high catalytic activity and supports high trehalose levels on expression in yeast

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Plants, such as *Arabidopsis thaliana* and *Selaginella lepidophylla*, contain genes homologous with the trehalose-6-phosphate synthase (TPS) genes of bacteria and fungi. Most plants do not accumulate trehalose with the desert resurrection plant *S. lepidophylla*, being a notable exception. Overexpression of the plant genes in a *Saccharomyces cerevisiae* *tps1Δ* mutant results in very low TPS-catalytic activity and trehalose accumulation. We show that truncation of the plant-specific N-terminal extension in the *A. thaliana* *AtTPS1* and *S. lepidophylla* *SITPS1* homologues results in 10–40-fold higher TPS activity and 20–40-fold higher trehalose accumulation on expression in yeast. These results show that the plant TPS enzymes possess a high-potential

catalytic activity. The growth defect of the *tps1Δ* strain on glucose was restored, however, the proper homeostasis of glycolytic flux was not restored, indicating that the plant enzymes were unable to substitute for the yeast enzyme in the regulation of hexokinase activity. Further analysis of the N-terminus led to the identification of two conserved residues, which after mutagenesis result in strongly enhanced trehalose accumulation upon expression in yeast. The plant-specific N-terminal region may act as an inhibitory domain allowing modulation of TPS activity.

Key words: glycolysis, *Saccharomyces cerevisiae*, sugar influx.

INTRODUCTION

Trehalose biosynthesis in bacteria and fungi is performed by two sequential reactions catalysed by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) [1,2]. Trehalose is now well known for its remarkable stress protection properties. It is widely present in nature in survival forms, such as spores, cysts and dry animal life stages, and is often accumulated in vegetative cells under stress conditions [3–5]. The stress-protective properties of trehalose *in vitro* on proteins, membranes and intact cells have been amply documented [5–7].

The presence of trehalose had not been reported in plants except for some unusual desiccation-tolerant plants, such as *Selaginella lepidophylla* and *Myrothamnus flabellifolia*, where trehalose is accumulated in large quantities apparently as stress protectant [8,9]. In *Arabidopsis thaliana*, *Nicotiana tabacum* and *Solanum tuberosum*, very low levels of trehalose can be detected when the plants are grown in the presence of the potent trehalase inhibitor validamycin A [10,11]. In spite of the absence of trehalose, numerous green plants have been found to contain homologues of the TPS genes of bacteria and fungi [9,12–14]. With the completion of the *Arabidopsis thaliana* genome-sequencing program, it has now been shown that there are 11 TPS homologous genes [15]. Based on the sequence similarity, these sequences can be subdivided into two classes, the first being

homologous with the yeast *TPS1* gene and the second being homologous with the yeast *TPS2* gene [15]. The function of the plant TPS gene products is not well understood, especially because of the absence of detectable levels of trehalose in nearly all the plants investigated. Functionality of the plant TPS genes has been demonstrated for *AtTPS1* from *A. thaliana* [12] and *SITPS1* from *S. lepidophylla* [16], mainly by heterologous expression in a *Saccharomyces cerevisiae* *tps1Δ* mutant. In both cases, however, only very low to undetectable TPS enzyme activity was present and there was also no accumulation of trehalose. The only indication for TPS activity was that overexpression of the plant genes could complement the growth defect on glucose of a *tps1Δ* strain. The Tps1 protein or its product trehalose 6-phosphate is thought to inhibit hexokinase, so that in TPS mutants there is a growth defect on glucose caused by an overactive influx of sugar into glycolysis, presumably due to the overactive hexokinase activity [17,18].

Expression of *Escherichia coli* and yeast TPS genes in transgenic plants with the aim of enhancing trehalose content and improving stress resistance had revealed unexpected effects on the morphology and physiology of such plants [19–22]. The overexpression of these heterologous genes also causes stimulation of photosynthetic activity, enhanced sugar partitioning to the storage organs and changes in the shape of the leaves [13,23,24]. This work has demonstrated that the plant TPS genes

Abbreviations used: Fru1,6bisP, fructose-1,6-bisphosphate; S, synthetic growth medium; TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase.

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might be involved in the control of sugar sensing and sugar partitioning. Recently [25], it has been shown that inactivation of *AtTPS1* in *A. thaliana* results in a lethal condition during embryo development, further supporting an important regulatory role for plant trehalose metabolism. This work has also provided preliminary evidence that plant *Tps1* might not act via the control of hexokinase activity, as opposed to yeast *Tps1* [17,18].

All results on TPS effects in transgenic plants have been obtained with expression of heterologous *TPS* genes. The question whether the plant TPS enzymes are capable of displaying significant catalytic activity remains [14,23]. This has hampered investigation into the physiological role of plant trehalose metabolism. The plant *TPS* genes contain specific N- and C-terminal extensions, not found in bacterial and fungal *TPS* genes. In the present study, we demonstrate that truncation of the plant-specific N-terminal extension of *AtTPS1* and *SITPS1* unlocks high catalytic activity on expression in yeast. This shows for the first time that the plant TPS enzymes have a high potential catalytic activity.

EXPERIMENTAL

Culture media and growth conditions

Yeast cells were grown at 30 °C in synthetic growth medium (S) [0.7% Bacto-yeast nitrogen base without amino acids (pH 6.0; Difco, Detroit, MI, U.S.A.), supplemented with 0.002% adenine, 0.002% histidine, 0.003% leucine, 0.003% tryptophan and 0.002% uracil] and 2% galactose (SGal), 2% glucose (SGlc) or 2% fructose was added.

Strains and genetic methods

The *E. coli* DH5 α -strain was used for subcloning and assembly of constructs. The yeast strains were: wild-type, W303-1A (*Mata leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2*) [26]; *tps1 Δ* , YSH290 (W303-1A, *tps1 Δ ::TRP1*) [27]; *tps2 Δ* , YSH450 (W303-1A, *tps2 Δ ::LEU2*) [28]; and *tps1 Δ tps2 Δ* , YSH652 (W303-1A, *tps1 Δ ::TRP1*, *tps2 Δ ::LEU2*) [28]. Yeast transformation was performed as described previously [29] and transformants were selected in minimal media without uracil for pSAL4 or without histidine for pSAL6.

Plasmid constructions

Recombinant DNA techniques such as bacterial transformation, isolation of DNA from plasmid or λ bacteriophage and labelling of radioactive fragments were performed according to the standard procedures [30]. The construction of the pSAL4 shuttle vector has previously been reported [16]. pSAL6 is similar to pSAL4, except that it has the *HIS3* marker instead of the *URA3* marker.

A fragment (3.1 kb) containing the full-length *SITPS1* cDNA was obtained after amplification by PCR (94 °C/3 min, 1 cycle; 94 °C/1 min, 50 °C/1 min, 72 °C/2 min, 40 cycles; 72 °C/10 min, 1 cycle) using Expand High Fidelity PCR System (Roche, Indianapolis, IN, U.S.A.). As oligonucleotides, SLTPS-S1 (Table 1) and universal (5'-GTAAACGACGGCCAGT-3') primers were used with *SITPS1* cDNA cloned in pBluescript SK (pIBT6 clone) as template [16]. The PCR fragment was digested with *NcoI* and *KpnI* and cloned in pSAL4 and the resulting con-

Table 1 Oligonucleotide primers used to clone the *TPS1* genes and to construct the different N-terminal deletions of the *AtTPS1* gene

Restriction endonuclease recognition sites (*NcoI*, *NotI* or *NheI*) have been underlined. Start and stop codons are indicated in boldface.

	SLTPS-S1	5'-CATGCC ATG GCTATGCCTCAGCCTTACC-3'
	SLTPS-100	5'-CATGCC ATG GGTCGAGGCCAGCGTTGC-3'
	SLDC400-3'	5'-CGGGGTACCT CA CTTTGACTCCGAGTACTTTGC-3'
	Ath/TPS-5'	5'-CATGCC ATG GCTATGCCTGGAAATAAGTACAACATGC-3'
	Ath/TPS-3'	5'-ATAGTTTTGCGGCCGCT TTA GGGTGAGGTGAGGAAGTGGTGTACAG-3'
	Ath/TPS- Δ N5'	5'-CATGCC ATG GCTTATAATAGGCAACGACTACTTGTATGTG-3'
	Ath/TPS- Δ N3'	5'-ATAGTTTTGCGGCCGCT TTA GGGTGAGGAAGTGGTGTACAG-3'
	INV-5'	5'-CAGCTAGCCATAGCCATGGCTCGAG-3'
Amino acids deleted	3' primer used	
Δ (2-5)	INV1-3'	5'-CTGCTAGCTACAACCTGCAGTTCTTCTC-3'
Δ (2-9)	INV2-3'	5'-CTGCTAGCTCTTCTCATATCCCACTC-3'
Δ (2-13)	INV3-3'	5'-CTGCTAGCCCACTCTCTCGAACAGAAC-3'
Δ (2-17)	INV4-3'	5'-CTGCTAGCACAGAAGCGCTCTTGAGAG-3'
Δ (2-21)	INV5-3'	5'-CTGCTAGCTTGAGAGATAGAGAGCTTAG-3'
Δ (2-25)	INV6-3'	5'-CTGCTAGCGAGCTTAGAGAGAAGAGG-3'
Δ (2-29)	INV7-3'	5'-CTGCTAGCAAGAGGAAGAGCAACCGAG-3'
Δ (2-33)	INV8-3'	5'-CTGCTAGCAACCGAGCTCGTAATCC-3'
Δ (2-37)	INV9-3'	5'-CTGCTAGCAATCCTAATGACGTTGCTG-3'
Δ (2-41)	INV10-3'	5'-CTGCTAGCGTTGCTGGCAGTTCCGAG-3'
Δ (2-52)	INV11-3'	5'-CTGCTAGCTTGCGTTTGAAGGTGAC-3'
Δ (2-63)	INV12-3'	5'-CTGCTAGCGTTGAACAGTACTTGGAAAG-3'
Δ (2-74)	INV13-3'	5'-CTGCTAGCATGGCGCAGCATGATGC-3'
Δ (2-85)	INV14-3'	5'-CTGCTAGCGTTAGGCCCTTATAATAGGC-3'
Δ (2-88)	INV Δ N-3'	5'-CTGCTAGCTATAATAGGCAACGACTAC-3'
Δ (2-96)	INV15-3'	5'-CTGCTAGCGTGGCTAACAGGCTCCC-3'
Δ (6-9)	INV2REV-3'	5'-CAGCTAGCCTTATTTCCAGGCATAGC-3'
Δ (10-13)	INV3REV-3'	5'-CAGCTAGCACTGCAGTTGTACTTATTTTC-3'
Δ (14-17)	INV4REV-3'	5'-CAGCTAGCGATATGAGAAGAACTGCAG-3'
Δ (30-33)	INV8REV-3'	5'-CAGCTAGCCTCTCTAAGCTCTCTATC-3'
	SEQINV	5'-CTTGTCTTGTATCAATTGC-3'
	AT1	5'-CTTCACTCTTCTCACGG-3'
	AT2	5'-CTTCTTGTAGTGAATATC-3'

struction was designated as pSTS1.1. For the N-terminal deletion constructs, the SLTPS-100 (Table 1) and universal primers were used, and pIBT6 was used as a template. A fragment (2.8 kb) was obtained after PCR amplification as described above, digested with *NcoI* and *KpnI*, and cloned in pSAL4 (plasmid pSTS1.2). An *SITPS1* gene truncated at both the 100-amino-acid N-terminal and 431-amino-acid C-terminal ends (Δ NSITPS1 Δ C) was constructed. The *SITPS1* region coding for the remaining 463-amino-acid part was amplified by PCR as described above with primers SLTPS-100 and SIDC400-3' (Table 1) with a TAG termination codon and cloned in pSAL4 (plasmid pSTS4.2). Construction of plasmid pSTS4, comprising the truncation of just the 431-amino-acid C-terminal end of *SITPS1* (*SITPS1* Δ C), has been described previously [16].

For the construction of yeast expression vectors containing the *A. thaliana AtTPS1* gene, reverse transcriptase-PCR was used. Total RNA (5 μ g) extracted from *A. thaliana* cv. Columbia seedlings grown for 2 weeks in liquid MS medium containing 100 mM NaCl was reverse-transcribed using SuperScript II (Gibco) with an oligo(dT) (25 mer) primer. PCR amplification was done as described above using Ath/TPS-5' and Ath/TPS-3' oligonucleotides (Table 1). A fragment (2.8 kb) was obtained with the expected size, digested with *NcoI* and *NotI*, and cloned in pSAL6 leading to plasmid pSAT1. For the N-terminal deletion constructs, the Ath/TPS- Δ N5' and Ath/TPS-3' primers were used to amplify by PCR, as described above, a 2.6 kb fragment, digested with *NcoI* and *NotI*, and cloned in pSAL6 (plasmid pSAT1.2). This construct misses the first 88 amino acids of the *AtTPS1* gene.

The *ScTPS1* yeast gene cloned in pSAL4 gave rise to plasmid pSTS2 and in pSAL6 to plasmid pSTS2.1 [16]. Yeast *tps1 Δ* and *tps1 Δ tps2 Δ* mutant strains were transformed with the different plasmid constructs and selected on SGal (–ura) or SGal (–His) plates. Complementation was assayed on SGlc (–ura) or SGlc (–His) plates plus 100 μ M CuSO₄.

Construction of N-terminal truncations

The N-terminal sequence of *AtTPS1* was gradually truncated by inverse PCR. Therefore, *AtTPS1* was subcloned into pBlueKSP with *ClaI*/*BamHI*, and pBlue/*AtTPS1* was used as a template. 5' primer INV-5' and 3' primers from INV1-3' to INV15-3' (Table 1) were used. PCR was conducted using the Expand Long Template PCR system (Roche). Reaction conditions were maintained as follows: 94 °C/4 min, 1 cycle; 92 °C/20 s, 54 °C/45 s, 68 °C/5 min, 30 cycles; 68 °C/10 min, 1 cycle). The PCR products were digested with *NheI* and religated. The N-terminus was exchanged with *NcoI*/*StuI* into pSal6/*AtTPS1*. To exchange deletion constructs 2–63, 2–74, 2–85, 2–88 and 2–96, a restriction site (*HpaI*) was made (T → C, 249) using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). All constructs were sequenced using primer SEQINV (Table 1).

Mutagenesis of the N-terminus

The N-terminus of *AtTps1* was random mutagenized by degenerate PCR amplification. Primers AT1 and AT2 (Table 1) were used, after 1 mM MnCl₂ was added. The pSal6/*AtTPS1* construct was used as a template. The PCR product was co-transformed with a gapped pSal6/*AtTPS1* plasmid (*NcoI*/*StuI*) in a yeast *tps1 Δ* strain and transformants were selected on SGal–His medium. Trehalose content was measured in 500 transformants. In transformants, with a 2-fold or higher increase in trehalose content, the N-terminus of *AtTPS1* on the plasmid was sequenced. For this purpose, the plasmid DNA was isolated using lyticase and phenol/chloroform/3-methylbutan-1-ol in the

ratio 50:49:1 treatment and transformed into *E. coli* by electroporation. The plasmid DNA was also re-transformed in a yeast *tps1 Δ* strain and trehalose was determined again. If two mutations were present, the contribution of the separate mutations was determined by using the Quikchange site-directed mutagenesis kit. All constructs were sequenced using primer SEQINV (Table 1).

Determination of trehalose

Trehalose was determined by hydrolysing to glucose using *Humicola* sp. trehalase and determination of the glucose by the glucose oxidase/peroxidase method as described previously [16]. *Humicola* sp. trehalase was purified according to Neves et al. [31].

Determination of TPS activity

TPS activity was measured by a coupled-enzyme assay as described previously [32]. Specific activity was expressed as μ kat/g of protein. Protein concentration was determined according to Bradford [33].

RESULTS

N-terminal truncated alleles of the plant TPS genes cause better complementation of the growth defect on glucose of the yeast *tps1 Δ* mutant

The plant TPS genes of *S. lepidophylla*, *SITPS1* and *A. thaliana*, *AtTPS1*, have N- and C-terminal extensions, which are not present in the *S. cerevisiae* TPS1 and *E. coli* *otsA* genes (Figure 1A). We have previously shown that full-length *SITPS1* could only complement the growth defect on glucose of the yeast *tps1 Δ* mutant when expressed from a strong promoter. With the weak Cu-inducible *CUP1* promoter, there was only complementation

Figure 1 Comparison of the structure of TPS proteins from different organisms

(A) Full-length TPS proteins (from top to bottom): *S. lepidophylla* (*SITPS1*), *A. thaliana* (*AtTPS1*), *E. coli* (*OtsA*) and *S. cerevisiae* (*ScTps1*). Protein size is shown in amino acid (aa) length. The conserved TPS1 domain is shown in black, the TPP-like region is hatched and the N-terminal region is white. (B) Diagram of plant TPS proteins (from top to bottom): truncated at the N-terminus, the C-terminus or at both ends.

Figure 2 The growth defect of the *S. cerevisiae tps1Δ* mutant on glucose is complemented by truncated Δ NSITPS1, but not by full-length SITPS1 or truncated Δ CSITPS1, expressed from the *CUP1* promoter

The *tps1Δ* mutant was transformed with the empty pSAL4 vector or with pSAL4 containing *SITPS1*, Δ NSITPS1, *SITPS1ΔC*, Δ NSITPS1ΔC or *ScTPS1*. The transformants were spread on agar plates with minimal medium without uracil (—ura) and supplemented with 2% SGal, 2% SGlc or 2% SGlc with 100 μ M copper sulphate. The wild-type strain W303-1A (WT) transformed with pSAL4 was included as a control.

of the *tps1Δtps2Δ* strain and not of the single *tps1Δ* mutant [16]. Complementation of the *tps1Δ* mutant by the *AtTPS1* gene expressed from the strong *PGK1* promoter has also been reported [12]. To investigate whether certain domains of the plant *TPS* genes were preventing complementation under conditions of normal-level expression, we constructed alleles with truncations either of the N- and C-terminus or of both (Figure 1B). For *SITPS1*, 100 amino acids were deleted from the N-terminus and 431 amino acids from the C-terminus, whereas for *AtTPS1* 88 amino acids were deleted from the N-terminus. The truncated plant *TPS* genes were generated by PCR and subcloned in pSAL-derived vectors under the control of *CUP1* promoter (see the Experimental section). The *CUP1* promoter is induced by copper sulphate but gives a low basic expression. The plasmids were then transformed into the *S. cerevisiae tps1Δ* mutant. As shown in Figure 2, N-terminal truncated *SITPS1*, Δ NSITPS1, was able to restore growth of the *tps1Δ* mutant on glucose, whereas the full-length *SITPS1* was not, irrespective of the presence of copper sulphate in the medium. This suggested that N-terminal deletion of *SITPS1* enhanced its activity. The C-terminal-truncated *SITPS1* allele, *SITPS1ΔC*, was not able to complement the *tps1Δ* mutant. Additional deletion of the C-terminus in the Δ NSITPS1 allele did not eliminate its ability to complement the *tps1Δ* mutant. This indicates that deletion of the C-terminus does not cause inactivation of the enzyme.

As opposed to *SITPS1*, complementation of the growth defect of the yeast *tps1Δ* mutant on glucose was observed with the

Figure 3 The growth defect of the *S. cerevisiae tps1Δ* mutant on glucose is better complemented by truncated Δ NSITPS1 than by full-length *AtTPS1* expressed from the *CUP1* promoter

The *tps1Δ* mutant was transformed with the empty pSAL6 vector or with pSAL6 containing *ScTPS1*, Δ NSITPS1 or *AtTPS1*. The transformants were spread on agar plates with minimal medium without His and supplemented with 2% SGal, 2% SGlc or 2% SGlc with 100 μ M copper sulphate. The WT strain W303-1A transformed with pSAL6 was included as a control.

full-length *AtTPS1* gene expressed from the *CUP1* promoter (Figure 3). However, in the absence of copper sulphate, truncation of the N-terminus resulted in much better restoration of growth (Figure 3).

N-terminal truncated plant *TPS* alleles cause high trehalose accumulation on expression in yeast

Although the full-length *A. thaliana* and *S. lepidophylla* *TPS* alleles were able to complement the growth defect on glucose of the yeast *tps1Δ* mutant on strong overexpression, the trehalose level in the transformants was very low [12,16]. Expression of the *AtTPS1* gene or the *SITPS1* gene from the *CUP1* promoter in the yeast *tps1Δ* mutant also resulted in a low trehalose level on the galactose medium (Table 2). However, upon expression of the N-terminal-truncated plant *TPS* genes, the trehalose level was approx. 10–20 times higher than with the corresponding full-length genes (Table 2). With both plant genes, the trehalose content was now in a similar range to that observed on expression of the yeast *TPS1* gene. Expression of C-terminal-truncated *SITPS1* resulted in the same very low trehalose level as with the full-length *SITPS1*. Truncation at the C-terminus in the Δ NSITPS1 allele did not give any additional increase in activity, over that seen with the N-terminal deletion alone (Table 2). These results support the inhibitory role of the N-terminus on the activity of the plant *TPS* alleles and show that deletion of the C-terminus apparently does not affect the activity.

Table 2 Trehalose accumulation and TPS activity in a wild-type yeast strain and in *tps1Δ* strains in which the *S. lepidophylla* or *A. thaliana* *TPS1* or N-terminal truncated *TPS1* gene is expressed

pSAL4, empty vector; *SITPS1* and Δ *NSITPS1*, wild-type and N-terminal truncated *S. lepidophylla* *TPS1*; *AtTPS1* and Δ *NAITPS1*, wild-type and N-terminal truncated *A. thaliana* *TPS1*; *ScTPS1*: *S. cerevisiae* *TPS1*; *SlTPS1ΔC* and Δ *NSITPS1ΔC*, C-terminal truncated *S. lepidophylla* *TPS1* and N- and C-terminal truncated *S. lepidophylla* *TPS1*.

	Tre	Trehalose (μ mol/g)	TPS activity (μ kat/g of protein)
WT + pSAL4	Galactose	66.5	0.77
	Glucose	52.4	0.65
	Fructose	72.5	0.58
<i>1Δ</i> + pSAL4	Galactose	3.2	0.05
	Glucose	NG	NG
	Fructose	NG	NG
<i>1Δ</i> + pSAL4:: <i>SITPS1</i>	Galactose	2.1	0.01
	Glucose	NG	NG
	Fructose	NG	NG
<i>1Δ</i> + pSAL4:: Δ <i>NSITPS1</i>	Galactose	43.5	0.41
	Glucose	49.2	0.65
	Fructose	89.2	0.43
<i>1Δ</i> + pSAL6:: <i>AtTPS1</i>	Galactose	4.1	0
	Glucose	NG	NG
	Fructose	NG	NG
<i>1Δ</i> + pSAL6:: Δ <i>NAITPS1</i>	Galactose	36.5	0.1
	Glucose	41.4	0.12
	Fructose	59	0.07
<i>1Δ</i> + pSAL4:: <i>ScTPS1</i>	Galactose	43.4	1.25
	Glucose	37.1	1.01
	Fructose	54.4	1.53
<i>1Δ</i> + pSAL4:: <i>SlTPS1ΔC</i>	Galactose	0	0
	Glucose	NG	NG
	Fructose	NG	NG
<i>1Δ</i> + pSAL4:: Δ <i>NSITPS1ΔC</i>	Galactose	42.3	0.12
	Glucose	32	0.69
	Fructose	39	0.14

Partial truncations and specific mutations in the N-terminus of *AtTps1* enhance trehalose accumulation capacity

We have performed a detailed mutational analysis of the N-terminus of *AtTps1* to identify amino acids that are important for this inhibitory role. In the first approach, we have gradually truncated the N-terminal DNA extension of *AtTps1* by inverse PCR. The different alleles were transformed into a yeast *tps1Δ* strain, and as a measurement for their activity, trehalose levels were determined (Figure 4A). Deletion of the first nine amino acids did not have any effect on the trehalose level. Further truncation of the N-terminus caused a stepwise increase in the trehalose content. Deletion of some amino acid regions (10–17, 30–33, 45–51, 86–88) caused a more pronounced increase in trehalose level, and these are assumed to have a more important inhibitory function. The increase in trehalose content observed on the additional deletion of amino acids 86–88 could be due to the deletion of Pro⁸⁸. Further truncation beyond amino acid 88 reduced trehalose production again. This is probably caused by the presence of an amino acid region conserved in all TPS genes (Figure 4D). Figure 4(B) shows the growth on glucose containing medium of yeast *tps1Δ* strains that express some of the truncation mutants. Truncation of up to 13 amino acids results in a growth curve that is similar to the one obtained when the full-length *AtTPS1* gene is expressed. Additional truncation of four more amino acid residues results in an allele (Δ 2–17) with a growth curve that resembles closely that of a strain expressing the

ΔNAITPS1 gene, which grows much better compared with a strain expressing the *AtTPS1* gene. This identifies amino acids 14–17 as being important for the inhibitory activity of the N-terminus (see below). Further truncations resulted in the alleles that grow in a similar way as the *ΔNAITPS1* allele (results not shown).

Secondly, we have performed random PCR mutagenesis of the N-terminus. This has resulted in three interesting mutants out of 500 screened. In the first mutant, a 3-fold increase in trehalose-producing capacity was observed compared with the full-length *AtTps1* protein (Figure 4C). In this strain, two mutations were present in the N-terminus [Arg¹⁷ → Gln (R17Q) and Asp⁵² → Gly (D52G)]. We have introduced, by site-directed mutagenesis, each of these mutations in a wild-type allele and showed that only amino acid 17 had an important role in the inhibitory function of the N-terminus. The second interesting mutant showed a 4-fold increase in trehalose content. This increase was due to only one mutation: amino acid 27 was changed from leucine to proline (Figure 4D). The third mutant also contained only one mutation, and this was the same mutation in amino acid 17 (Arg → Gln) as found in mutant 1. This confirmed the importance of this specific amino acid. Interestingly, both amino acids 17 and 27 are conserved in the *Selaginella lepidophylla* *Tps1* protein (Figure 4D, indicated in boldface). The results that we have obtained with the mutagenesis strategy support the possible importance of the inhibitory activity of the N-terminus. Interaction of other proteins with critical amino acid residues in the N-terminus may modulate activity of the protein.

N-terminal truncated plant *TPS* alleles cause high TPS activity on expression in yeast

So far, the significant TPS activity in the plant *TPS* gene products has not been directly demonstrated. All evidence for TPS activity was indirect, based on the restoration of growth on glucose of a *S. cerevisiae* *tps1Δ* mutant. Also, on expression of the full-length *SITPS1* allele in a yeast *tps1Δ* mutant only very low activity could be detected [16]. Expression of the N-terminal-truncated *SITPS1* allele, on the other hand, results in a 40-fold higher TPS activity (Table 2). The levels obtained were approx. 30–50 % of the activity obtained with the yeast *TPS1* allele expressed from the same promoter and plasmid, depending on the carbon source used for growth. With the N-terminal-truncated *AtTPS1* allele, TPS activity was 15–25 % of that obtained with the N-terminal-truncated *SITPS1* allele and approx. 10 % of the activity obtained with the yeast *TPS1* allele (Table 2). With the full-length *AtTPS1* allele also, the TPS activity could not be detected. C-terminal truncation did not significantly affect enzymic activity of the N-terminal-truncated *S. lepidophylla* allele (Table 2). This is consistent with the absence of any effect of the C-terminal deletion of this *ΔNSITPS1* allele for complementation of the growth defect on glucose and for trehalose accumulation in the *tps1Δ* mutant.

The plant TPS proteins do not completely restore the control of glucose influx into glycolysis in a yeast *tps1Δ* mutant

Yeast *tps1Δ* mutants show hyperaccumulation of sugar phosphates, especially fructose-1,6-bisphosphate (Fru1,6bisP), and depletion of ATP and free phosphate after addition of glucose [18]. We have measured Fru1,6bisP, ATP and free phosphate levels as a function of time after addition of glucose to cells of *tps1Δ* strains, expressing the full-length or N-terminal-truncated *S. lepidophylla* or *A. thaliana* *Tps1* protein (Figure 5).

Figure 4 Mutational analysis of the N-terminus of *AtTPS1*

(A) Different alleles of the *AtTPS1* gene with various truncations or internal deletions of the N-terminus were constructed as indicated in the Experimental section and were transformed in a yeast *tps1Δ* strain. The transformants were grown in 5 ml of SGal-His for 48 h and trehalose levels were determined. The black bars indicate the full-length and the *ΔNAtTPS1* genes. (B) Growth curve on SGlc-His medium of yeast *tps1Δ* strains with expression vectors containing different N-terminal truncated alleles of the *AtTPS1* gene. The following alleles are shown: ●, *AtTPS1*; ▲, *Δ(2-5)*; △, *Δ(2-9)*; ■, *Δ(2-13)*; □, *Δ(2-17)*; ◆, *Δ(2-21)*; ○, *ΔNAtTPS1*. (C) Different point mutants in the N-terminus of *AtTPS1* were obtained by PCR mutagenesis and were expressed in a *tps1Δ* strain. The transformants were grown in 5 ml of SGal-His for 48 h and trehalose levels were determined. (D) Comparison of the N-terminal region of the *A. thaliana AtTPS1* with other TPS protein sequences. Amino acids 17, 27 and 52 identified with PCR mutagenesis are indicated in boldface. Identical residues are indicated in white with black background. Amino acid sequences of TPS from *A. thaliana* (*AtTPS1*; accession no. Y08568); *S. lepidophylla* (*SlTPS1*; accession no. U96736); *S. cerevisiae* (*ScTPS1*; accession no. X68214) and *E. coli* (*OtsA*; accession no. X69160).

Figure 5 Fru1,6bisP, ATP and free phosphate level (mM) in the first few minutes after addition of glucose to yeast cells pre-grown on glycerol

●, Wild-type strain (W303-1A); ○, *tps1Δ*; ▲, *tps1Δ* + *SITPS1*; △, *tps1Δ* + *ΔNSITPS1*; ■, *tps1Δ* + *AtTPS1*; □, *tps1Δ* + *ΔNAAtTPS1*.

Fru1,6bisP hyperaccumulation in the *tps1Δ* strain was not suppressed by expression of the full-length or the N-terminal-truncated plant proteins (Figure 5). Also the glucose 6-phosphate and fructose 6-phosphate levels showed a partial hyperaccumulation (results not shown). The ATP level did not fall as low as in the *tps1Δ* strain, but it was still considerably lower compared with the control wild-type strain, consistent with the hyperaccumulation of sugar phosphates (Figure 5). The somewhat higher ATP level is in agreement with the restoration of growth on glucose in these strains. The free phosphate level showed a similar fall compared with the *tps1Δ* strain, i.e. it decreased more significantly than in the wild-type strain (Figure 5). These results show that the plant TPS enzymes, neither the full-length allele with low activity nor the N-terminal truncated allele with high activity, are able to restore proper control of glucose influx into glycolysis. Recently [34], we have shown a similar phenotype for a yeast strain where the *TPS1* gene had been replaced by its *E. coli* homologue *OtsA*. In this study too, growth on glucose was restored, but not proper control of glucose influx into glycolysis.

DISCUSSION

N-terminal truncation reveals high potential TPS activity in plant *TPS1* gene products

Several results indicate that truncation of the N-terminus of both the *A. thaliana* and *S. lepidophylla* TPS enzymes strongly enhances their catalytic activity and their capacity to enhance trehalose content when expressed in yeast. The growth defect on glucose of the yeast *tps1Δ* mutant was much better suppressed by the N-terminal-truncated alleles than by the full-length alleles. The accumulation of trehalose was much higher and the trehalose levels actually approached the levels obtained with the yeast Tps1 enzyme. Finally, direct measurements of TPS activity in the cell extracts of yeast *tps1Δ* transformants resulted in much higher activity with the truncated plant alleles compared with the full-length alleles.

The low activity of the plant TPS enzymes on expression in yeast could have been due to several factors. First, the problem could have been caused in some way by the expression in the heterologous system. The expression level in yeast could have been too low, the proper post-translational modifications could have been absent, or the yeast cells might contain components inhibiting the plant enzyme. Secondly, the problem could have been specific for trehalose metabolism. It is well known that yeast TPS activity is much lower in mutants lacking proper formation of the trehalose synthase complex, e.g. due to absence of Tps2 or of both Tps3 and Tsl1 [35,36]. Hence, inability of the plant TPS protein to form a trehalose synthase complex with the yeast Tps2, Tps3 and Tsl1 subunits could also have been a cause. Thirdly, the plant TPS enzymes could have had an inherently much lower activity than the yeast enzyme. This would not have been surprising, at least in the case of *A. thaliana*, since it accumulates trehalose only at very low levels and only on growth in the presence of validamycin A, a potent trehalase inhibitor [10,11] (N. Avonce, P. Van Dijck, J. M. Thevelein and G. Iturriaga, unpublished work). Trehalose has been detected in a few plants, such as the two desiccation-tolerant plants, *S. lepidophylla* and *M. flabellifolia*. However, in spite of the high trehalose accumulation in *S. lepidophylla* (even under non-stressed conditions), the activity of its TPS enzyme was equally low compared with that of the *A. thaliana* enzyme.

Our results with the N-terminal-truncated plant TPS enzymes appear to exclude the possibility that the low activity was due to unspecific problems, such as those associated with the expression in a foreign host. In addition, we could not show differences in TPS complex formation between the full-length and the N-terminal-truncated plant TPS enzymes (results not shown). Hence, all results seem to be consistent with the conclusion that the full-length plant TPS enzymes have a much lower inherent activity than the N-terminal-truncated enzymes and that the N-terminus inhibits in some way the activity of the catalytic domain. This suggests that the N-terminus could be a target for modulation of plant TPS activity. This is supported by the identification of specific amino acid residues in the N-terminus important for the inhibition of TPS activity. Mutagenesis of the amino acids 17 (arginine) and 27 (leucine) resulted in a significant release of inhibition, and their importance is further underscored by their conservation in the *Selaginella* sp. Tps1 protein. These results suggest that these amino acids could act as potential interaction sites for other proteins modulating TPS activity. One class of proteins that may interact with the Tps proteins are the 14-3-3 proteins, of which one has previously been shown to interact with a cauliflower class II TPS [37]. 14-3-3 proteins are homodimers or heterodimers that bind to phosphopeptide motifs in target proteins [38]. That 14-3-3 proteins can influence the catalytic

activity of enzymes has recently been shown for sucrose phosphate synthase which is inhibited when it is bound by 14-3-3 proteins [39].

Apart from *AtTPS1*, two other *A. thaliana* genes with homology to yeast *TPS1* (*AtTPS7* and *AtTPS8*) have recently been expressed in yeast cells [11]. In this study too, it was not possible to demonstrate any TPS or TPP activity for these class B genes. Also in this case, it is not excluded that specific modifications may result in alleles with high activity. The very low activity of the plant TPS enzymes has made the examination of the role of trehalose metabolism in plants difficult. Determination of TPS activity in plant extracts is not possible, which has hampered purification of the enzyme. The very low activity would also impede proper characterization of the purified enzyme. Our results create new possibilities for investigation of the plant TPS enzyme in plants. First, it might be possible to enhance TPS activity in plant extracts by limited proteolysis. This might also facilitate detection of TPS activity. Secondly, the N-terminal truncated plant TPS alleles can be expressed in transgenic plants, and their activity can then be assessed in cell extracts. In addition, these much more active alleles might cause similar effects on the physiology and morphology of the plants to those described for expression of the heterologous *E. coli* and yeast *TPS* genes [19–22].

Possible functions for the plant TPS enzyme

In many bacteria, fungi and other lower eukaryotes, significant levels of trehalose are accumulated either in vegetative cells or in developmental stages [1,3,40]. The absence of trehalose in most of the higher plants is not in agreement with a simple house-keeping function for the plant TPS enzymes in trehalose accumulation. In spite of this, we now demonstrate that the plant TPS enzymes do have a high potential catalytic activity. This appears to be highly significant for elucidation of their function.

The unusually high potential activity of the plant TPS enzymes, in view of the absence of trehalose, could be explained in several ways. First, one could suggest that the plant TPS enzymes do have a house-keeping function, but that trehalose is only accumulated in very specific plant tissues or subcellular compartments and/or under specific conditions and that this has largely gone unnoticed. A study on salinity-induced trehalose accumulation in roots of rice seems to be consistent with this idea [41]. We have fused the N-terminal region of *SlTPS1* to green fluorescent protein and expressed it under appropriate strong promoters in yeast and tobacco protoplasts. In both cases, fluorescence was present in the whole cell rather than discrete or compartmentalized (results not shown). These results suggest that the N-terminal sequence is probably not an intracellular targeting sequence. A purely house-keeping function for plant TPS in trehalose biosynthesis does not seem to be very consistent with the dramatic and predictable effects of modification of plant trehalose metabolism on important physiological properties of the whole plant [13,23,25]. Secondly, the high potential activity could be indicative of a signalling function for the plant TPS enzyme. In most circumstances, the activity would be low, but occasionally and possibly very transiently, the activity could be enhanced drastically during signal transduction. Such a situation is observed, e.g. in yeast adenylate cyclase, whose potential activity is much higher than that expected from the low basal cAMP level in the cells. It is so high that in feedback-inhibition-deficient mutants it allows rapid accumulation of cAMP to levels that are 100–1000-fold higher than the basal cAMP level in the cells [42,43]. Interestingly, also yeast adenylate cyclase has a N-terminal inhibitory domain, which might be a target for activation

of the enzyme by regulatory proteins [44]. Interaction of the N-terminus of the plant TPS enzymes with a regulatory protein might relieve the inhibition on the catalytic domain and cause rapid accumulation of trehalose-6-phosphate or trehalose.

The N-terminal truncated TPS enzyme of *S. lepidophylla* showed 5-fold higher activity than that of *A. thaliana*. This might also indicate that the full-length *SlTps1* enzyme is intrinsically more active in the plant than the *AtTps1* enzyme. This difference could offer an (partial) explanation for the high accumulation of trehalose in *S. lepidophylla* as opposed to *A. thaliana*. Otherwise, the properties of the two TPS enzymes appear similar. Both full-length enzymes have very low activity and both truncated enzymes have much higher activity. Even though the difference in activity between the two N-terminal-truncated plant enzymes *in vitro* reflected a real difference in activity between the full-length enzymes *in vivo*, it does not seem to be a sufficient explanation for the huge difference in trehalose accumulation in the two plants. Hence, the presence or absence of trehalose accumulation in *S. lepidophylla* and *A. thaliana* appears to be mainly determined by regulation of the TPS enzyme rather than by a very large difference in intrinsic activity. On the other hand, differences in trehalase activity in the two plants could also be involved.

Role of Tps1 in control of carbon metabolism

In a previous study, a strong interest has been raised in the role of glucose-sensing mechanisms in plants and their crucial role in regulating a variety of plant physiological properties, in particular source/sink sugar allocation [45]. Because of the close connection between trehalose biosynthesis and glucose catabolism, and the similarity of phenotypic properties regulated by glucose sensing and those affected by modification of plant trehalose metabolism, the latter has been proposed as possibly playing a role in the glucose-sensing process [13,23]. Interestingly, in yeast, *Tps1* is closely involved in the control of hexokinase activity, an important player in glucose sensing, by either its product trehalose-6-phosphate and/or the protein itself [17,18,34]. In plants too, extensive evidence has been obtained for a role of hexokinase in sugar sensing [46]. On the other hand, a recent work [25] has shown that *A. thaliana* hexokinase *AtHxk1* is not sensitive to inhibition by trehalose 6-phosphate *in vitro*, and hence it is questionable whether trehalose metabolism acts on plant carbon metabolism via an effect on hexokinase. In the present study, we have shown that the plant *TPS1* gene products are unable to control glucose influx into yeast glycolysis in the same way as the yeast *TPS1* gene product. This appears to support that the plant *Tps1* proteins act at least partially in a different way compared with the yeast *Tps1* protein. Since the plant *Tps1* proteins do restore growth on glucose of yeast *tps1Δ* mutants, they are likely to share at least one target with the yeast *Tps1* protein.

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