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Functional screening of a cDNA library from the desiccation-tolerant plant *Selaginella lepidophylla* in yeast mutants identifies trehalose biosynthesis genes of plant and microbial origin

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Abstract Trehalose is a non-reducing disaccharide that accumulates to large quantities in microbial cells, but in plants it is generally present in very low, barely-detectible levels. A notable exception is the desiccation-tolerant plant *Selaginella lepidophylla*, which accumulates very high levels of trehalose in both the hydrated and dehydrated state. As trehalose is known to protect membranes, proteins, and whole cells against dehydration stress, we have been interested in the characterization of the trehalose biosynthesis enzymes of *S. lepidophylla*; they could assist in engineering crop plants towards better stress tolerance. We previously isolated and characterized trehalose-6-phosphate synthases from *Arabidopsis thaliana* (desiccation sensitive) and *S. lepidophylla* (desiccation tolerant) and found that they had similar enzymatic characteristics. In this paper, we describe the isolation and characterization of trehalose-6-phosphate phosphatase from *S. lepidophylla* and show that its catalytic activities are also similar to those of its homolog in *A. thaliana*. Screening of an *S. lepidophylla* cDNA library using yeast trehalose biosynthesis mutants resulted in the isolation of a large number of trehalose biosynthesis genes that were of microbial rather than plant origin. Thus, we suggest that the high trehalose levels observed in *S. lepidophylla* are not the product of the plant but that of endophytes, which are known to be present in this plant. Additionally, the high trehalose levels in *S. lepidophylla* are unlikely to account for its desiccation tolerance, because its drought-stress-sensitive relative, *S. moellendorffii*, also accumulated high levels of trehalose.

Keywords Desiccation tolerance · *Selaginella lepidophylla* · TPS · TPP · Trehalose

Introduction

Trehalose is a non-reducing disaccharide (α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside) present in bacteria, yeast, fungi, invertebrates, and desiccation-tolerant plants. In these different organisms, trehalose functions as a reserve carbohydrate as well as an important stress-protecting molecule (Elbein et al. 2003). The hydrolysis of trehalose is a major event during fungal spore germination (Thevelein 1984) and insect flight (Becker et al. 1996), where trehalose presumably supplies glucose as a carbon and energy source. In mycobacteria and corynebacteria, trehalose is also an important structural component of the cell wall (Lederer 1976). Finally, trehalose, trehalose 6-phosphate (T6P; the intermediate molecule during trehalose biosynthesis), or the enzymes involved in its biosynthesis have also been shown to act as regulatory molecules, e.g., by controlling the influx of glucose into glycolysis in yeast cells (Bonini et al. 2003; Hohmann et al. 1996).

Desiccation-tolerant organisms are able to lose 80–95 % of their water content and stay dormant in the dehydrated state until water becomes available (Crowe et al. 1992).

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A common characteristic in most of these organisms is the rapid increase in compatible solutes, such as trehalose, during dehydration. High trehalose contents have been measured during desiccation in some 'resurrection' plants, yeast and bacterial cells, fungal spores, and also in microscopic animals such as nematodes, rotifers and, tardigrades (Hengherr et al. 2008; Oliver et al. 2000). In the course of desiccation, trehalose contributes to membrane stability by preventing the lipid phase transition and fusion of drying vesicles (Crowe 2007). Furthermore, the accumulation of trehalose in response to stress in Archaebacteria suggests that the protective role of trehalose during cell dehydration might be an ancient adaptation that is evolutionarily preserved in desiccation-tolerant plants (Zaparty et al. 2013). Among vascular plants, high amounts of trehalose are detected in only a few desiccation-tolerant plants, such as *Myrothamnus flabellifolius*, *Sporobolus atrovirens*, and *Selaginella lepidophylla* (Müller et al. 1995).

Selaginella lepidophylla is a desiccation-tolerant plant from the Chihuahuan desert of North America. This lycophyte is able to lose 80–95 % of its protoplasmic water during a dry period and revive when water becomes available again (Yobi et al. 2012). During the dehydration–hydration cycles, the stems of *S. lepidophylla* curl and uncurl. Ultrastructural and biochemical analyses of the microphyll behavior during rehydration uncurling showed that the cellular and basal enzymatic integrity of the desiccated microphylls was conserved (Adams et al. 1990; Bergtrom et al. 1982; Yobi et al. 2013). Exceptionally for plants, the trehalose content in *S. lepidophylla* exceeds those of sucrose and glucose; however, in its desiccation-sensitive relative *Selaginella moellendorffii*, even higher levels of trehalose were detected (Yobi et al. 2012, 2013). We recently summarized the characteristics of *S. lepidophylla* that may be involved in its extreme drought-stress tolerance (Pampurova and Van Dijck 2014).

Because of the very low trehalose content in most vascular plants, they were thought to have lost the capacity to produce this molecule and that it was replaced by sucrose. However, most or all desiccation-intolerant vascular plants, such as *Arabidopsis thaliana* and *Oryza sativa*, harbor large trehalose-biosynthetic gene families in their genomes (Avonce et al. 2010; Leyman et al. 2001; Lunn 2007). In plants, trehalose biosynthesis involves a two-step pathway catalyzed by T6P synthase (TPS; EC 2.4.1.15) and T6P phosphatase (TPP; EC 3.1.3.12) (Avonce et al. 2006). In the first step, TPS catalyzes the transfer of glucose from UDP-glucose to glucose 6-phosphate forming T6P and UDP, while in the second step TPP dephosphorylates T6P to trehalose and inorganic phosphate (Elbein et al. 2003). Trehalose is catabolized by trehalase (EC 3.2.1.28) into two glucose moieties (Avonce et al. 2006). In the model plant *A. thaliana*, there are 11 genes encoding TPS- or TPS-like proteins, 10 encoding TPPs, and one encoding trehalase (Leyman et al. 2001).

Given the high trehalose content in *S. lepidophylla*, the characterization of its trehalose biosynthetic genes might provide insights into the evolutionary preservation of trehalose in a desiccation-tolerant plant. Until now, only one TPS-encoding gene (designated *SlTPS1*) has been reported from *S. lepidophylla* (Márquez-Escalante et al. 2006; Valenzuela-Soto et al. 2004; Van Dijck et al. 2002). Similar to *At*TPS1, this *Sl*TPS1 is able to complement a yeast *tps1Δ* mutant growing on glucose, but only when its inhibitory *N*-terminal domain is removed (Van Dijck et al. 2002). The removal of this domain results in very high enzymatic activity upon expression in yeast (Van Dijck et al. 2002). One hypothesis that may support high trehalose levels in *S. lepidophylla* is that in this plant the *N*-terminal domain may be inhibited by a protein or metabolite or removed by a specific protease, resulting in high enzymatic activity. To investigate this possibility, we generated an *S. lepidophylla* cDNA library in a yeast expression vector. A large number of screenings in yeast resulted in the identification of many TPS-encoding genes that showed more homology with fungal TPS than with plant TPSs. Because *S. lepidophylla* harbors many endophytes (Brighigna et al. 2002) and is unable to regenerate from spores without them, i.e., when grown in sterile conditions (personal communication, G. Iturriaga), we here suggest that the high trehalose levels present in *S. lepidophylla* originate from the endophytes and are required for normal growth of *S. lepidophylla*.

Materials and methods

Plant material

Desiccated *S. lepidophylla* plants were obtained from Prof. G. Iturriaga (Centro de Investigación en Biotecnología—UAEM, Morelos, Mexico) and from the companies Floréac (Lochristi, Belgium) and Livo B.V. (Overijssel, The Netherlands). To construct the cDNA library, plant samples were taken at four different time points during a rehydration/dehydration cycle. *Selaginella lepidophylla* plants used for real-time quantitative reverse transcription PCR (RT-qPCR) analysis were completely hydrated (4 days hydration after the initial rehydration/dehydration cycle) and completely dehydrated (3 days dehydrated after the initial rehydration/dehydration cycle).

Selaginella lepidophylla cDNA library construction

RNA samples were collected from fully-desiccated, hydrated (24 h after watering), partly-dried plants (8 h

drying), and completely-dried plants (24 h drying). RNA extraction was performed as previously described (Valenzuela-Avendano et al. 2005). Complementary DNA was generated using the cDNA library construction kit (Invitrogen, Carlsbad, CA, USA), cloned in the Gateway donor vector pDONR222, and transferred to the yeast expression vector pVV214 (Van Mullem et al. 2003). The resulting cDNA library was composed of 1.019×10^{11} colony forming units ml^{-1} with an average cDNA insert size of 1.16 kb.

Isolation of proteins interacting with the *N*-terminus of *Sl*TPS1

Expression of *Sl*TPS1 in a yeast *tps1Δ* mutant does not restore growth on glucose (Van Dijck et al. 2002). However, expression of a construct in which the first 300 nucleotides (100 amino acids) of *SlTPS1* were removed resulted in the production of a protein with high enzymatic activity and was able to restore the mutant's growth on glucose. To identify proteins that might interact with the *N*-terminus and remove its inhibitory activity on the catalytic domain, we transformed the *S. lepidophylla* cDNA library into a *Saccharomyces cerevisiae* strain (Gietz et al. 1995) expressing the full-length *SlTPS1* and selected transformants that could grow on glucose. Plasmids from positive clones were isolated, amplified in *E. coli*, and retransformed into the same yeast strain for confirmation. The inserts of the positive clones were sequenced.

Isolation of *TPP* and *TPS* genes from *S. lepidophylla* cDNA library

To isolate *TPP* and *TPS* genes, we transformed the cDNA library into yeast *tps1Δ* or *tps2Δ* strains, which lack TPS or TPP activity, respectively. Complemented transformants that could grow on glucose (*tps1Δ*) or at 38 °C (*tps2Δ*) were retested in a spot assay. For the spot assays, overnight liquid cultures were adjusted to an optical density at 600 nm (OD_{600}) = 0.1, diluted tenfold, and spotted (5 µL) on synthetic solid medium without uracil and with glucose or galactose as carbon source. Transformants in the *tps1Δ* background were selected for growth on glucose, and transformants in the *tps2Δ* background were selected for growth at 38 °C. Plasmids from positive clones were isolated, amplified in *E. coli*, and retransformed into the same yeast strain for confirmation. The inserts of the positive clones were sequenced.

In an alternative approach to isolate these genes, we used the degenerate-oligonucleotide PCR approach. Based on sequences obtained from searches of the *S. lepidophylla* EST library (Iturriaga et al. 2006) and homologous *TPS* and *TPP* genes from other species, degenerate primers (see Table S1) were designed and combined with primers specific to the AttB-sites on the pVV214 vector. After obtaining specific cDNA fragments, a second set of primers was designed to obtain the complete cDNA sequence. The following PCR program was used: initial denaturation at 95 °C for 5 min, five cycles of (95 °C for 1 min, 30 °C for 1 min, 72 °C for 2 min), 30 cycles of (95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min), and final extension step at 72 °C for 7 min. To obtain the full cDNA sequence with the specific primers, after initial denaturation for 5 min at 95 °C, the reaction was as follows: 35 cycles (95 °C for 40 s, 65 °C for 1 min, 72 °C for 1 min) and final extension at 72 °C for 7 min.

Phylogenetic analysis

The sequences used for the phylogenetic reconstruction of the *TPP* gene family were collected using the PLAZA 1.0 platform (Proost et al. 2009) as previously described (Avonce et al. 2010). The gene families from *Physcomitrella patens*, *A. thaliana*, and *S. moellendorffii* were included in the analysis (supplementary Table 2). Multiple sequence alignment of the deduced protein sequences was performed with Clustal Omega (Sievers et al. 2011), and ClustalW2 (Larkin et al. 2007) was used to calculate a distance matrix with the neighbor-joining algorithm. The estimated phylogenetic trees were drawn with NJplot (Perrière and Gouy 1996).

Growth conditions, yeast strains and transformation

Yeast cells were grown in synthetic growth medium (1.7 g Bacto-yeast nitrogen base without amino acids (pH 6.0), 5 g ammonium sulfate; supplemented with 50 mg adenine, 100 mg histidine, 250 mg leucine, 100 mg tryptophan, and 50 mg uracil in 1 L) and 2% galactose (SGal) or 2% glucose (SGlc). The yeast strains were: wild-type (WT), W303-1A (Mata *leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100 GAL SUC2*) (Thomas and Rothstein 1989); *tps1Δ*, YSH290 (W303-1A, *tps1Δ::TRP1*) (Hohmann et al. 1993); and *tps2Δ*, YSH488 and YSH587 (W303-1A, *tps2Δ::LEU2*) (Neves et al. 1995). Shuttle vectors pSAL4 (Zentella et al. 1999) and pYX212 (Novagen) were used to express the genes of interest in yeast. Yeast transformation was performed according to Elble (1992), and transformants were selected on plates containing minimal medium without uracil.

Trehalose measurements

Trehalose was measured as previously described using *Humicola* sp. trehalase and glucose oxidase/peroxidase assays (Zentella et al. 1999).

TPP activity

TPP activity was determined as previously described (De Virgilio et al. 1993; Zentella et al. 1999). Protein concentrations were measured with the Bradford method (Bradford 1976). Enzyme activity is expressed as nanokatals per gram of protein (nkat g protein^{-1}).

qRT-PCR analysis

Gene expression was tested with RT-qPCR. RNA was extracted according to Valenzuela-Avendano et al. (2005), and cDNA was prepared from 2 µg of total RNA using a commercial cDNA synthesis kit (Invitrogen) before analysis on an ABI PRISM 7000 thermocycler using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to manufacturer's instructions. All reactions were done in triplicate. Relative expression levels of target genes were quantified with specific primer sets (Table S1), and *26S rRNA* gene expression was used as an internal standard. To calculate the transcript quantity relative to the hydrated state, the $2^{\Delta\Delta C_t}$ method was used. Statistical significance was calculated using Student's *t* test.

Results

Initial screening of *S. lepidophylla* cDNA library for *TPS* and *TPP* genes

We used *S. cerevisiae tps1Δ* and *tps2Δ* strains to isolate *TPS* or *TPP* genes from the desert resurrection plant *S. lepidophylla*. A total of 30,000 transformants was screened for growth on glucose (*tps1Δ* complementation) and at 38 °C (*tps2Δ* complementation). Plasmid DNA from positive transformants was isolated and retested. Interestingly, screening for genes that suppressed the glucose growth defect of the *tps1Δ* strain led to the identification of several genes with higher sequence similarities to fungal TPS than to plant TPS proteins (Table 1). Furthermore, the positive clones that allowed growth at 38 °C of the *tps2Δ* strain harbored genes encoding stress proteins, such as LEA proteins and dehydrins, instead of the expected *TPP* genes.

Isolation of active microbial TPS genes from the *S. lepidophylla* cDNA library

According to Adams et al. (1990) the content of trehalose in *S. lepidophylla* is much higher than in other plants. We have previously shown that an *N*-terminal truncation of *SlTPS1* (or *AtTPS1*) results in a much higher enzymatic activity and very good complementation of the yeast *tps1Δ* mutant (Van Dijck et al. 2002). One possibility for the high trehalose content in *S. lepidophylla* is that the *N*-terminal domain is non-functional or removed in this plant, possibly by being bound or cut by another protein. To isolate such proteins, we used the *S. lepidophylla* cDNA library to screen a *tps1Δ* yeast strain expressing the full-length *SlTPS1* gene for transformants that could grow on glucose. Such transformants may express a protein that releases high TPS catalytic activity.

One hundred and forty colonies that were obtained grew on glucose. The positive plasmids were re-tested by retransformation in the *tps1Δ* strain expressing the full-length *SlTPS1* gene. As in the first screen, the positive clones contained sequences with the closest similarity to TPS enzymes of microbial origin, indicating that the glucose growth complementation was a result of expressing heterologous *TPS* genes. The two most frequently isolated *TPS* genes, *glu13* and *glu15*, had highest homology to *TPS* genes from the rock-inhabiting fungus *Coniosporium apollinis* (81.68 % amino acid identity) and the amoebozoan *Polysphondylium pallidum* (61.05 %), respectively (Fig. 1a). The microbial genes *glu13* and *glu15* encode for active TPS enzymes, because their expression could complement the growth defect of the yeast *tps1Δ* mutant on glucose, even in the absence of the *SlTPS1* gene (Fig. 1b). As in the first screening, no plant *TPS1* homologs were isolated.

Table 1 Genes isolated by screening for *Selaginella lepidophylla* proteins that can complement the growth defects of the yeast *tps1Δ* (glucose tolerance) or the yeast *tps2Δ* mutant (growth at high temperature) mutants

Growth conditions	Strain	Gene	Species	Kingdom
Glucose	*tps1Δ*	TPS	*Coniosporium apollinis*[a]	Fungi
Glucose	*tps1Δ*	TPS	*Polysphondylium pallidum*[a]	Amoebozoa
Glucose	*tps1Δ*	TPS	*Leptosphaeria maculans*[a]	Fungi
Glucose	*tps1Δ*	TPS	*Exophiala dermatitidis*[a]	Fungi
38 °C	*tps2Δ*	DHN	*Selaginella lepidophylla*[b]	Plantae
38 °C	*tps2Δ*	LEA	*Selaginella lepidophylla*[b]	Plantae
38 °C	*tps2Δ*	ELIP	*Selaginella lepidophylla*[b]	Plantae
38 °C	*tps2Δ*	GST	*Selaginella lepidophylla*[b]	Plantae

TPS T6P synthase, *DHN* dehydrin, *LEA* late embryogenesis abundant protein, *ELIP* early light inducible protein, *GST* glutathione-*S*-transferase

[a] The closest homologs to the isolated TPS genes are from the indicated species

[b] The isolated genes are complete matches with already-identified genes from *S. lepidophylla*

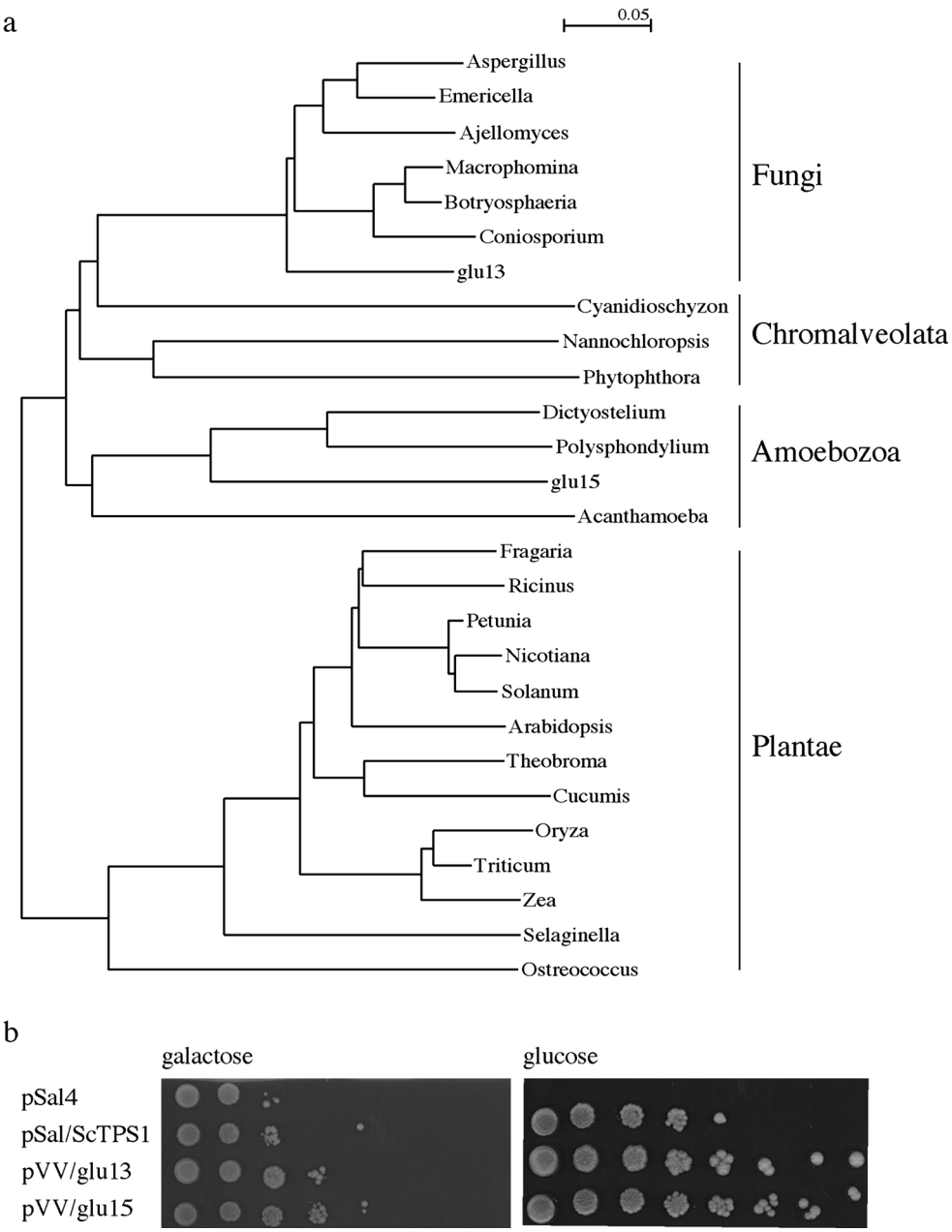

Fig. 1 Screening for *Selaginella lepidophylla* trehalose biosynthetic genes resulted in the isolation of a number of microbial *TPS* genes. **a** Phylogenetic analysis of the isolated *TPS* genes (see Table S2 for information on the genes used in this analysis). The most frequently-isolated transformants, *glu15* and *glu13*, were most homologous with TPS genes from Amoebozoa and Fungi, respectively. **b** The microbial *glu15* and *glu13* genes are active *TPS* genes. Expression of *glu15* and *glu13* under the control of a yeast constitutive promoter (*PGK* promoter) results in complementation of the yeast *tps1Δ* mutant for growth on glucose. The yeast *ScTPS1* gene under control of the *CUP1* promoter, which is less active than the *PGK* promoter, was used as a control

Isolation and characterization of *S. lepidophylla* TPPs

SlTPP genes were not isolated during the *S. lepidophylla* library screenings, so a degenerate-oligonucleotide-based PCR approach was used. Degenerate oligonucleotides were designed according to sequences from the available *S. lepidophylla* EST library and from *TPP* genes from other plant species. Based on the sequences of the primary PCR products, a second set of primers was designed and used in combination with primers annealing to the vector sequence to amplify the complete cDNA inserts. Three *SlTPP* genes, named *SlTPPA*, *SlTPPB*, and *SlTPPC*, were isolated. To phylogenetically characterize them, the *TPP* gene families from three selected species were obtained from PLAZA 1.0 (Proost et al. 2009): *Physcomitrella patens* had eight *TPP* genes (*PP00032G01010*, *PP00046G00150*, *PP00047G00570*, *PP00056G01260*, *PP00141G00660*, *PP00201G00540*, *PP00227G00210*, and *PP00333G00270*), *S. moellendorffii* had two (*SM00030G00440* and *SM00032G01340*), and *A. thaliana* had 10 (*AT1G22210*, *AT1G35910*, *AT1G78090*, *AT2G22190*, *AT4G12430*, *AT4G22590*, *AT4G39770*, *AT5G10100*, *AT5G51460*, and *AT5G65140*). According to our phylogenetic analysis, *SlTPPA* and *SlTPPC* were orthologous *SM00032G01340* and *SM00030G00440*, respectively, inferring a common

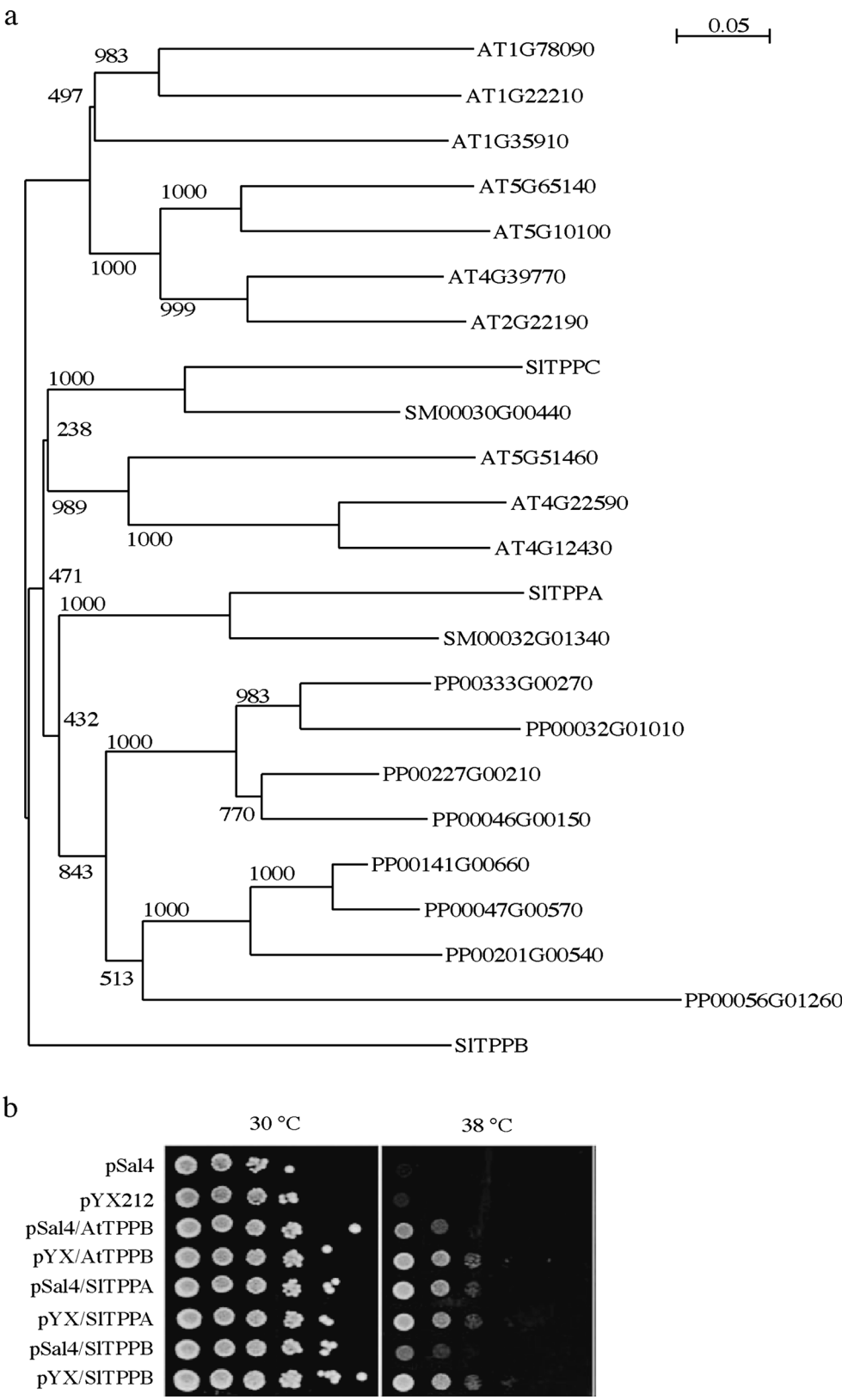

Fig. 2 Phylogenetic analysis and functional characterization of *Selaginella lepidophylla TPP* genes. **a** Phylogenetic analysis of *SlTPP* and *TPP* genes from *Arabidopsis thaliana*, *Physcomitrella patens*, and *Selaginella moellendorffii* (see Table S2 for information on the genes used in this analysis). *SlTPPA* and *SlTPPC* are clear orthologs of the two *S. moellendorffii* genes, while. *SlTPPB* did not seem to have an ortholog in this species. **b** *SlTPPA* and *SlTPPB* are active *TPP* genes. Expression of *SlTPPA* and *SlTPPB* in a yeast *tps2Δ* strain restores growth at 38 °C, whereas strains transformed with empty plasmids do not grow at this temperature. The *A. thaliana TPPB* gene was used as a control

ancestor gene prior to the speciation event separating *S. moellendorffii* and *S. lepidophylla*. The *SlTPPB* gene did not have an ortholog in *S. moellendorffii* (Fig. 2a).

To investigate the functional activity of the SlTPPA (pI 5.36, 45.87 kDa) and SlTPPB (pI 5.65, 34.39 kDa) proteins, the genes were cloned into pSal4 and pYX212 vectors

Table 2 Trehalose levels and TPP enzymatic activity of wild type and *tps2Δ* yeast strains expressing the *Selaginella lepidophylla TPPA* or *TPPB* gene

Strain	Plasmid	Trehalose (μmol g wet weight^{-1})	TPP activity (nkat g protein^{-1})
Wild type	pYX212	99.3 ± 1.1	799.6
tps2Δ	pYX212	38 ± 4.4	0
tps2Δ	pYX212/ SlTPPA	52 ± 4.6	3.2
tps2Δ	pYX212/ SlTPPB	61.3 ± 3.8	13.6
tps2Δ	pYX212/ AtTPPB	57.7 ± 5.1	0

pYX212 empty vector, *SlTPPA and SlTPPB S. lepidophylla* TPPs, *AtTPPB Arabidopsis thaliana* TPPB

and introduced into yeast. Complementation assays showed that SlTPPA and SlTPPB were active TPPs, able to complement the growth of a yeast *tps2Δ* at 38 °C (Fig. 2b). The trehalose measurements indicated a subtle increase in trehalose content for SlTPPA and SlTPPB, with 52 ± 4.6 and 61.3 ± 3.8 μmol g^{-1}, respectively, compared with 38 ± 4.3 and 99.3 ± 1.1 μmol g^{-1} measured in the *tps2Δ* and the WT strains, respectively. This correlated with a measurable level of TPP activity, 3.2 and 13.6 nkat g protein^{-1} measured in *SlTPPA* and *SlTPPB* transformants, respectively, compared with the control *tps2Δ* strain, in which no TPP enzymatic activity could be measured. Compared with the 799.6 nkat g protein^{-1} measured in the WT strain, the enzymatic activities obtained with the *S. lepidophylla TPP* genes were low (Table 2).

Isolation of an *S. lepidophylla* class II *TPS* gene

Apart from the active TPS and TPP enzymes (SlTPS1 and SlTPPA, SlTPPB, and SlTPPC in *S. lepidophylla*), all plants also have genes that encode for so-called class II TPS enzymes. They are very similar to the class I active TPS enzymes but lack the *N*-terminal extension, and some crucial amino acids in the catalytic domains are not conserved. Most species have more class II than class I enzymes (e.g., *A. thaliana* has four and seven, respectively); only in the primitive alga *Ostreococcus tauri* is there an equal number (one each) (Avonce et al. 2010). The *S. lepidophylla* EST library (Iturriaga et al. 2006) screening resulted in the isolation of a putative *SlTPS* class II fragment. Based on this fragment, specific primers were designed (supplementary Table 1), and the *SlTPS* class II cDNA clone was amplified. This class II enzyme had 849 amino acids and, similar to the class II proteins of other plants, could not complement yeast *tps1* or *tps2* mutants (data not shown). Phylogenetic analysis with homologous TPS class II genes from *A. thaliana*, *P. patens*, and *S. moellendorffii* (Fig. 3a) indicated that the closest homolog for *SlTPS* class II was the TPS class II *SM0003G06280* gene from *S. moellendorffii* (88.9 % identity). The second TPS class II gene from *S. moellendorffii*, *SM0057G00180*, had 71.84 % identity with our *SlTPS* class II gene. Shared identity with the four *P. patens* TPS II genes was 73.37, 74.46, 69.14 and 70.93 % for *PP0081G00980*, *PP00088900870*, *PP00080G00530*, and *PP00153G00600*, respectively. Among the seven *AtTPS* class II genes, *SlTPS* class II shared closest homology with *AtTPS5* (67.78 % identity, AT4G17770). Because the whole genome sequence is not yet available, how many class II enzymes there are in *S. lepidophylla* is not clear.

Relative transcript levels of *SlTPSI*, *SlTPPA*, and *SlTPPB* during hydrated and dehydrated states of *S. lepidophylla*

The expression levels of three trehalose biosynthetic genes were measured. The expression level of *SlTPSI* did not change significantly between the hydrated and dehydrated states of *S. lepidophylla* (Fig. 4). In the case of the *TPP* genes, *SlTPPA* expression was significantly decreased during dehydration, while there was no significant difference in the expression level of *SlTPPB* between the two states. As mentioned above, trehalose levels do not differ significantly between the hydrated and dehydrated states of this species (Adams et al. 1990; Yobi et al. 2013).

Discussion

The high trehalose levels in *S. lepidophylla* have always been linked to its high drought-stress tolerance. One of the first genes isolated from this plant was *SlTPS1*, encoding a trehalose-6-phosphate synthase (Zentella et al. 1999). However, heterologous expression in yeast showed that this enzyme was even less active than AtTPS1 (Van Dijck et al. 2002). If trehalose biosynthesis was linked to drought-stress tolerance, one would expect the enzymatic activity and trehalose levels to be higher in the dry state. However, SlTPS1 is active in both the dry and hydrated states, with fully-hydrated plants having the highest SlTPS1 activity (Márquez-Escalante et al. 2006). Additionally, the levels of trehalose were not significantly different between the dry and hydrated states. The level of trehalose in *S. lepidophylla* corresponded to the levels measured in microbes, which are 3,000 times higher than in plants such as rice, tomato, and potato. Although high trehalose levels have been linked specifically with the desiccation-tolerance of *S. lepidophylla*, a large-scale comparative metabolomics study revealed that *S. moellendorffii*, the

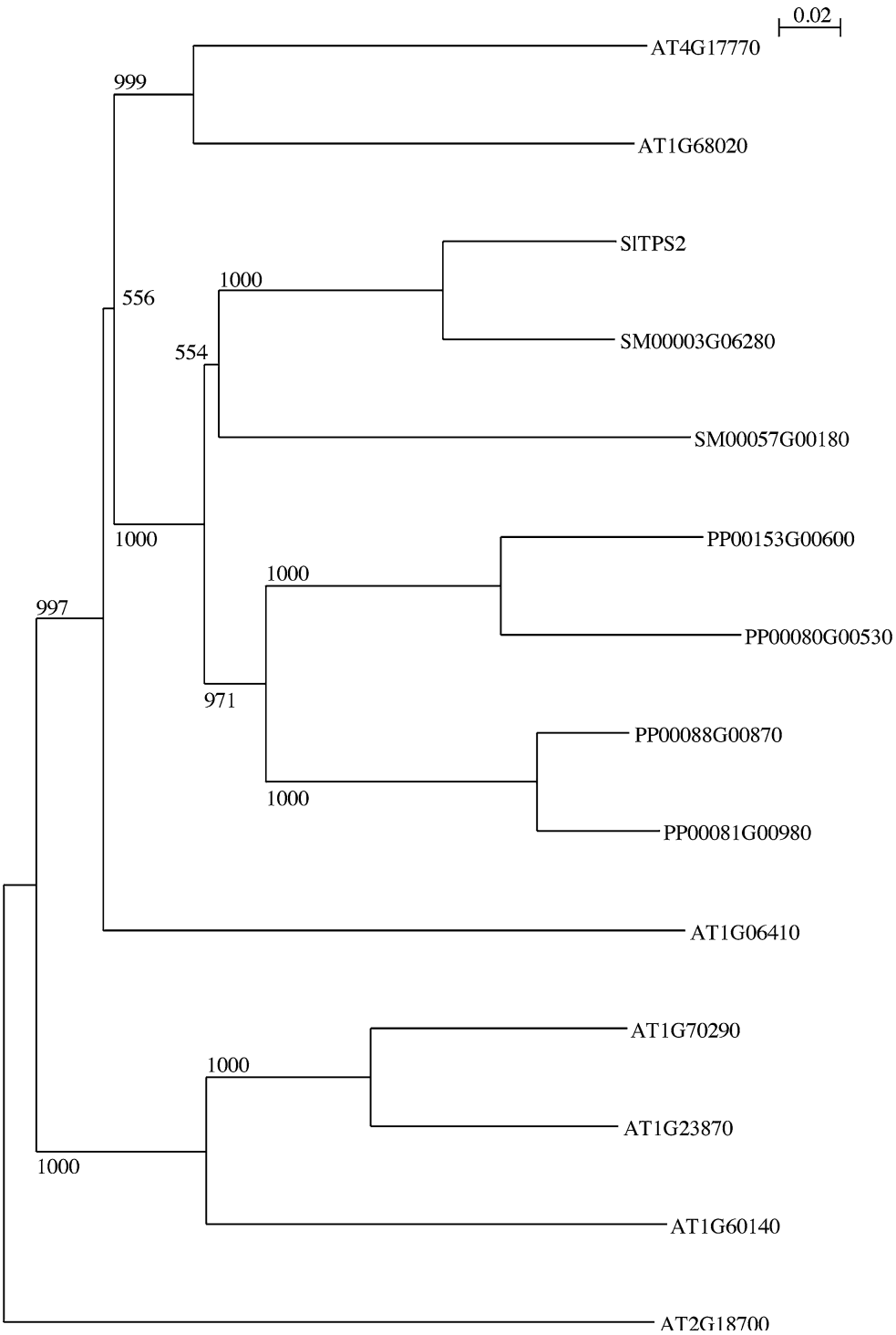

Fig. 3 Phylogenetic analysis of an *SlTPS* class II gene. A full-length *TPS* class II gene of *Selaginella lepidophylla* was isolated. Phylogenetic analysis shows that it shares most homology with a class II enzyme of *S. moellendorffii*

drought-sensitive relative of *S. lepidophylla,* had even higher levels of trehalose than *S. lepidophylla* (Yobi et al. 2012). The high trehalose levels detected in both lycophytes represent an evolutionary-preserved metabolic feature that requires further study. The results obtained here question the origin of trehalose in *S. lepidophylla*, because the *S. lepidophylla* cDNA library screenings resulted in the isolation of T6P synthases of microbial origin (Table 1; Fig. 1a, b). This finding may explain the similar trehalose concentrations in the hydrated and dry states. Furthermore, ultrastructural analysis of *S. lepidophylla* during rehydration clearly showed the presence of fungi and bacteria in the dry microphylls (Brighigna et al. 2002). These facts, together with the isolation of *TPS* genes of microbial origin and the

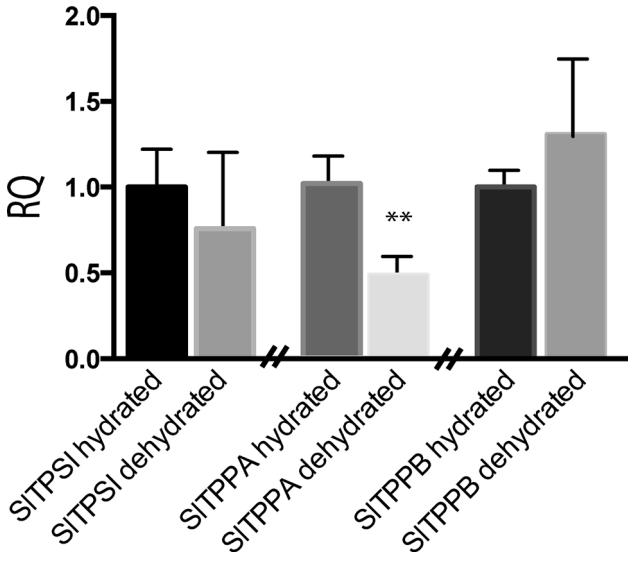

Fig. 4 Relative transcript levels of *SlTPS1*, *SlTPPA*, and *SlTPPB* in the hydrated and dehydrated states of *Selaginella lepidophylla*. *SlTPPA* relative transcript levels are significantly lower in the dehydrated state ($P < 0.01$; Student's t test, $n = 9$). There is no significant difference in the expression levels of *SlTPS1* and *SlTPPB* in the two states

absence of expression differences in *S. lepidophylla TPS* and *TPP* genes strongly support the hypothesis that trehalose in this plant is of microbial origin. Recently, the *S. moellendorffii* genome was shown to also contain DNA from nucleocytoplasmic large DNA viruses, probably a result of horizontal gene transfer (Maumus et al. 2014). The endogenous origin of trehalose in *S. lepidophylla* could be proven by measuring trehalose content in sterile in vitro cultures of the plant. However, attempts to grow *S. lepidophylla* in vitro from spores have failed, probably because of the lack of symbiotic bacteria/fungi necessary for this lycophyte to grow (G. Iturriaga, personal communication), further supporting the microbial origin of trehalose production.

As the cDNA library screening was not successful in isolating *SlTPP* genes, degenerate-oligonucleotide PCR approaches were used to isolate three *SlTPP* genes (*SlTPPA*, *SlTPPB* and *SlTPPC*) from the cDNA library. The phylogenetic analysis showed that *SlTPPA* and *SlTPPC* were orthologs of two *S. moellendorffii* genes, suggesting common ancestor genes prior to speciation, while *SlTPPB* did not appear to have an ortholog in *S. moellendorffii* (Fig. 2a). The complementation of the yeast *tps2Δ* mutant and enzymatic activity determinations proved that both *SlTPPA* and *SlTPPB* were active TPP proteins, although their activity (nkat g protein^{-1}) values were very low compared with that of ScTPS2 (WT control) and slightly higher than that of the *tps2Δ* strain (the negative control) (Table 2) (*SlTPPC* was not tested in this assay). The low enzymatic activity of the SlTPP enzymes did not differ significantly from that of AtTPPB, which was used as a control (Fig. 2b; Table 2). Again, the data indicate that the basic enzymatic activity of TPP in *S. lepidophylla* did not differ from that of other plants, so it probably cannot account for the higher presence of trehalose.

Finally, similar to all other plant species, *S. lepidophylla* has at least one class II enzyme, which we isolated using a PCR approach. This means that all three groups of enzymes (or regulatory proteins in the case of the class II protein) are present in this resurrection plant. Sequencing of the *S. lepidophylla* genome would provide an opportunity to characterize the complete trehalose biosynthetic gene family in this desiccation-tolerant plant. In the case of *S. moellendorffii*, in silico evolutionary studies of the gene family are possible, because its genome has already been sequenced (Banks et al. 2011), and in silico studies of other gene families have already been performed (Saha et al. 2013). Evolutionary studies of the *S. lepidophylla* trehalose biosynthetic genes might provide important hints into their specific functions under different conditions. Our expression analysis already suggests diverse regulation of *SlTPPA* and *SlTPPB* during hydration and dehydration. *SlTPPA* transcript levels were significantly lower during dehydration, while the expression of *SlTPPB* did not change significantly during this process.

The fact that *S. moellendorffii* has similarly high trehalose levels to *S. lepidophylla* but is drought-stress sensitive seems to suggest that other characteristics (maybe together with trehalose) are involved in the latter's desiccation tolerance. An overview of these characteristics can be found in the review by Pampurova and Van Dijck (2014).

Conclusions

The isolation and characterization of three *SlTPPs* genes and one *SlTPS* class II gene, together with the previously characterized *SlTPS1*, showed that this plant also has all three groups of trehalose biosynthesis enzymes. The most striking result of our work was the isolation of many TPS genes of microbial origin from our cDNA library. Additionally, the *S. lepidophylla* TPPA and TPPB enzymes were not more active than *A. thaliana* TPPB. Taken together, the results suggest that the high trehalose content in this plant (and probably also in *S. moellendorffii*) is of microbial origin.

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References

Adams RP, Kendall E, Kartha KK (1990) Comparison of free sugars in growing and desiccated plants of *Selaginella lepidophylla*. Biochem Syst Ecol 18:107–110

Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G (2006) Insights on the evolution of trehalose biosynthesis. BMC Evol Biol 6:109

Avonce N, Wuyts J, Verschooten K, Vandesteene L, Van Dijck P (2010) The *Cytophaga hutchinsonii* ChTPSP: first characterized bifunctional TPS-TPP protein as putative ancestor of all eukaryotic trehalose biosynthesis proteins. Mol Biol Evol 27:359–369

Banks JA, Nishiyama T, Hasebe M, Bowman JL, Gribskov M, dePamphilis C, Albert VA, Aono N, Aoyama T, Ambrose BA, Ashton NW, Axtell MJ, Barker E, Barker MS, Bennetzen JL, Bonawitz ND, Chapple C, Cheng C, Correa LG, Dacre M, DeBarry J, Dreyer I, Elias M, Engstrom EM, Estelle M, Feng L, Finet C, Floyd SK, Frommer WB, Fujita T, Gramzow L, Gutensohn M, Harholt J, Hattori M, Heyl A, Hirai T, Hiwatashi Y, Ishikawa M, Iwata M, Karol KG, Koehler B, Kolukisaoglu U, Kubo M, Kurata T, Lalonde S, Li K, Li Y, Litt A, Lyons E, Manning G, Maruyama T, Michael TP, Mikami K, Miyazaki S, Morinaga S, Murata T, Mueller-Roeber B, Nelson DR, Obara M, Oguri Y, Olmstead RG, Onodera N, Petersen BL, Pils B, Prigge M, Rensing SA, Riano-Pachon DM, Roberts AW, Sato Y, Scheller HV, Schulz B, Schulz C, Shakirov EV, Shibagaki N, Shinohara N, Shippen DE, Sorensen I, Sotooka R, Sugimoto N, Sugita M, Sumikawa N, Tanurdzic M, Theissen G, Ulvskov P, Wakazuki S, Weng JK, Willats WW, Wipf D, Wolf PG, Yang L, Zimmer AD, Zhu Q, Mitros T, Hellsten U, Loque D, Otillar R, Salamov A, Schmutz J, Shapiro H, Lindquist E, Lucas S, Rokhsar D, Grigoriev IV (2011) The *Selaginella* genome identifies genetic changes associated with the evolution of vascular plants. Science 332:960–963

Becker A, Schlöder P, Steele JE, Wegener G (1996) The regulation of trehalose metabolism in insects. Experientia 52:433–439

Bergtrom G, Schaller M, Eickmeier WG (1982) Ultrastructural and biochemical bases of resurrection in the drought-tolerant vascular plant, *Selaginella lepidophylla*. J Ultrastruct Res 78:269–282

Bonini BM, Van Dijck P, Thevelein JM (2003) Uncoupling of the glucose growth defect and the deregulation of glycolysis in *Saccharomyces cerevisiae* Tps1 mutants expressing trehalose-6-phosphate-insensitive hexokinase from *Schizosaccharomyces pombe*. Biochim Biophys Acta 1606:83–93

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brighigna L, Bennici A, Tani C, Tani G (2002) Structural and ultrastructural characterization of *Selaginella lepidophylla*, a desiccation-tolerant plant, during the rehydration process. Flora 197:81–91

Crowe JH (2007) Trehalose as a "chemical chaperone": fact and fantasy. Adv Exp Med Biol 594:143–158

Crowe JH, Hoekstra FA, Crowe LM (1992) Anhydrobiosis. Annu Rev Physiol 54:579–599

De Virgilio C, Bürckert N, Bell W, Jenö P, Boller T, Wiemken A (1993) Disruption of *TPS2*, the gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae*, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity. Eur J Biochem 212:315–323

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. Glycobiology 13:17R–27R

Elble R (1992) A simple and efficient procedure for transformation of yeasts. Biotechniques 13:18–20

Gietz RD, Schliestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360

Hengherr S, Heyer AG, Köhler HR, Schill RO (2008) Trehalose and anhydrobiosis in tardigrades-evidence for divergence in responses to dehydration. FEBS J 275:281–288

Hohmann S, Neves MJ, de Koning W, Alijo R, Ramos J, Thevelein JM (1993) The growth and signalling defects of the *ggs1* (*fdp1/byp1*) deletion mutant on glucose are suppressed by a deletion of the gene encoding hexokinase PII. Curr Genet 23:281–289

Hohmann S, Bell W, Neves MJ, Valckx D, Thevelein JM (1996) Evidence for trehalose-6-phosphate-dependent and -independent mechanisms in the control of sugar influx into yeast glycolysis. Mol Microbiol 20:981–991

Iturriaga G, Cushman MAF, Cushman JC (2006) An EST catalogue from the resurrection plant *Selaginella lepidophylla* reveals abiotic stress-adaptive genes. Plant Sci Int J Exp Plant Biol 170:1173–1184

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948

Lederer E (1976) Cord factor and related trehalose esters. Chem Phys Lipids 16:91–106

Leyman B, Van Dijck P, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. Trends Plant Sci 6:510–513

Lunn JE (2007) Gene families and evolution of trehalose metabolism in plants. Funct Plant Biol 34:550–563

Márquez-Escalante JA, Figueroa-Soto CG, Valenzuela-Soto EM (2006) Isolation and partial characterization of trehalose-6-phosphate synthase aggregates from *Selaginella lepidophylla* plants. Biochimie 88:1505–1510

Maumus F, Epert A, Nogué F, Blanc G (2014) Plant genomes enclose footprints of past infections by giant virus relatives. Nat Commun 5:4268

Müller J, Boller T, Wiemken A (1995) Trehalose and trehalase in plants: recent developments. Plant Sci 112:1–9

Neves MJ, Hohmann S, Bell W, Dumortier F, Luyten K, Ramos J, Cobbaert P, Dekoning W, Kaneva Z, Thevelein JM (1995) Control of glucose influx into glycolysis and pleiotropic effects studied in different isogenic sets of *Saccharomyces cerevisiae* mutants in trehalose biosynthesis. Curr Genet 27:110–122

Oliver MJ, Tuba Z, Mishler BD (2000) The evolution of vegetative desiccation tolerance in land plants. Plant Ecol 151:85–100

Pampurova S, Van Dijck P (2014) The desiccation tolerant secrets of *Selaginella lepidophylla*: what we have learned so far? Plant Physiol Biochem 80:285–290

Perrière G, Gouy M (1996) WWW-query: an on-line retrieval system for biological sequence banks. Biochimie 78:364–369

Proost S, Van Bel M, Sterck L, Billiau K, Van Parys T, Van de Peer Y, Vandepoele K (2009) PLAZA: a comparative genomics resource to study gene and genome evolution in plants. Plant Cell 21:3718–3731

Saha J, Gupta K, Gupta B (2013) In silico characterization and evolutionary analyses of CCAAT binding proteins in the lycophyte plant *Selaginella moellendorffii* genome: a growing comparative genomics resource. Comput Biol Chem 47:81–88

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7:539

Thevelein JM (1984) Regulation of trehalose mobilization in fungi. Microbiol Rev 48:42–59

Thomas BJ, Rothstein RJ (1989) Elevated recombination rates in transcriptionally active DNA. Cell 56:619–630

Valenzuela-Avendano JP, Estrada Mota IA, Lizama G, Souza Perera R, Valenzuela-Soto EM, Zuniga Aguilar JJ (2005) Use of a simple method to isolate intact RNA from partially hydrated *Selaginella lepidophylla* plants. Plant Mol Biol Rep 23:199a–199g

Valenzuela-Soto EM, Márquez-Escalante JA, Iturriaga G, Figueroa-Soto CG (2004) Trehalose 6-phosphate synthase from *Selaginella lepidophylla*: purification and properties. Biochem Biophys Res Commun 313:314–319

Van Dijck P, Mascorro-Gallardo JO, De Bus M, Royackers K, Iturriaga G, Thevelein JM (2002) Truncation of *Arabidopsis thaliana* and *Selaginella lepidophylla* trehalose-6-phosphate synthase (TPS) unlocks high catalytic activity and supports high trehalose levels upon expression in yeast. Biochem J 366:63–71

Van Mullem V, Wery M, De Bolle X, Vandenhaute J (2003) Construction of a set of *Saccharomyces cerevisiae* vectors designed for recombinational cloning. Yeast 20:739–746

Yobi A, Wone BW, Xu W, Alexander DC, Guo L, Ryals JA, Oliver MJ, Cushman JC (2012) Comparative metabolic profiling between desiccation-sensitive and desiccation-tolerant species of *Selaginella* reveals insights into the resurrection trait. Plant J 72:983–999

Yobi A, Wone BW, Xu W, Alexander DC, Guo L, Ryals JA, Oliver MJ, Cushman JC (2013) Metabolomic profiling in *Selaginella lepidophylla* at various hydration states provides new insights into the mechanistic basis of desiccation tolerance. Mol Plant 6:369–385

Zaparty M, Hagemann A, Bräsen C, Hensel R, Lupas AN, Brinkmann H, Siebers B (2013) The first prokaryotic trehalose synthase complex identified in the hyperthermophilic crenarchaeon *Thermoproteus tenax*. PLoS ONE 8:e61354

Zentella R, Mascorro-Gallardo JO, Van Dijck P, Folch-Mallol J, Bonini B, Van Vaeck C, Gaxiola R, Covarrubias AA, Nieto-Sotelo J, Thevelein JM, Iturriaga G (1999) A *Selaginella lepidophylla* trehalose-6-phosphate synthase complements growth and stress-tolerance defects in a yeast *tps1* mutant. Plant Physiol 119:1473–1482