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A *Selaginella lepidophylla* Trehalose-6-Phosphate Synthase Complements Growth and Stress-Tolerance Defects in a Yeast *tps1* Mutant[1]

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The accumulation of the disaccharide trehalose in anhydrobiotic organisms allows them to survive severe environmental stress. A plant cDNA, *SlTPS1*, encoding a 109-kD protein, was isolated from the resurrection plant *Selaginella lepidophylla*, which accumulates high levels of trehalose. Protein-sequence comparison showed that SlTPS1 shares high similarity to trehalose-6-phosphate synthase genes from prokaryotes and eukaryotes. *SlTPS1* mRNA was constitutively expressed in *S. lepidophylla*. DNA gel-blot analysis indicated that *SlTPS1* is present as a single-copy gene. Transformation of a *Saccharomyces cerevisiae tps1*Δ mutant disrupted in the *ScTPS1* gene with *S. lepidophylla SlTPS1* restored growth on fermentable sugars and the synthesis of trehalose at high levels. Moreover, the *SlTPS1* gene introduced into the *tps1*Δ mutant was able to complement both deficiencies: sensitivity to sublethal heat treatment at 39°C and induced thermotolerance at 50°C. The osmosensitive phenotype of the yeast *tps1*Δ mutant grown in NaCl and sorbitol was also restored by the *SlTPS1* gene. Thus, SlTPS1 protein is a functional plant homolog capable of sustaining trehalose biosynthesis and could play a major role in stress tolerance in *S. lepidophylla*.

An amazing adaptation that allows survival under complete dehydration is present in yeast cells, fungal spores, certain invertebrate species, and resurrection plants that resume their vital functions as soon as they resume contact with water (Clegg, 1965; Gaff, 1971; Thevelein, 1984). These anhydrobiotic organisms also withstand strong vacuum, high doses of ionizing radiation, and extreme temperatures without suffering damage. In addition, many of these organisms accumulate the nonreducing disaccharide trehalose (Weisburd, 1988; Crowe et al., 1992).

Among several well-characterized osmoprotectors (Yancey et al., 1982), trehalose seems to be one of the most efficient at maintaining lipids in a fluid phase in the absence of water, thus avoiding phase separation, leakage, and membrane fusion (Crowe et al., 1984, 1987). Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside), like other polyols, plays a key role in the structural and functional stabilization of membranes and proteins in the anhydrous state, apparently by means of water replacement of osmolyte molecules (Clegg, 1985) or formation of a glassy state (Burke, 1985).

The biosynthesis of trehalose consists of two enzymatic steps catalyzed by the oligomeric subunits TPS, which synthesizes trehalose-6-P from Glc-6-P and UDP-Glc, and TPP, which forms trehalose (Cabib and Leloir, 1958; Vandercammen et al., 1989; Londesborough and Vuorio, 1993). The genes encoding both enzymes from bacteria and yeast have already been isolated and sequenced (Luyten et al., 1993; Kaasen et al., 1994). Deletion mutants in the trehalose pathway in these microorganisms cause a reduction in osmotolerance (Giaever et al., 1988; Mackenzie et al., 1988) and thermotolerance (Hengge-Aronis et al., 1991; De Virgilio et al., 1994).

The yeast *tps1*Δ mutant and its alleles are unable to grow in Glc as the sole carbon source. There is strong evidence to suggest that this defect is attributable to the additional role of the TPS1 subunit in regulating the flow of Glc into the cell (Van Aelst et al., 1993; Neves et al., 1995; Thevelein and Hohmann, 1995; Hohmann et al., 1996).

Here we report the isolation and molecular characterization of a full-length cDNA encoding the enzyme TPS (*SlTPS1*) from the resurrection plant *Selaginella lepidophylla*, which is one of the organisms that accumulates trehalose at higher levels (Adams et al., 1990; Müller et al., 1995). We show that *SlTPS1* cDNA encodes a functional TPS able to

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Abbreviations: TPP, trehalose-6-P phosphatase; TPS, trehalose-6-P synthase.

restore growth in fermentable sugars of a yeast *tps1*Δ mutant. Furthermore, *SlTPS1* synthesizes high levels of trehalose and complements osmotolerance and thermotolerance deficiencies in a *tps1*Δ mutant.

MATERIALS AND METHODS

Plant Material

Selaginella lepidophylla (Hook. & Grev. Spring.) plants were collected from arid regions of Morelos state in Mexico. Plants were rehydrated and maintained in controlled conditions (24°C and 16 h of light with an average of 50% humidity) in growth chambers (Conviron, Asheville, NC). Subsequently, *S. lepidophylla* microphyll fronds were air dried at the indicated times by placing them on 3MM filter paper (Whatman).

Strains

The cDNA bank was plated in the *Escherichia coli* strain XL-1-Blue MRF′, and the strain SOLR was used to excise the pBluescript from the λ phage, following the manufacturer's instructions (Stratagene). The *E. coli* DH5α strain was used to subclone and make constructs. The yeast strains were wild type, W303-1A (*Mat a leu2-3, 112 ura3-1, trp1-1, his3-11, 15 ade2-1, can1-100, GAL, SUC2*) (Thomas and Rothstein, 1989); *tps1*Δ, YSH290 (W303-1A, *ggs1/tps1*Δ::*TRP1*) (Hohmann et al., 1993); *tps2*Δ, YSH450 (W303-1A, *tps2*Δ::*LEU2*) (Neves et al., 1995); and *tps1*Δ*tps2*Δ, YSH652 (W303-1A, *ggs1/tps1*Δ::*TRP1*, *tps2*Δ::*LEU2*) (Neves et al., 1995).

Construction of the cDNA Bank of *S. lepidophylla*

An expression bank was prepared with mRNA isolated from *S. lepidophylla* microphylls dehydrated for 2.5 h using the ZAP cDNA synthesis kit, the Uni-ZAP XR vector, and the Gigapack II Gold packaging extracts following the manufacturer's instructions (Stratagene). The initial titer of the bank was 2×10^6 plaques of bacteriophage/mL and 1.5×10^{11} plaques of bacteriophage/mL after bank amplification. After 4×10^5 recombinant bacteriophages from the amplified cDNA bank of *S. lepidophylla* were plated, 13 plaques were obtained that hybridized with a mixture of the five oligonucleotides TPS5′-1, TPS5′-2, TPS5′-3, TPS3′-1, and TPS3′-2 (see Fig. 1A). In a second screening round, only 6 of the initial 13 plaques hybridized with a mixture of the oligonucleotides TPS5′-1 and TPS5′-2. In a third step, pIBT6 was the only clone that hybridized with the oligonucleotide TPS5′-1, corresponding to the most 5′ end of the selected region for isolation of the cDNA. Plaques were converted into plasmid by in vivo excision according to the manufacturer's instructions (Stratagene). Plasmid DNA was digested with *Eco*RI and *Xho*I to excise the corresponding insert, transferred to a Hybond N^+ nylon membrane (Amersham), and hybridized to oligonucleotides ^{32}P labeled with T4 polynucleotide kinase.

The following degenerated oligonucleotides were synthesized for screening of the cDNA bank: TPS5′-1, 5′-YTNTGGCCNBCNTTYCAYTAY-3′; TPS5′-2, 5′-GGNTKBTTYYTNCAYAYNCCNTTYCC-3′; TPS5′-3, 5′-MGNYTNGAYTAYWBNAARGGNBTNCC-3′; TPS3′-1, 5′-SWNACNARRTTCATNCCRTCNCK-3′; and TPS3′-2, 5′-CCRWANTKNCCRTTDATNCKNCC-3′ (single-letter abbreviations for wobble nucleotides are B, C, G, or T; K, G or T; M, A or C; N, A or C or G or T; R, A or G; S, C or G; W, A or T; D, A, G, or T; and Y, C or T). These oligonucleotides were designed based on conserved domains of *TPS* sequences (depicted in Fig. 1A) using the following accession numbers: *E. coli*, X69160; *Schizosaccharomyces pombe*, Z29971; *Aspergillus niger*, U07184; *Saccharomyces cerevisiae*, X68214; and *Kluyveromyces lactis*, X72499.

Hybridization of Nucleic Acids

To screen the cDNA bank, the bacteriophage plaques were transferred to a nylon membrane and the filter was hybridized with oligonucleotides and labeled with the ^{32}P isotope by means of T4 polynucleotide kinase using 6× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) at 37°C. The filter was washed three times at the same temperature for 10 min each under the following conditions: 6× SSC, 4× SSC, and 2× SSC.

Southern- and northern-blot techniques were used according to standard protocols (Sambrook et al., 1989) with the following modifications. For the genomic Southern-blot analysis, the DNA was fractionated on a 0.8% agarose gel in TBE (0.09 M Tris-borate and 0.002 M EDTA) buffer and transferred to a nylon membrane. The filter was hybridized using *SlTPS1* cDNA labeled with ^{32}P isotope as a probe, using 2× SSC at 65°C. The filter was washed three times at the same temperature for 20 min each time under the following conditions: 2× SSC, 1× SSC, and 0.5× SSC. For the northern-blot analysis, a 1.2% agarose gel was used in a Mops-formaldehyde buffer and a Hybond N^+ nylon membrane was also used for the transfer. Hybridization conditions were in 50% formamide and 2× SSC at 42°C. The three successive filter washings were performed with 2× SSC, 2× SSC, and 1× SSC at 55°C.

DNA Sequencing

Nested deletions of the insert were created with the enzymes exonuclease III and nuclease S1 from the selected clone to subsequently determine the nucleotide sequence of both strands using Sequenase version 2.0 (United States Biochemical). Protein sequence alignments were analyzed using the CLUSTAL W software program (Thompson et al., 1994).

DNA Manipulation and Constructs

Recombinant DNA techniques such as bacterial transformation, isolation of DNA from plasmid, λ bacteriophage, and labeling of radioactive fragments were carried out according to standard procedures (Sambrook et al., 1989). For expression in yeast, two new shuttle-expression vectors

named pSAL4 and pRS6 were constructed. pSAL4 is similar to pSAL1 (Mascorro-Gallardo et al., 1996), but it has the pRS426 backbone (Christianson et al., 1992) with the 2 μ origin of replication and the *URA3* marker. The pRS6 vector was constructed using the *PMA1* gene promoter from the pRS699 vector (Serrano and Villalba, 1995), a polylinker, and the *CYC1* terminator. This cassette was cloned in the pRS423 backbone (Christianson et al., 1992) with the 2 μ origin of replication and the *HIS3* marker.

Two out-of-frame ATG triplets in *SlTPS1* cDNA leader (see Fig. 1D) were removed by digesting the pIBT6 clone with *Nco*I and treatment with nuclease S1. The intact leader precludes expression of SlTPS1 in yeast (data not shown). The 3.2-kb *SlTPS1* cDNA was subcloned into pSAL4 or pRS6, and the resulting constructions were designated pSTS1 and pRTS1, respectively. The *ScTPS1* and *ScTPS2* yeast genes were isolated from genomic yeast DNA by PCR using primers ScTPS1-5′ (5′-CCGCTCGAGGGTACTCACATACAGAC-3′), ScTPS1-3′ (5′-ATAGTTTTGCGGCCGCATCGGGTTCATCAG-3′), ScTPS2-5′ (5′-CCGCTCGAGCACTATTTCTGTGCCG-3′), and ScTPS2-3′ (5′-CGGGGTACCATGGTGGGTTGAGAC-3′). Amplified fragments were cloned into pSAL4 to give plasmids pSTS2 and pSTS3 or into pRS6 to give plasmids pRTS2 and pRTS3. A truncated *SlTPS1* (*SlTPS1*Δ*C*) was constructed by deleting the DNA sequence coding for the 431-amino acid C terminus. The *SlTPS1* region coding for the resting 563-amino acid N terminus was amplified by PCR with primers SlDC400-5′ (5′-CATGCCATGGCTATGCCTCAGCCTTACC-3′) and SlDC400-3′ (5′-CGGGGTACCTCACTTTGACTCCGAGTACTTTGC-3′) with TAG termination codon. A deletion of *SlTPS1* (*SlTPS1*Δ*N*) comprising the 396-amino acid C terminus was constructed by PCR with primer SlDN600-5′ (5′-CCGCTCGAGCCATGGTGCATATTCCGCCTCAATTGCC-3′) and universal primer (5′-GTAAACGACGGCCAGT-3′). Both *SlTPS1*Δ*C* and *SlTPS1*Δ*N* were amplified using pIBT6 as a template. The PCR products were subcloned into pSAL4 vector to give plasmid pSTS4 or into pRS6 vector to give plasmid pRTS4. PCR was conducted using the Expand High Fidelity PCR System (Boehringer Mannheim), and reaction conditions were 1 cycle at 94°C for 3 min; 30 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 2 min; and 1 cycle at 72°C for 10 min.

Transformation, Complementation, and Stress Assays in Yeast

Yeast was grown at 30°C in minimal medium (0.7% Bacto-yeast nitrogen base without amino acids, pH 6.0, supplemented with 0.002% adenine, 0.002% His, 0.003% Leu, 0.003% Trp, and 0.002% uracil) plus 2% Gal. Transformation was performed as described previously (Elble, 1992), and transformants were selected in medium without uracil for pSAL4 or in medium without His for pRS6. For each construct, at least three independent transformants were chosen to test their ability to restore the growth defect on minimal medium plus 2% Glc. The candidates were streaked on agar plates with minimal medium with 2% Gal, Glc, or Fru. As a control, the same strains were transformed with the pSAL4 or the pRS6 vector alone. After 3 d, growth was visible in positive control and complemented mutants.

For the thermotolerance assays, liquid cultures were grown at 25°C in minimal medium plus 2% Gal up to the mid-log phase (4×10^6 colony-forming units/mL, corresponding to 0.4 A_{600}), then shifted to 39°C for 1 h for thermoinduction, and further incubated at 50°C for different times to evaluate induced thermotolerance. Decimal dilutions were plated in solid YPGal (2% Bacto-peptone, 1% yeast extract, 2% Gal, and 2% agar) and grown at 25°C for 3 d before colony counting. The level of thermotolerance was expressed as the ratio (percentage) of viability after the 50°C treatment and immediately before the 50°C treatment.

For the osmotolerance assays, exponential cultures were first diluted until A_{600} was 0.1 and then serially diluted 5-fold at each step. Spots were made with 4 μL of each dilution and plated in solid YPGal supplemented with 0.9 M NaCl or 1.6 M sorbitol. Growth was recorded after 3 d for the control plates and after 4 d for the osmotic stress treatments.

Trehalose Determination

Essentially, yeast cells (25–50 mg fresh weight) were collected by vacuum filtration through 0.22- to 0.45-μm membranes (Gelman Sciences, Ann Arbor, MI) and washed several times with ice-cold water to remove external Glc. Yeast cells were scraped from the filter membrane before being frozen in liquid nitrogen and stored at −80°C or immediately transferred to a screw-capped tube containing 1 mL of 0.25 M Na_2CO_3 and boiled in a water bath for 20 min. After cooling, samples were centrifuged in a microfuge and 0.2 mL of supernatant was mixed with 0.1 mL of 1 M acetic acid to neutralize before the addition of 0.1 mL of buffer T (0.3 M sodium acetate plus 0.03 M $CaCl_2$, pH 5.5). For trehalose quantification, 0.05 mL of samples or trehalose standards and 0.05 mL of *Humicola grisea* trehalase (or water to determine Glc not derived from trehalose) were incubated for 45 min at 40°C. One milliliter of Tris-HCl buffer, pH 8.0, containing 100 units each of Glc oxidase and peroxidase (Sigma) plus 0.1 mg of *o*-dianisidine (Sigma) was added. Incubation was for 1 h at 30°C, and the reaction was stopped with 0.5 mL of 56% (v/v) sulfuric acid. The A_{546} was determined before 1 h. *H. grisea* trehalase was purified according to the method of Neves et al. (1994).

RESULTS

Cloning and Sequence Analysis of *S. lepidophylla SlTPS1*

To isolate a gene involved in trehalose synthesis, a comparison of the deduced amino acid sequences of TPS was made from the reported nucleotide sequences from bacteria and yeast (see "Materials and Methods"). Highly conserved regions were selected to synthesize degenerated oligonucleotides (Fig. 1A), which were used to screen a cDNA library from *S. lepidophylla* (see "Materials and Methods"). The largest clone isolated, pIBT6 (3.2 kb), contains a 110-nucleotide 5′ leader sequence, a 96-nucleotide

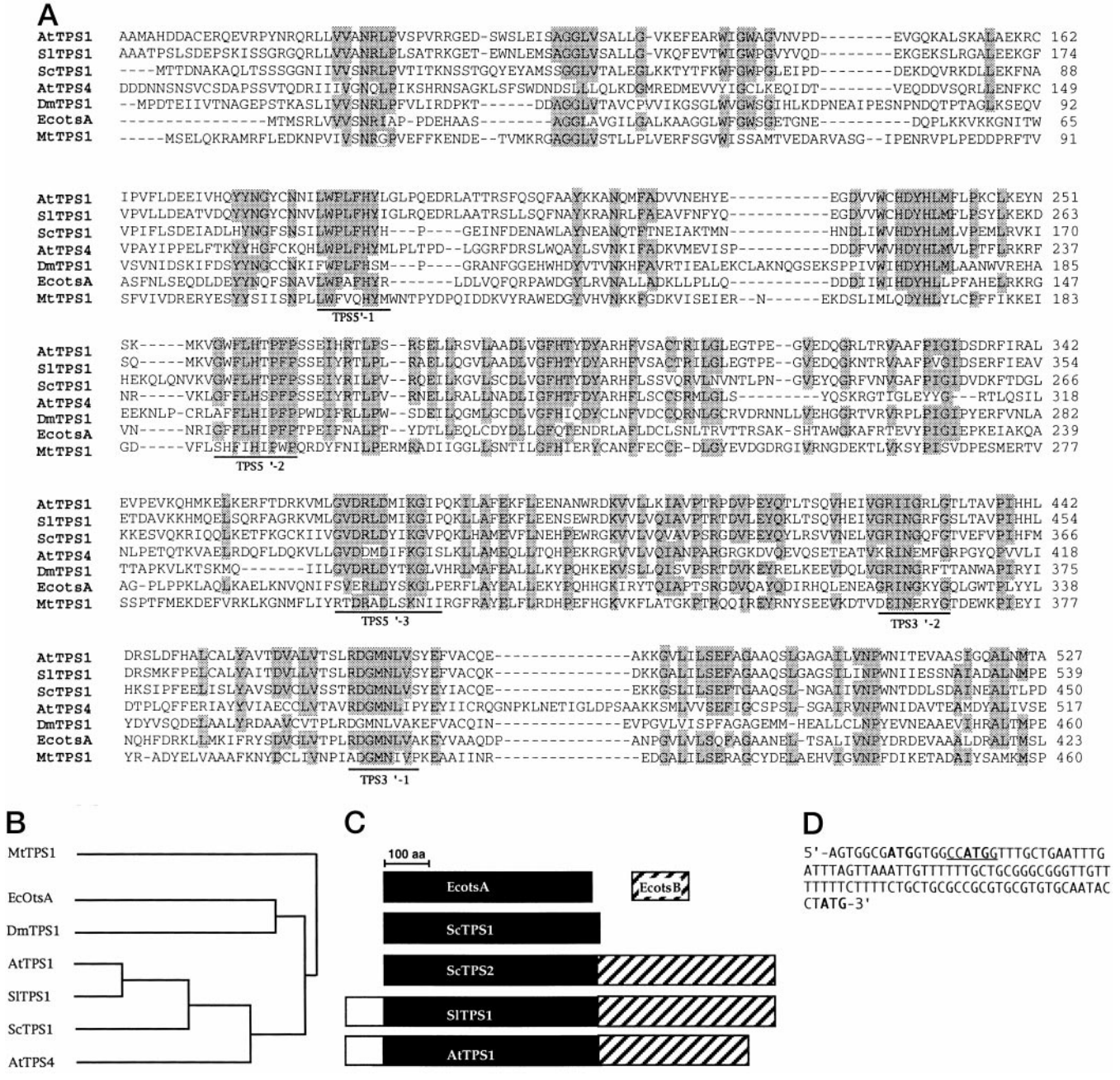

Figure 1. Comparison of *S. lepidophylla* SlTPS1 with other TPS protein sequences. A, Alignment of an internal region of plant TPS amino acid sequences with bacteria, yeast, and animal sequences. Gaps that were introduced to optimize the alignment are indicated by dashes; identical residues are shaded. B, Dendrogram of TPS sequences. Amino acid sequences of TPS from (top to bottom) *M. thermoautotrophicum* (MtTPS1; accession no. AE000931); *E. coli* (EcOtsA; accession no. X69160); *D. melanogaster* (DmTPS1; accession no. AC004373); *A. thaliana* (AtTPS1; accession no. Y08568); *S. lepidophylla* (SlTPS1; accession no. U96736); *S. cerevisiae* (ScTPS1; accession no. X68214); and *A. thaliana* (AtTPS4; accession no. Z97344). DmTPS1 and AtTPS4 expressed sequence tags were found in databases from the respective systematic genome-sequencing programs. C, Diagram of TPS and TPP protein sequences. A comparison of protein size and structure is shown based on sequence-alignment data presented in A and compared with *E. coli* (EcotsB) and *S. cerevisiae* (ScTPS2) sequences. Protein size is shown in amino acid (aa) length. D, The 5′ leader sequence of the *SlTPS1* gene is shown. Three ATG triplets are present (boldface), but only the one close to the 3′ end is in frame with the open reading frame sequence; thus, it is considered to be the putative initiation codon. The *Nco*I restriction site is underlined.

3′-untranslated sequence, and an open reading frame coding for a protein of 994 amino acids with a calculated molecular mass of 109 kD; it was designated SlTPS1. The alignment of deduced amino acid sequences of TPS from bacteria, yeast, plants, and animals showed several regions of homology (Fig. 1A). Only the most conserved region in all of the compared TPS sequences is shown. These deduced amino acid sequences were plotted in a dendrogram (Fig. 1B). The most distant sequence from SlTPS1 was the archaebacterium *Methanobacterium thermoautotrophicum* MtTPS1, which is only 27% identical to *S. lepidophylla* SlTPS1. The *E. coli* EcOtsA and the *Drosophila melanogaster* DmTPS1 sequences shared 35% and 40% identity to SlTPS1, respectively. Plant and yeast TPS sequences were found closely related to each other. SlTPS1 is the most related to the recently reported AtTPS1 sequence from Arabidopsis (Blázquez et al., 1998), and both sequences share an identity of 67%, whereas the *S. cerevisiae* ScTPS1 is 50% identical to both SlTPS1 and AtTPS1. It is interesting that another Arabidopsis TPS homolog, AtTPS4, is more closely related to ScTPS1 (41% identity) than to SlTPS1 and AtTPS1 (37% identity). *AtTPS4* may represent a divergent TPS gene that probably arose by duplication of the ScTPS1 ancestor gene.

An additional feature of plant TPS sequences is the presence of two putative domains (Fig. 1C). Only the N-terminal sequence of SlTPS1, which contains 563 amino acids, has similarity to TPS sequences (Fig. 1, A and B), whereas the remaining 431-amino acid sequence of SlTPS1, which constitutes its C-terminal region, is also present in AtTPS1 and AtTPS4 but is absent in yeast and *E. coli* TPS sequences (Fig. 1C). This C-terminal region of SlTPS1 shows a certain degree of identity to sequences encoding TPP: 29% to the yeast ScTPS2 subunit and 22% to the *E. coli* EcOtsB enzyme.

Expression Pattern and Copy Number of *SlTPS1*

RNA-blot analysis was performed to investigate the expression pattern of the *SlTPS1* gene. Poly(A^+) RNA was isolated from *S. lepidophylla* microphylls (lycophyte leaves) that were fully turgid or desiccated for different lengths of time. *SlTPS1* mRNA is expressed as a single band corresponding to a 3.2-kb transcript present in fully hydrated and dehydrated *S. lepidophylla* microphylls at similar levels (Fig. 2). This constitutive expression of the *S1TPS1* gene is in agreement with the comparable levels of trehalose in both nonstressed and desiccated *S. lepidophylla* plants (Adams et al., 1990).

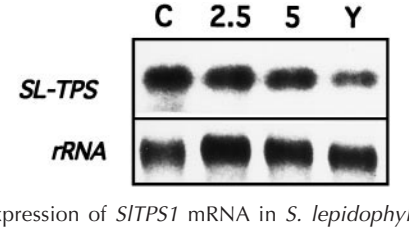

Figure 2. Expression of *SlTPS1* mRNA in *S. lepidophylla*. Poly(A^+) RNA (2 μg) was extracted from fully hydrated (lane C) *S. lepidophylla* microphylls or dehydrated for 2.5 h (lane 2.5), 5 h (lane 5), or 1 year (lane Y). The RNA blot was hybridized with ^{32}P-labeled *SlTPS1* cDNA (top row). An rRNA gene fragment was hybridized to the same filter as a loading control (bottom row).

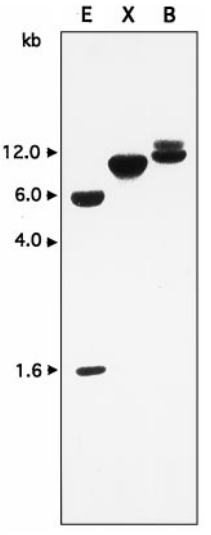

Figure 3. DNA gel-blot analysis of the *SlTPS1* gene. Genomic DNA (20 μg) of *S. lepidophylla* was digested with *Eco*RI (lane E), *Xba*I (lane X), or *Bam*HI (lane B). The filter was probed with ^{32}P-labeled *SlTPS1* cDNA. The positions of the DNA markers are indicated on the left.

The copy number of the *SlTPS1* gene was determined by DNA gel-blot analysis. *S. lepidophylla* genomic DNA was digested with *Eco*RI, which cuts internally, and *Bam*HI and *Xba*I, which do not cut the cDNA. After probing with the full-length *SlTPS1* insert, two expected bands were obtained with *Eco*RI (Fig. 3). Digestion with *Xba*I corresponded to a single band, but two bands were obtained using *Bam*HI, suggesting either that there are two *SlTPS1* genes or that the *Bam*HI site is present in an intron. To test this latter possibility, we used specific oligonucleotides matching the coding 5′ and 3′ ends of *SlTPS1* to amplify genomic homologs by PCR. A single fragment was obtained that led to two bands after digestion with *Bam*HI, thus suggesting the possibility of a *Bam*HI site in an intron. Partial nucleotide sequence of these PCR fragments matched exactly the cDNA sequence (data not shown). Therefore, according to these data, *SlTPS1* seems to be a single-copy gene.

Functional Analysis of *SlTPS1* in Yeast

To determine whether the *SlTPS1* gene can complement the physiological defects of mutant yeast cells devoid of trehalose biosynthesis genes, the corresponding 3.2-kb cDNA was subcloned into the yeast pSAL4 vector behind a *CUP1* promoter that is inducible by copper ions (Mascorro-Gallardo et al., 1996). The resulting plasmid pSTS1 was used to transform the yeast *tps1*Δ and *tps1*Δ*tps2*Δ mutants. These mutants are unable to grow with Glc or Fru as the carbon source, apparently because of the role of TPS1 in regulating the flow of Glc in glycolysis (Van Aelst et al., 1993; Thevelein and Hohmann, 1995). After transformation with pSTS1 containing the *SlTPS1* gene, the *tps1*Δ*tps2*Δ mutant was able to grow in Glc in the presence of copper

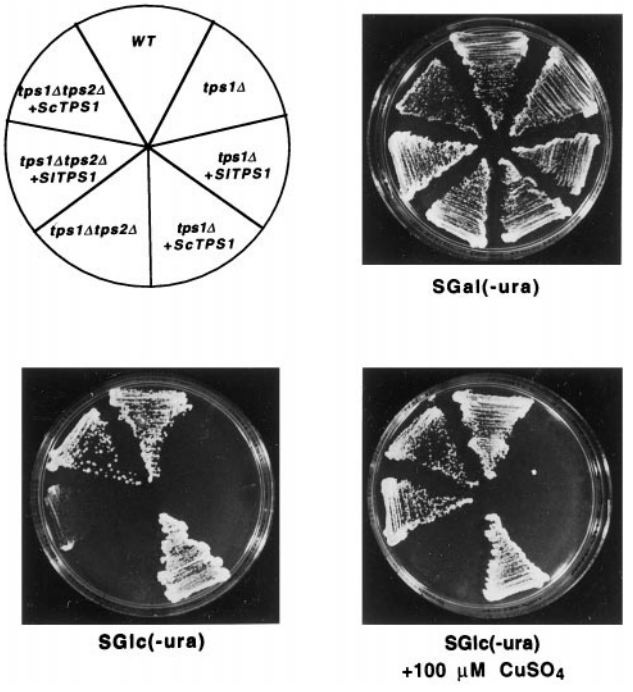

Figure 4. Complementation of the *tps1*Δ*tps2*Δ mutant by *SlTPS1* under the control of the *CUP1* promoter. Yeast mutants lacking the TPS1 protein (*tps1*Δ) or both TPS1 and TPS2 (*tps1*Δ*tps2*Δ) were transformed with pSTS1 (pSAL4 containing *SlTPS1*), pSTS2 (pSAL4 containing *ScTPS1*), or pSAL4 vector alone and spread on 2% agar plates in minimal medium without uracil (−ura) supplemented with 2% Gal (SGal), 2% Glc (SGlc), or 2% Glc with copper sulfate (SGlc +100 μM $CuSO_4$). The wild-type control strain W303-1A (WT) was also transformed with pSAL4.

ions (Fig. 4), whereas no growth was observed for the *tps1*Δ mutant. All strains grew well in Gal, as expected. Transformation of the *tps1*Δ mutant with plasmid pSTS2, which harbors the homologous *ScTPS1* gene, restored growth for both the *tps1*Δ and *tps1*Δ*tps2*Δ mutants (Fig. 4). Trehalose levels were measured in the *tps1*Δ and *tps1*Δ*tps2*Δ mutants transformed with pSTS1 plasmid (containing *SlTPS1*) grown in different carbon sources (Table I). Neither the *tps1*Δ nor the *tps1*Δ*tps2*Δ mutant transformed with *SlTPS1* accumulated trehalose above the level detected for the same mutants transformed with the pSAL4 vector alone. We decided to express *SlTPS1* cDNA under the control of a stronger promoter to observe a possible accumulation of trehalose. Thus, the promoter of the *PMA1* gene that encodes the H^+-ATPase (Serrano and Villalba, 1995) was subcloned in a multicopy vector to give plasmid pRS6 (see "Materials and Methods"). After *SlTPS1* was subcloned under the control of the *PMA1* promoter (plasmid pRTS1), this construction was used to transform the yeast *tps1*Δ mutant. It was observed that the *tps1*Δ mutant transformed with pRTS1 plasmid was able to grow in Glc (Fig. 5).

The trehalose content was determined for *tps1*Δ mutant strains transformed with the pRTS1 (containing *SlTPS1*) and pRTS2 (harboring *ScTPS1*) plasmids. Trehalose was detected in the *tps1*Δ mutant complemented with *SlTPS1* grown in different carbon sources (Table II). The trehalose levels were higher when the *tps1*Δ mutant was complemented with the *S. cerevisiae ScTPS1* gene than when *SlTPS1* was used. These results indicate that heterologous *S. lepidophylla* SlTPS1 is not completely fulfilling the function of the yeast ScTPS1 protein, considering the sequence divergence and the fact that the SlTPS1 polypeptide is larger than ScTPS1. Therefore, we constructed a deletion mutant of *SlTPS1*, named *SlTPS1*Δ*C*, to remove the sequence encoding its C-terminal domain of 431 amino acids, which lacks similarity to ScTPS1 or EcOtsA (Fig. 1C). Plasmid pRTS4 containing *SlTPS1*Δ*C* was used to transform the *tps1*Δ and *tps1*Δ*tps2*Δ mutants. Growth in Glc was complemented in both mutants by the C-terminal deletion of *SlTPS1*. When *SlTPS1*Δ*C* was cloned under control of the *CUP1* promoter (plasmid pSTS4), only partial complementation was observed in the *tps1*Δ*tps2*Δ mutant: Growth in Glc was slow compared with that in the same mutant transformed with the full-length *SlTPS1* gene in pSAL4 (data not shown). Trehalose levels in the *tps1*Δ mutant transformed with pRTS4 were 2.5 times lower than the levels with the full-length *SlTPS1* gene but significantly higher than in the *tps1*Δ mutant transformed with vector alone (Table II). These results show that the C-terminal region of SlTPS1 is required for full activity.

Given that the C-terminal region of SlTPS1 shares relative similarity to TPPs (Fig. 1C), we tested the ability of full-length *SlTPS1* and its C-terminal domain (*SlTPS1*Δ*N*) to complement the defect to grow at 37.5°C associated with the yeast *tps2*Δ and *tps1*Δ*tps2*Δ mutants. pRTS1 (containing *SlTPS1*) and pRTS5 (harboring *SlTPS1*Δ*N*) were used to transform these mutants. It is known that the *tps2*Δ mutant

Table I. *Trehalose content of the transformed tps1*Δ *and tps1*Δ*tps2*Δ *mutants with pSAL4-derived vectors*

Three independent transformants of wild-type yeast, the *tps1*Δ mutant, or the *tps1*Δ*tps2*Δ mutant transformed with pSAL4 vector alone or harboring the *SlTPS1, SlTPS1*Δ*C,* or *ScTPS1* genes were grown in minimal medium with the indicated carbon source plus 100 μM $CuSO_4$. Trehalose content was determined in the stationary phase (7.0 A_{600}). Values are means ± SD.

Carbon Source	Strain	Trehalose	Trehalose Content
		μmol g⁻¹ yeast wet wt	*%*
Gal	Wild type (pSAL4)	71.70 ± 25.18	100
	*tps1*Δ*tps2*Δ (pSAL4)	5.25 ± 6.77	7
	*tps1*Δ*tps2*Δ (*SlTPS1*)	6.32 ± 6.23	9
	*tps1*Δ*tps2*Δ (*SlTPS1*Δ*C*)	2.13 ± 0.52	3
	*tps1*Δ*tps2*Δ (*ScTPS1*)	19.27 ± 14.93	27
	*tps1*Δ (pSAL4)	4.80 ± 4.90	7
	*tps1*Δ (*S1TPS1*)	2.83 ± 1.65	4
	*tps1*Δ (*ScTPS1*)	48.43 ± 21.16	67
Glc	Wild type (pSAL4)	50.90 ± 9.02	100
	*tps1*Δ*tps2*Δ (*SlTPS1*)	5.42 ± 4.58	11
	*tps1*Δ*tps2*Δ (*ScTPS1*)	18.50 ± 12.93	36
	*tps1*Δ (*ScTPS1*)	36.48 ± 10.69	72
Fru	Wild type (pSAL4)	81.77 ± 31.03	100
	*tps1*Δ*tps2*Δ (*SlTPS1*)	6.95 ± 5.01	7
	*tps1*Δ*tps2*Δ (*ScTPS1*)	21.13 ± 13.04	26
	*tps1*Δ (*ScTPS1*)	54.40 ± 24.92	66

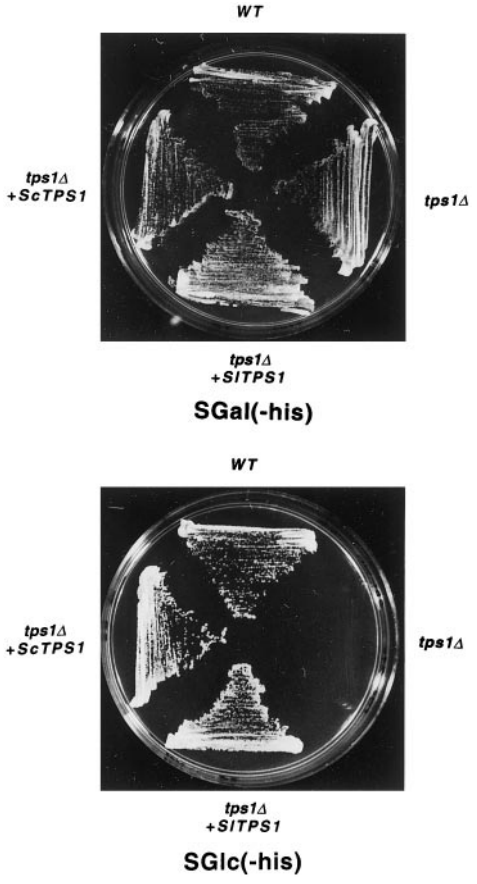

Figure 5. Complementation of the *tps1*Δ mutant by *SlTPS1* under the control of the *PMA1* promoter. The yeast mutant lacking TPS1 protein (*tps1*Δ) was transformed with pRTS1 (pRS6 containing *SlTPS1*), pRTS2 (pRS6 containing *ScTPS1*), or pRS6 vector alone and spread on 2% agar plates in minimal medium without His (−his) supplemented with 2% Gal (SGal) or 2% Glc (SGlc). The wild-type control strain W303-1A (WT) was also transformed with pRS6.

grows normally in Glc but is unable to grow continuously at 37.5°C (De Virgilio et al., 1993). The *tps1*Δ*tps2*Δ mutant has both growth defects. *SlTPS1* was not able to restore the growth of the *tps2*Δ and *tps1*Δ*tps2*Δ mutants at 37.5°C. Transformation with pRTS3, which harbors the homologous *ScTPS2* gene, complemented both mutants (data not shown).

Trehalose is required for the acquisition of thermotolerance in yeast and *E. coli* (Hengge-Aronis et al., 1991; De Virgilio et al., 1994). The yeast *tps1*Δ, *tps2*Δ, and *tps1*Δ*tps2*Δ deletion mutants are deficient in both induced and noninduced thermotolerance. The phenotypes are restored by complementation with the corresponding homologous gene. To determine whether the *SlTPS1* gene can also complement these deficient stress responses, the yeast *tps1*Δ mutant was transformed with plasmid pRTS1 (harboring *SlTPS1*) or pRTS2 (containing *ScTPS1*). We assayed the ability to survive both a sublethal heat shock of 39°C for 1 h (thermoinduction) and a lethal heat shock of 50°C for 20 min after an acclimation treatment of 39°C for 1 h (induced thermotolerance). The viability of the *tps1*Δ mutant after

Table II. *Trehalose content of the transformed tps1*Δ *mutant with pRS6-derived vectors*

Three independent transformants of wild-type yeast or the *tps1*Δ mutant transformed with pRS6 vector alone or harboring the *SlTPS1, SlTPS1*Δ*C,* or *ScTPS1* genes were grown in minimal medium with the indicated carbon source.

Carbon Source	Strain	Trehalose[a]	Trehalose Content
		μmol g^{-1} *yeast wet wt*	%
Gal	Wild type (pRS6)	16.45 ± 4.59	100
	*tps1*Δ (pRS6)	1.45 ± 0.07	9
	*tps1*Δ (*SlTPS1*)	16.55 ± 0.35	100
	*tps1*Δ (*SlTPS1*Δ*C*)	6.59 ± 0.72	40
	*tps1*Δ (*ScTPS1*)	26.20 ± 2.97	159
Glc	Wild type (pRS6)	29.45 ± 0.64	100
	*tps1*Δ (*SlTPS1*)	18.95 ± 5.87	64
	*tps1*Δ (*ScTPS1*)	27.85 ± 7.42	95
Fru	Wild type (pRS6)	29.00 ± 7.07	100
	*tps1*Δ (*SlTPS1*)	20.40 ± 3.39	70
	*tps1*Δ (*ScTPS1*)	34.05 ± 4.59	117

[a] Trehalose content was determined in the stationary phase (7.0 A_{600}). Values are means ± SD.

thermoinduction decreased to 30%, but almost complete viability was recovered after transformation with the *SlTPS1* or the *ScTPS1* gene (Table III). After lethal heat shock, *S. lepidophylla SlTPS1* restored the induced thermotolerance as effectively as yeast *ScTPS1* in *tps1*Δ mutant cells compared with the level in the wild-type cells (Fig. 6). Both *SlTPS1*- and *ScTPS1*-transformed cells displayed a 10-fold higher induced thermotolerance than the *tps1*Δ mutant cells transformed with the pRS6 vector alone after 20 min at 50°C.

Finally, it has been shown that the *tps1*Δ mutant is osmosensitive (Hounsa et al., 1998). To determine whether *SlTPS1* could restore the osmotolerance defect of the *tps1*Δ mutant, this strain was transformed with the plasmids pRTS1 and pRTS2 and growth was evaluated under osmotic stress conditions. The *tps1*Δ mutant transformed either with the *SlTPS1* or the *ScTPS1* gene was able to grow in medium containing 1.6 M sorbitol or 0.9 M NaCl (Fig. 7). As a positive control, we used the wild-type strain, which also grew normally. In contrast, the *tps1*Δ mutant transformed with the vector alone was unable to grow in high-osmoticum conditions. All of these results suggest that the

Table III. *Viability of tps1*Δ *mutant cells transformed with SlTPS1 gene after thermoinduction*

Thermoinduction was performed by incubating yeast cells in liquid SGal (-His) medium up to approximately 0.4 A_{600} at 25°C, and then shifted for 1 h at 39°C. Aliquots for colony counting were taken just before and after thermoinduction. Data represent the mean of three independent transformants and their SD.

Strain	Surviving Cells
	%
Wild type (pRS6)	127.00 ± 19.52
*tps1*Δ (pRS6)	29.67 ± 6.66
*tps1*Δ (SlTPS1)	80.00 ± 21.16
*tps1*Δ (ScTPS1)	96.00 ± 2.65

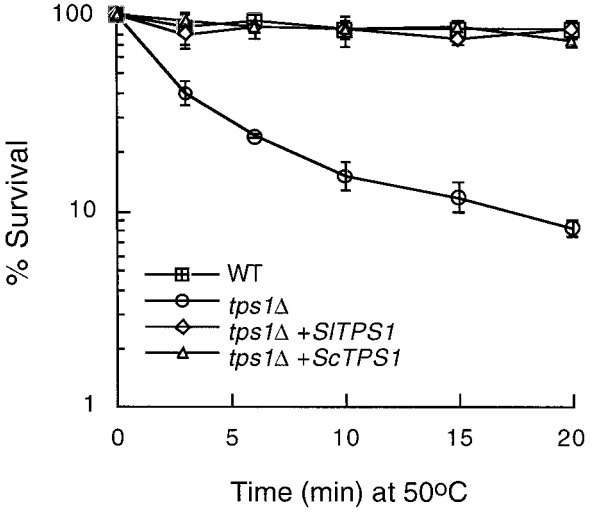

Figure 6. Complementation of thermotolerance deficiency of the *tps1*Δ deletion mutant transformed with the *SlTPS1* gene. Transformed strains were grown in liquid culture and assayed for induced thermotolerance (see "Materials and Methods"). Values shown for each construct are the averages of three independent transformants. WT, Wild-type cells transformed with the pRS6 vector alone; *tps1*Δ, mutant transformed with the pRS6 vector alone; *tps1*Δ+*SlTPS1*, mutant transformed with plasmid pRTS1; *tps1*Δ+*ScTPS1*, mutant transformed with plasmid pRTS2. Error bars denote ±SD.

levels of trehalose accumulated in the *tps1*Δ mutant transformed with *SlTPS1* are sufficient to restore growth under osmotic and heat stress.

DISCUSSION

Trehalose is one of the most efficient osmoprotectors and thermoprotectors in nature (Colaço et al., 1992; Crowe et al., 1992). Although much experimental work concerning trehalose has been done in bacteria and yeast, little information has been reported from animals or plants. Here we report the molecular and functional characterization of a full-length cDNA encoding a novel TPS (SlTPS1) from the resurrection plant *S. lepidophylla*. This plant is known to accumulate trehalose at levels comparable to yeast and other fungi and at levels much higher than those found in other plants (Adams et al., 1990; Müller et al., 1995). It is likely that a major factor determining the anhydrobiotic ability of resurrection plants is their capacity to accumulate high levels of osmoprotectors such as trehalose. Until a few years ago, it was thought that only resurrection plants had the capacity to synthesize trehalose (Müller et al., 1995). Recently, the presence of trehalose was reported in tobacco, potato (Goddijn et al., 1997), and rice (Garcia et al., 1997), but at levels about 3000-fold less than in *S. lepidophylla* (Adams et al., 1990). Although the actual level of trehalose in Arabidopsis has not yet been reported, presumably it is not significant because *AtTPS1* mRNA is expressed at very low levels (Blázquez et al., 1998). Additionally, it is possible that the low levels of trehalose in most higher plants could be the result of very active trehalase. Nevertheless, when Goddijn et al. (1997) incubated tobacco and potato plants in the presence of the trehalase inhibitor validamycin A, trehalose did not reach concentrations comparable to those in a resurrection plant.

Another possibility is that substitutions in the amino acid sequence that occurred during evolution in different plant TPS have an important influence on trehalose synthesis among plant species.

In this work, we present evidence that the expression of the *SlTPS1* gene in the yeast *tps1*Δ mutant under control of the strong *PMA1* promoter leads to an accumulation of trehalose in the stationary phase equivalent to 64% to 100% of the levels reached by wild-type yeast, depending on the carbon source. Similarly, Blázquez et al. (1998) used the *PGK1* promoter to express *AtTPS1*, which resulted in 25% of the trehalose found in the wild-type yeast grown in Gal. These results might be attributable to either a difference between *PGK1* and *PMA1* promoter strength or an increased capacity of SlTPS1 to synthesize trehalose. Therefore, it remains to be shown if the difference in the treha-

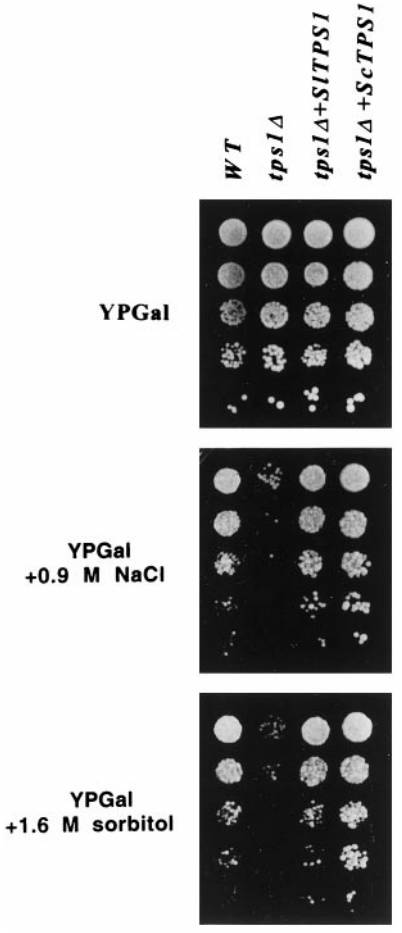

Figure 7. *SlTPS1* restores the osmotolerance defect of a yeast *tps1*Δ deletion mutant. Osmotolerance assay of a *tps1*Δ deletion mutant transformed with the *SlTPS1* gene. Yeast mutants lacking the protein TPS1 (*tps1*Δ) were transformed with pRTS1 (containing *SlTPS1*), pRTS2 (containing *ScTPS1*), or pRS6 vector alone and spotted on 2% agar plates in rich YPGal medium supplemented with 0.9 M NaCl or 1.6 M sorbitol. The wild-type control strain W303-1A (WT) was also transformed with pRS6.

lose content between *S. lepidophylla* and nonresurrection plants is the result of transcriptional control and/or balance between trehalase and TPS enzyme activities.

Transformation of the yeast *tps1*Δ mutant with the *SlTPS1* gene under the control of a moderate promoter such as *CUP1* did not restore the ability of the wild-type phenotype to grow in Glc, whereas the growth of the *tps1*Δ*tps2*Δ mutant was complemented. Nevertheless, trehalose levels in both the *tps1*Δ and *tps1*Δ*tps2*Δ mutants transformed with *SlTPS1* were negligible, thus suggesting that restoration of growth in Glc of the *tps1*Δ*tps2*Δ mutant by *SlTPS1* is independent of the presence of trehalose. This observation is interesting because *SlTPS1* expressed in a moderate promoter, such as *CUP1*, allows separate analyses of both TPS functions, i.e. trehalose synthesis capacity and regulation of Glc influx in glycolysis (Van Aelst et al., 1993; Thevelein and Hohmann, 1995).

Moreover, the fact that *tps1*Δ was not complemented by the *SlTPS1* gene expressed under the control of the *CUP1* promoter led us to suggest that maybe SlTPS1 was somehow inhibited by ScTPS2. One possible explanation for these results is a negative interaction or sequestration of SlTPS1 by ScTPS2. In yeast, ScTPS1, ScTPS2, ScTPS3, and ScTSL1 interact with each other to form the holoenzyme complex (Reinders et al., 1997). Thus, given the sequence similarity between yeast and plant TPS1, ScTPS2 and SlTPS1 may interact with each other, resulting in a structural constraint of SlTPS1 enzyme activity.

The *SlTPS1* cDNA encodes a 109-kD protein that shares strong similarity to TPS sequences and has a C-terminal extension with some similarity to TPP. We tested whether *SlTPS1* had TPP activity by transforming the yeast *tps2*Δ and *tps1*Δ*tps2*Δ mutants with the full-length *SlTPS1* gene or just its C-terminal region under the control of the strong *PMA1* promoter. However, the lack of complementation for growth at 37.5°C suggested that SlTPS1 does not have TPP activity. It has been shown that all TPP enzymes from bacteria to plants have two short and well-conserved regions of homology (Vogel et al., 1998). These sequences are absent in SlTPS1 and AtTPS1, providing further evidence for the absence of TPP activity in SlTPS1.

In yeast, it is well established that trehalose is involved in acquired thermotolerance and tolerance to continuous growth at sublethal temperatures (De Virgilio et al., 1994; Elliot et al., 1996). It has been shown that the *tps1*Δ mutant is deficient in induced thermotolerance, and this phenotype can be restored after complementation with the homologous *ScTPS1* gene (De Virgilio et al., 1994). Here we show that *SlTPS1* is able to restore the yeast capacity for both thermoinduction and induced thermotolerance. Another aspect of *SlTPS1* that we addressed was its capacity to confer osmotolerance. A recent work (Hounsa et al., 1998) analyzed the osmosensitive phenotype *tps1*Δ mutant under moderate and severe osmotic stress, revealing the strong correlation between the presence of trehalose in yeast and survival under osmotic stress conditions. In the present study, we showed that both *SlTPS1* and *ScTPS1* are able to complement and restore growth of yeast cells under osmotic stress. These data suggest that trehalose may play a similar role in *S. lepidophylla* as a stress protectant.

A few TPS homologs have been cloned from bacteria, fungi, and higher eukaryotes (Fig. 1). It is possible that TPS1 is present in most organisms, although not necessarily involved in the synthesis of significant amounts of trehalose. That plant or animal TPS might have a role similar to that of yeast ScTPS1 as a controller of the influx of sugar into glycolysis is an intriguing possibility. The fact that both *SlTPS1* and *AtTPS1* are able to complement the growth defect on fermentable sugars of the yeast *tps1*Δ mutant suggests this possibility and opens a new perspective on the regulation of glycolysis in plants and animals that should be explored.

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