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ORIGINAL ARTICLE

A bifunctional TPS–TPP enzyme from yeast confers tolerance to multiple and extreme abiotic-stress conditions in transgenic *Arabidopsis*

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Abstract Improving stress tolerance is a major goal for agriculture. Trehalose is a key molecule involved in drought tolerance in anhydrobiotic organisms. Here we describe the construction of a chimeric translational fusion of yeast trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. This construct was overexpressed in yeast cells displaying both TPS and TPP enzyme activities and trehalose biosynthesis capacity. In *Arabidopsis thaliana*, the gene fusion was overexpressed using either the *35S* promoter or the stress-regulated *rd29A* promoter. Transgene insertion in the genome was checked by PCR and transcript expression by RT-PCR. Several independent homozygous lines were selected in the presence of kanamycin and further analyzed. Trehalose was accumulated in all these lines at low levels. No morphological or growth alterations were observed in lines overexpressing the *TPS1–TPS2* construct, whereas plants overexpressing the *TPS1* alone under the control of the 35S promoter had aberrant growth, color and shape. *TPS1–TPS2* overexpressor lines were glucose insensitive, consistent with a suggested role of trehalose/T6P in modulating sugar sensing and carbohydrate metabolism. Moreover, *TPS1–TPS2* lines displayed a significant increase in drought, freezing, salt and heat tolerance. This is the first time that trehalose accumulation in plants is shown to protect against freezing and heat stress. Therefore, these results demonstrate that engineering trehalose metabolism with a yeast TPS–TPP bifunctional enzyme confers multiple stress protection in plants, comprising a potential tool to improve stress-tolerance in crops.

Keywords *Arabidopsis* · Stress-tolerance · Trehalose

Abbreviations

T6P	Trehalose-6-phosphate
TPS	Trehalose-6-phosphate synthase
TPS1	Yeast gene encoding TPS
TPP	Trehalose-6-phosphate phosphatase
TPS2	Yeast gene encoding TPP

Introduction

Abiotic stress is the most limiting constraint for crop productivity and yield, compromising food supply for a further growing human population in the coming decades. Drought and salinity affect more than 10% of arable land declining yield for most crops by around 50% and this figure is expected to rise as Global Warming is increasing steadily (Bray et al. 2000). Therefore, there is a strong need to improve stress tolerance in crops against adverse environmental conditions. Decades of plant breeding attempts revealed its limited use to improve tolerance to drought, salinity or other abiotic stresses. Therefore, genetic engineering

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of stress tolerance has become one of the major goals of agricultural research.

Exposure to drought, salt or extreme temperatures triggers many common responses in plants. All lead to cellular dehydration and build-up of reactive oxygen species, which affect subcellular structures and proteins. A major adaptive response of several plants to environmental stress is the synthesis of compatible solutes (osmoprotectants). These molecules can effectively counteract dehydration and free-radical species by lowering osmotic potential and protecting membranes, enzymes and other structures against irreversible damage and denaturation (Bartels and Sunkar 2005). Among the different classes of osmoprotectants trehalose stands as one of the most effective against extreme environmental stress. This non-reducing disaccharide (α-D-glucopyranosyl-1, 1-α-D-glucopyranoside) is found in several groups of eubacteria, archaea, fungi, plants and invertebrates, namely anhydrobiotic organisms. These can recover after long periods of survival in a dehydrated state in a matter of hours after coming again in contact with water (Elbein et al. 2003).

There are at least five routes for trehalose biosynthesis (Avonce et al. 2006) and the most widely distributed has been reported in several species of bacteria including *E. coli*, yeast, insects, and plants such as *Arabidopsis thaliana* and the so-called "resurrection plant" *Selaginella lepidophylla* (Zentella et al. 1999). Trehalose metabolism has been studied in most detail in *Saccharomyces cerevisiae*. In this microorganism, trehalose is synthesized by a multienzyme complex comprising trehalose-6-phosphate synthase (TPS) encoded by *TPS1*, trehalose-6-phosphate phosphatase (TPP) encoded by *TPS2* and two regulatory subunits encoded by the *TSL1* and *TPS3* genes. Although the latter genes do not play a catalytic role, they share significant homology with TPS and TPP (Reinders et al. 1997; Bell et al. 1998). Trehalose-6-phosphate (T6P) is synthesized from UDP-glucose and glucose-6-phosphate substrates by TPS and dephosphorylated to trehalose by TPP (Cabib and Leloir 1958; Vandercammen et al. 1989; Londesborough and Vuorio 1993). Deletion of the *TPS1* or *TPS2* gene caused an impairment of stress tolerance (Mackenzie et al. 1988; De Virgilio et al. 1994). Additionally, the yeast *tps1*Δ mutant and its alleles are unable to grow in glucose as the sole carbon source. It has been suggested that this defect may be due to the additional role of the TPS subunit in regulating the flow of glucose into glycolysis (Van Aelst et al. 1993; Thevelein and Hohmann 1995).

The bioengineering of trehalose biosynthetic genes in plants has led to an improvement of stress tolerance, although it caused striking morphological changes when a TPS coding gene was expressed alone (Goddijn et al. 1997; Pilon-Smits et al. 1998; Yeo et al. 2000). Expression in rice of a gene fusion of *otsA* (*TPS1* homologue) and *otsB* (*TPS2* homologue) driven by a stress-regulated promoter conferred resistance to several abiotic stresses without causing morphological changes (Garg et al. 2002; Jang et al. 2003).

Here we report the construction of a bifunctional yeast *TPS1–TPS2* fusion which restored growth and stress tolerance in *S. cerevisiae*. In transgenic *Arabidopsis* plants, the chimeric fusion improved extreme drought-, heat-, freezing- and salt-stress conditions without causing morphological abnormalities.

Materials and methods

Gene constructs

To construct the *TPS1–TPS2* translational gene-fusion, a 1,526-bp *TPS1* gene fragment, including 41-bp of leader sequence, was amplified by PCR using 100 ng of yeast genomic DNA, the High Expand DNA polymerase (Roche, Indianapolis, IN, USA) and the following conditions: one cycle at 94°C, 3 min; and 30 cycles of 94°C, 1 min; 55°C, 1 min; and 72°C, 1.5 min. Oligonucleotides (5′-CCGCTC GAGGGTACTCACATACAGAC and 5′-CGGGATCCG GTGGCAGAGGAGCTTGTTGAGC) were used to insert a *Xho*I site at its 5′-end, and a *Bam*HI site (encodes for Gly and Ser) at its 3′-end, removing the two last encoded amino acids (Lys and Asn) and the stop codon. A 1353-bp region of *TPS2* gene, which encodes the phosphatase domain at its carboxy-terminal part and 153 bp of the non-translated 3′-end was amplified by PCR as before using oligonucleotides (5′-CGGGATCCGCTAACT AATCTATTAACATGG and 5′-CGGGGTACCATGGTGGGTTGAGAC) which allowed inserting a *Bam*HI site at its 5′-end, adding encoded Gly and Ser, and a *Kpn*I site at its 3′-end. Both fragments when fused comprised a 2,873-kb chimeric gene and were subcloned in the pBluescript SK vector (Invitrogen, Carlsbad, CA, USA) and sequenced.

Yeast complementation and growth

The *TPS1–TPS2* gene fusion was subcloned in the yeast pSal4 expression vector that uses the cupper-inducible *CUP1* promoter (Mascorro-Gallardo et al. 1996). The subcloning of *TPS1* or *TPS2* yeast genes in pSal4 has already been described (Zentella et al. 1999). Yeast growth was at 30°C and the culture media and yeast strains (W303-1A wild type, *tps1Δ*, *tps2Δ* and *tps1tps2Δ*) were described before (Zentella et al. 1999). Transformation was performed as described previously (Elble 1992), and transformants were selected in minimal media without uracil. For each construct, at least 5 independent transformants were chosen to test their ability to restore the growth defect on

minimal medium plus 2% glucose. The candidates were streaked on agar plates with minimal medium and 2% galactose or glucose. As a control, the same strains were transformed with the pSAL4 vector alone. After 3 days, growth was visible in positive control and complemented mutants. To assay thermotolerance, strains were grown at 38.5°C for 3 days in solid YP media supplemented with either 2% galactose or glucose.

Plant material and growth conditions

Arabidopsis thaliana Col-0 ecotype (seeds originally obtained from the Arabidopsis Biological Resource Centre, Ohio State University, Columbus, OH, USA) was used for overexpressing the *TPS1–TPS2* or *TPS1* gene constructs. Plants were routinely grown on Metro-Mix 200 (Grace Sierra, Milpitas, CA, USA) soil at 24/20°C with 16-h light/8-h dark cycle. Surface-sterilized seeds were germinated on MS media: 1× Murashige and Skoog basal salt mixture medium (Invitrogen) supplemented with 1% (w/v) sucrose, B5 vitamins, 0.05% Mes (w/v) and 0.8% (w/v) phytoagar. To break dormancy, seeds were incubated at 4°C for 4 days with cool-white illumination (20 µmol photons m^{-2} s^{-1}). For glucose sensitivity experiments, wild type Arabidopsis, *TPS1–TPS2* overexpressor lines, *abi4* mutant (Arenas-Huertero et al. 2000) or the *12.3* line overexpressing *35S::AtTPS1* (Avonce et al. 2004) were germinated on MS medium supplemented with 6% (w/v) glucose.

Plant transformation

The *TPS1–TPS2* gene fusion or the *TPS1* gene, were subcloned in the pBin19 vector (Bevan 1984) containing either the 0.8 kb *35S* or the 1 kb *rd29A* promoter (Yamaguchi-Shinozaki and Shinozaki 1994) with its corresponding leader, and the 0.3-kb *NOS* polyadenylation site. The constructs were introduced by electroporation in *Agrobacterium tumefaciens* C58C1 strain containing the pGV2260 plasmid. The resulting bacteria were used to transform *A. thaliana* by the floral dip method (Clough and Bent 1998). Transgenic seedlings were selected on MS media containing 50 µg ml^{-1} kanamycin (Sigma, St. Louis, MO, USA). One-week-old seedlings were transferred to pots under the indicated conditions until plants formed seeds. Homozygous lines from the T_3 generation were used in the present work except for the *35S::TPS1* transgenic lines, which were not viable in homozygous condition.

Genomic and RT-PCR analysis

Genomic DNA from Arabidopsis was isolated with a DNA isolation kit (Puregene Gentra System, Minneapolis, MN, USA) and 100 ng were used for a PCR reaction using oligonucleotides corresponding to *TPS2* gene region of *TPS1–TPS2* chimeric gene (see above). PCR conditions were: one cycle at 94°C, 3 min; 35 cycles: 94°C, 1 min; 57°C, 1 min; 72°C, 1.5 min; and finally one cycle at 72°C, 5 min. RT-PCR experiments were performed using 2 µg of total RNA extracted from 2 weeks old *A. thaliana* plants using TRIZOL reagent (Invitrogen) according to manufacturer's instructions and used for 1st strand cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen) and oligo dT. PCR was conducted using oligonucleotides corresponding to the 5′- and 3′-ends of *TPS1–TPS2* (5′-CCGCTCG AGGGTACTCACATACAGAC and (5′-CGGGGTACC ATGGTGGGTTGAGAC) and the program was as described above, except that extension at 72°C was during 3 min, and only 25 cycles were used which corresponded to the linearity phase of the exponential reaction after comparison of the PCR products at different cycles. As RT-PCR controls, the constitutive *APT1* gene or the stress-inducible *rd29A* gene were used for each transgenic line. PCR products were resolved in 1× TAE, 1% agarose gels stained with EtBr.

Trehalose determination

To determine trehalose content in yeast cells, the method reported by Neves et al. (1994) was used where the disaccharide was first hydrolyzed to glucose with trehalase and then glucose concentration was determined by a coupled reaction of glucose oxidase and peroxidase in the presence of O-dianisidine. Reactions were stopped by sulfuric acid before reading at 546 nm. Glucose was used as a standard to determine the concentration (2 mol of glucose = 1 mol of trehalose). For determination of trehalose in plants, extracts were prepared as described before (Avonce et al. 2004) except that samples were analyzed by HPLC using a ZORBAX carbohydrate analysis column (Agilent Technologies, Santa Clara, CA, USA) eluted with acetonitrile:water (65:35, v/v). Trehalose was used as a standard to determine the concentration.

Determination of enzyme activity

Activity of TPS was measured by a coupled-enzyme assay as described previously (Hottiger et al. 1987). Activity of TPP was determined as reported before (De Virgilio et al. 1993). Specific activity was expressed as µkat/g of protein. Protein concentration was determined according to a well-known method (Bradford 1976).

Plant stress tolerance

Four-weeks old *Arabidopsis* plants grown on Metro-Mix 200 (Grace Sierra) were used for all the different stress

tolerance experiments. For drought tolerance, watering was withhold for 2 weeks and then plants were rewatered, and allowed to recover for 1 day before being photographed (Avonce et al. 2004). For salinity tests, plants were irrigated with MS media supplemented with increasing concentrations of NaCl every 4 days, starting from 50 mM and up to 350 mM. After the highest concentration was reached (50, 100, 150, 200, 250, 300 or 350 mM), plants were rinsed in MS media and left 2 weeks without NaCl for recovery. Freezing stress was conducted on plants pre-adapted 3 days at 4°C before subjected to freezing stress at −15°C for 2 days. Plants were left at 24°C during 2 weeks for recovery. Heat-shock experiments were conducted by subjecting plants to 56°C during 3 h, without prior heat pre-adaptation, and allowing 2 weeks of recovery.

Results

Bifunctional TPS–TPP enzyme is active in yeast

To construct a chimeric gene encoding the yeast TPS–TPP bifunctional enzyme, the full-length *TPS1* gene was fused to the 3′-end part of *TPS2*. It is known that when the first 504 amino acids are removed from *TPS2* encoded protein the truncation allele is still fully active since the remaining 392-amino-acid carboxy-terminus encompasses the homology blocks present in all TPP enzymes (Vogel et al. 1998). Therefore, we fused a *TPS1* 1,526-bp fragment (encoding 493 amino acids plus 2 linker amino acids) to a 1353-bp fragment corresponding to the carboxy-end of *TPS2* (encoding 397 amino-acid plus 2 linker amino acids) to yield a 2873-bp DNA fragment fused through the *Bam*HI linker site, which showed a continuous open reading frame encoding the predicted 892 amino-acid sequence after DNA sequencing. This fusion gene was subcloned in the pSal4 vector behind a *CUP1* promoter that is inducible by copper ions (Mascorro-Gallardo et al. 1996) and tested in different yeast mutant cells in order to determine if a functional enzyme was obtained. The glucose-sensitive *tps1Δ*, thermosensitive *tps2Δ* and double *tps1Δtps2Δ* mutants were transformed with pSal4::*TPS1–TPS2* construct and checked for complementation (Fig. 1). The *TPS1* and *TPS2* genes alone were subcloned in the same vector and used as controls.

The *TPS1–TPS2* gene was able to restore growth in glucose of *tps1Δ* and *tps1Δ tps2Δ* mutants (Fig. 1b). These mutants are unable to grow with glucose as the carbon source due to the role of TPS in regulating the flow of glucose into glycolysis (Van Aelst et al. 1993; Thevelein and Hohmann 1995). Also, the *tps2Δ* and *tps1Δtps2Δ* mutants transformed with *TPS1-TPS2* gene were able to grow at 38.5°C, restoring yeast thermotolerance (Fig. 1d). All these

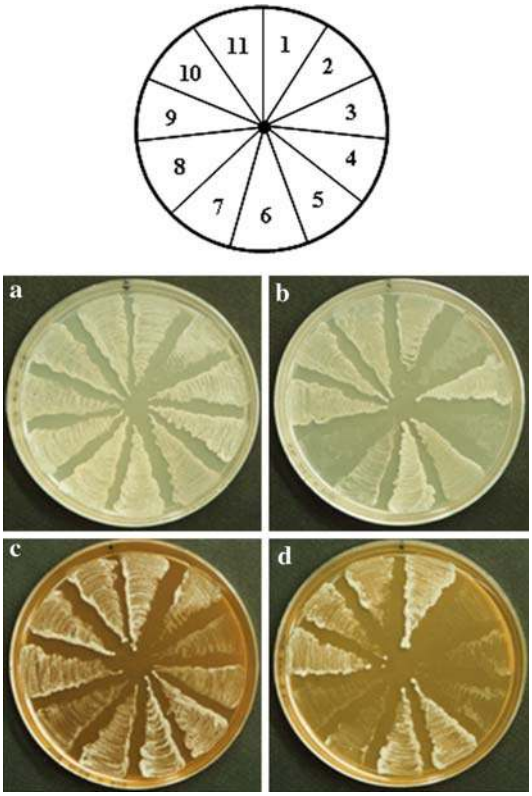

Fig. 1 **a–d** Yeast complementation by a trehalose biosynthesis bifunctional enzyme. Yeast mutants lacking the TPS protein (*tps1Δ*), TPP protein (*tps2Δ*) or both TPS and TPP proteins (*tps1Δtps2Δ*) were transformed with pSal4 containing *TPS1*, pSal4 containing *TPS2*, pSal4 containing the *TPS1-TPS2* gene fusion or pSal4 vector alone and spread on 2% agar plates in minimal medium without uracil (ura), with 100 μM $CuSO_4$, supplemented with 2% galactose (**a**) or 2% glucose (**b**) and grown at 30°C. For thermotolerance assays yeast mutants were spread on YP rich media, with 100 μM $CuSO_4$, supplemented with 2% galactose (**c**) or 2% glucose (**d**) and grown at 38.5°C. The wild type control strain W303-1A (WT) was also transformed with pSal4. Numbers inside circle in top panel denote: *1* Wt pSal4; *2 tps1Δ* pSal4; *3 tps2Δ* pSal4; *4 tps1Δ tps2Δ* pSal4; *5 tps1Δ* pSal4::*TPS1*; *6 tps2Δ* pSal4::*TPS2*; *7 tps1Δtps2Δ* pSal4::*TPS1*; *8 tps1Δtps2Δ* pSal4::*TPS2*; *9 tps1Δ* pSal4::*TPS1–TPS2*; *10 tps2Δ* pSal4::*TPS1–TPS2*; *11 tps1Δtps2Δ* pSal4::*TPS1–TPS2*

results suggest that the *TPS1-TPS2* gene fusion encodes an active TPS–TPP enzyme. As expected, all strains grew in galactose as a carbon source (Fig. 1a, c).

To corroborate the biosynthesis capacity of TPS–TPP fusion enzyme, the complemented strains were grown in liquid media to measure trehalose content. As shown in Table 1, this bifunctional enzyme expressed in the *tps1Δtps2Δ*, *tps1Δ* or *tps2Δ* mutants is able to synthesize trehalose at similar levels than wild type strain or *tps1Δ* and *tps2Δ* mutants complemented with their corresponding homologous gene. However, if the *tps1Δtps2Δ* double mutant is complemented just with *TPS1* the trehalose levels are about half than if using the *TPS1–TPS2* gene fusion, or very low levels of trehalose accumulate if complemented

Table 1 Trehalose content and TPS and TPP specific activities in wild type yeast and mutant strains complemented with the pSal4 plasmid expressing the *TPS1–TPS* gene fusion

Strain	Trehalose content in galactose (μmol/g FW)	Trehalose content in glucose (μmol/g FW)	TPS specific activity in galactose (*μ*kat/g protein)	TPS activity in glucose (*μ*kat/g protein)	TPP activity in galactose (*μ*kat/g protein)	TPP activity in glucose (*μ*kat/g protein)
WT + pSAL4	59.88 ± 26.3	54.36 ± 7.42	0.91 ± 0.21	1.31 ± 0.31	36.19 ± 6.10	41.12 ± 7.64
tps1Δ + pSAL4	4.99 ± 0.8	NG	0.00	NG	28.05 ± 5.40	NG
tps2Δ + pSAL4	6.99 ± 4.3	3.6 ± 1.70	0.00	0.19 ± 0.03	0.00	0.00
tps1Δtps2Δ + pSAL4	5.98 ± 4.1	NG	0.00	NG	0.00	NG
tps1Δ + pSAL4::*TPS1*	44.07 ± 4.6	48.45 ± 8.13	2.72 ± 0.16	2.4 ± 0.27	39.14 ± 4.92	33.61 ± 9.11
tps2Δ + pSAL4::*TPS2*	47.35 ± 21.6	38.75 ± 15.06	1.05 ± 0.13	1.12 ± 0.20	243.12 ± 63.72	252.49 ± 78.63
tps1Δtps2Δ + pSAL4::*TPS1*	24.43 ± 11.9	22.3 ± 0.57	0.21 ± 0.01	0.34 ± 0.03	4.80 ± 1.12	6.90 ± 2.34
tps1Δtps2Δ + pSAL4::*TPS2*	6.44 ± 2.5	NG	0.00	NG	171.87 ± 54.23	NG
tps1Δ + pSAL4::*TPS1-TPS2*	44.27 ± 6.8	54.2 ± 4.10	1.35 ± 0.40	1.43 ± 0.57	542.64 ± 85.21	495.00 ± 160.27
tps2Δ + pSAL4::*TPS1-TPS2*	56.13 ± 16.5	48.9 ± 0.85	1.3 ± 0.15	1.22 ± 0.30	604.46 ± 68.54	561.49 ± 79.34
tps1Δtps2Δ + pSAL4::*TPS1-TPS2*	45.93 ± 4.8	51.6 ± 9.05	1.46 ± 0.33	1.55 ± 0.52	512.23 ± 101.42	522.16 ± 42.14

Three independent transformants of wild-type yeast, the *tps1*Δ mutant, *tps2*Δ or the *tps1tps2*Δ mutant transformed with pSal4 vector alone or harboring *TPS1*, *TPS2*, or *TPS1–TPS2* genes were grown in minimal medium with the indicated carbon source plus 100 μM $CuSO_4$. Trehalose content and enzyme activity were determined in the stationary phase. Values are means ± SD

NG No growth

with *TPS2* alone. These results suggest that *TPS1–TPS2* is restoring both gene functions.

In order to estimate correctly TPS and TPP enzyme activities, it is important to remark that the expression of *TPS1*, *TPS2*, or *TPS1–TPS2* genes under the control of the *CUP1* promoter yielded in general a higher TPS or TPP enzyme activity than in the wild type strain, probably because *CUP1* is stronger than *TPS1* or *TPS2* endogenous promoters (Table 1). In this regard, the TPS activity of the TPS-TPP protein fusion was about half compared to that of the *tps1Δ* mutant transformed with the pSal4::*TPS1* gene alone. However, the *tps1Δtps2Δ* mutant transformed with either pSal4::*TPS1* or pSal4::*TPS2* had a lower enzyme activity for both TPS and TPP, apparently because the holoenzyme complex is required in order to have a full activity (Bell et al. 1998). Surprisingly, the TPP activity of the TPS–TPP fusion protein was consistently higher than the activity of the TPP alone (Table 1). The fusion enzyme had about 2-times higher TPP activity when expressed either in *tps1Δ*, *tps2Δ* or *tps1Δtps2Δ* mutant backgrounds than when the *TPS2* was expressed under the same *CUP1* promoter in *tps2Δ* or *tps1Δtps2Δ* mutants. Therefore, all these results strongly suggest that the *TPS1-TPS2* gene fusion encodes an active TPS–TPP bifunctional enzyme.

Transformation and molecular analysis of transgenic *Arabidopsis* plants

To determine the effect of overexpressing the yeast TPS-TPP bifunctional enzyme in plants, the 2.9-kb *TPS1–TPS2* gene fusion was subcloned in the pBin19 vector downstream of the constitutive *35S* promoter or under the control of the stress-inducible *rd29A* promoter. Also, a construct of the *TPS1* gene alone under the 35S promoter was obtained. Twenty *35S::TPS1-TPS2*, sixteen *rd29A::TPS1-TPS2* and ten *35S::TPS1* independent *Arabidopsis thaliana* transgenic lines were obtained after transformation with the *Agrobacterium tumefaciens* system. After genetic analysis using kanamycin to score a 3:1 segregation ratio, suggesting a single gene-insertion, only nine *35S::TPS1-TPS2* and eight *rd29A::TPS1-TPS2* homozygous plants were obtained. In the case of plants transformed with the *35S::TPS1* construct, no homozygous lines were viable and only seven heterozygous lines segregating in 3:1 ratio were obtained.

The integration event from each *35S::TPS1-TPS2* and *rd29A::TPS1-TPS2* transgene was determined by genomic PCR (Fig. 2a, b), using *TPS2* gene as a target since it lies in the left border of the T-DNA, which can yield imprecise excision. All transgene insertions were of the expected 1.3 kb size. To assay for gene expression of *TPS1–TPS2* in transgenic plants, we used RT-PCR. *35S::TPS1-TPS2* lines expressed the 2.9-kb *TPS1–TPS2* at similar high levels

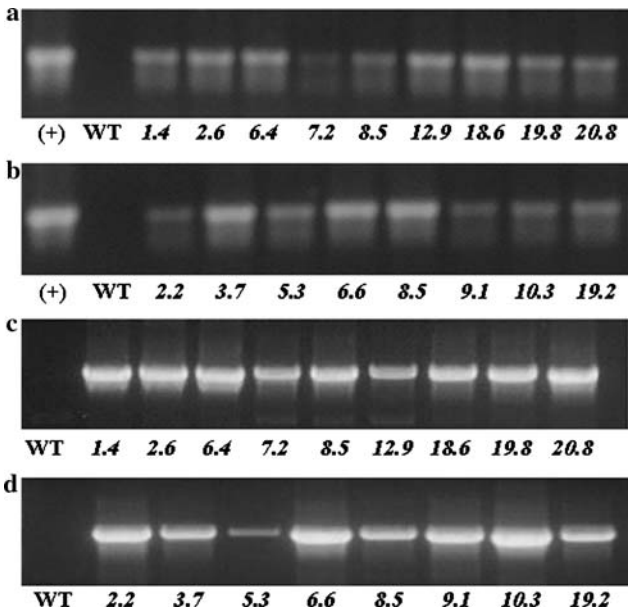

Fig. 2 **a**–**d** Molecular analyses of transgenic plants. Genomic PCR of *Arabidopsis* lines transformed with the *35S::TPS1–TPS2* (**a**) or *rd29A::TPS1–TPS2* constructs (**b**). Gene-expression analysis by RT-PCR of *Arabidopsis* lines transformed with the *35S::TPS1–TPS2* (**c**) or *rd29A::TPS1–TPS2* construct (**d**). Genomic DNA extracted from 2-week-old plantlets (**a**, **b**) or total RNA extracted from untreated 2-week-old plantlets (**c**) or dehydrated for 1 h (**d**). A 1.3-kb PCR fragment corresponding to the *TPS2* part of the gene-fusion (**a**, **b**) or 2.9-kb PCR fragment corresponding to the full-length *TPS1–TPS2* is shown (**c**, **d**). (+) is an amplification control from a plasmid containing the *TPS1–TPS2* construct. RT-PCR was normalized after comparison with the constitutive expression of the *APT1* gene for plants expressing *35S::TPS1–TPS2* or with the stress-inducible *rd29A* gene for *rd29A::TPS1-TPS2* expression (data not shown)

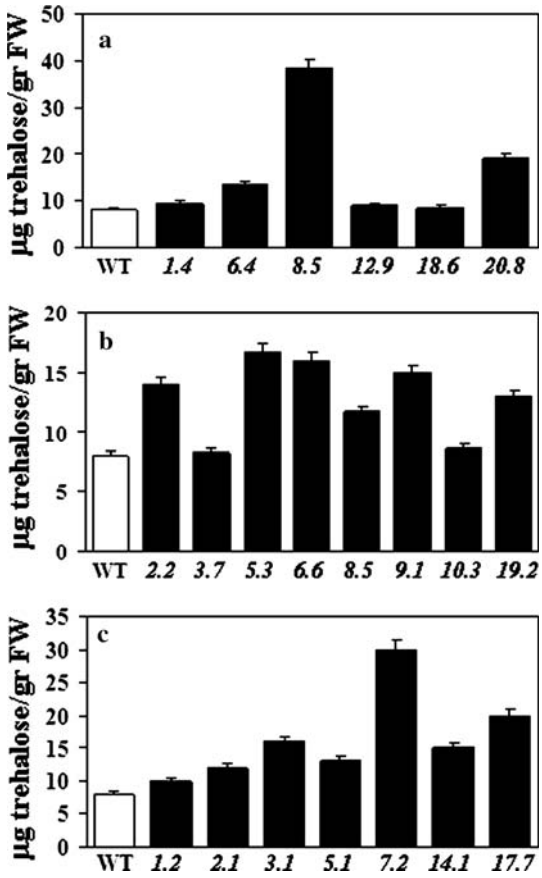

Fig. 3 **a**–**c** Trehalose accumulation in transgenic Arabidopsis. Trehalose content in wild type (WT) or transgenic lines overexpressing the *TPS1–TPS2* gene fusion by means of the *35S* (**a**) or the stress-inducible *rd29A* promoter (**b**) and the *TPS1* gene alone with the *35S* promoter (**c**). Trehalose in *rd29A::TPS1–TPS2* plants was quantified after dehydrating tissue for 1 h. *FW* is fresh weight

(Fig. 2c) after comparison to *APT1*, which encodes adenine ribosyl phosphotransferase. The *APT1* gene was used as a constitutive control and showed comparable levels of expression also in the wild type plants (data not shown). To examine the inducible expression of *rd29A::TPS1-TPS2*, plants were untreated or dehydrated for 1 h and compared to the expression of endogenous *rd29A* gene (data not shown). *TPS1–TPS2* expression was detected only after drought stress treatment and varied within a fivefold range among the different lines (Fig. 2d). The molecular analysis of the seven heterozygous *35S::TPS1* lines also revealed the presence of the transgene inserted in the genome and expression at comparable high levels (data not shown).

Arabidopsis *TPS1-TPS2* lines accumulate trehalose and are glucose-insensitive

To analyze the accumulation of trehalose in transgenic plants, carbohydrate analysis was performed by HPLC (Fig. 3). Trehalose was detected in the different *35S::TPS1–TPS2* transgenic lines at levels of 8.5–38.4 µg/g fresh weight, compared to 7.6 µg/g fresh weight in the wild type (Fig. 3a). A relatively similar range of trehalose accumulation was found in *35S::TPS1* lines, from 10 to 29.9 µg/g fresh weight (Fig. 3c). In contrast, the transgenic *rd29A::TPS1-TPS2* lines accumulated trehalose at lower levels which varied from 8.2 to 16.7 µg/g fresh weight (Fig. 3b). The *TPS1–TPS2* or *TPS1* expression levels, as judged by RT-PCR, did not correlate with those of trehalose content in the different lines.

In the present study we also analyzed whether the overexpression of a yeast *TPS1–TPS2* gene fusion in *Arabidopsis* would lead to a sugar response phenotype. To quantify the effect of glucose on plant development, the germination rate was determined. Different transgenic lines expressing *TPS1–TPS2* and wild-type seeds were germinated on MS media containing 6% glucose and scored at 1-day intervals. *35S::TPS1-TPS2* lines germinated and developed at a normal rate with well-expanded green leaves, in contrast to wild-type seedlings which developed poorly displaying

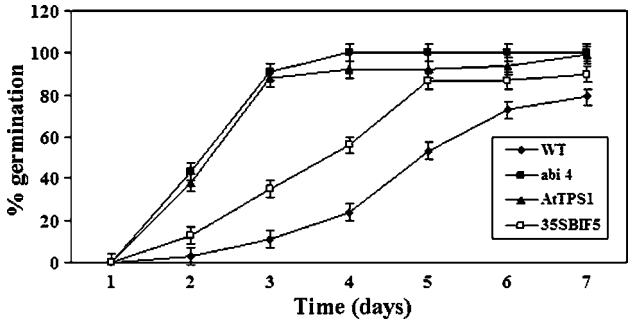

Fig. 4 Glucose sensitivity of *35S::TPS1–TPS2* plants. Germination kinetics of a representative transgenic line overexpressing *TPS1–TPS2* (*35SBIF5*) in comparison to wild type (*WT*), an abscisic-acid insensitive mutant line (*abi4*) and the *12.3* line overexpressing *AtTPS1* (Avonce et al. 2004), growing on MS media supplemented with 6% glucose. Germination was defined as complete protrusion of the radicule. The data are the mean of 3 independent experiments (±SD) evaluating 100 seeds per data point

minimal leaf and root elongation (data not shown). The *35S::TPS1-TPS2* line *8.5* was chosen for further experiments as a representative line and the *abi4* mutant (Arenas-Huertero et al. 2000) and *12.3* line overexpressing *AtTPS1* (Avonce et al. 2004) were used as positive controls since they are glucose-insensitive. Four days after sowing in MS media with 6% glucose, the *abi4* mutant and line *12.3* have completely germinated, in contrast to *35S::TPS1-TPS2* line *8.5* and wild-type plants (Fig. 4). However, the line *8.5* germinated 60% compared to only 20% of the wild type. Therefore, the glucose insensitive phenotype of plants expressing the *35S::TPS1-TPS2* was lower than *35S::AtTPS1* plants but still remained higher than wild type Arabidopsis. These results might be explained by a lower accumulation of T6P in the *35S::TPS1-TPS2* plants.

Transgenic *TPS1–TPS2* lines have normal growth and morphology

Several reports have documented that overexpression of *TPS* genes of different origin in plants provoke growth and morphological abnormalities with variable severity (Holmström et al. 1996; Goddijn et al. 1997; Romero et al. 1997; Yeo et al. 2000; Avonce et al. 2004; Cortina and Culiáñez-Macià 2005). Therefore, we analyzed possible growth and shape abnormalities in transgenic plants overexpressing yeast TPS–TPP enzyme. Representative *35S::TPS1-TPS2* line *8.5* and *rd29A::TPS1-TPS2* line *5.3* showed normal vegetative growth, organ shape and fertility, similarly to untransformed plants (Fig. 5). In contrast, the *35S::TPS1* plants (line *7.2*) grew slowly, had a smaller and dark-green rosette, and were sterile or produced a limited amount of seeds, after comparison to the wild type and *35S::TPS1-TPS2* and *rd29A::TPS1-TPS2* plants (Fig. 5). In fact, it was not possible to obtain homozygous lines, suggesting that gene-dosage hampered viability since the probably higher T6P levels in the *35S::TPS1* plants are interfering with normal plant development.

35S::TP1S-TPS2 lines tolerate several abiotic stresses

To assess whether the accumulation of trehalose in transgenic lines overexpressing *TPS1–TPS2* gene fusion correlated with improved stress tolerance, different stress treatments were applied on transgenic plants using ten 4-week-old individuals from several independent *35S::TPS1-TPS2* (*1.4, 6.4, 8.5, 12.9, 18.6, 20.8*) and *rd29A::TPS1-TPS2* (*2.2*, *3.7, 5.3, 6.6, 8.5, 9.1, 10.3, 19.2*) lines and wild type plants, grown in pots with soil. First of all, plants were subjected to drought tolerance tests consisting of depriving fully watered plants of water for 2 weeks. Most transgenic lines recovered from water deprivation after rewatering for 1 day, whereas wild type plants did not survive the same dehydration treatment (Fig. 6a). After rewatering, transgenic plants continued their normal growth. The survival rate of the different transgenic lines after drought stress was determined in 10 individual per line (Fig. 6d, e). The majority of the lines survived at significantly higher rate than wild type plants.

We tested freeze stress in *35S::TPS1–TPS2* and *rd29A::TPS1–TPS2* lines by subjecting transgenic plants to

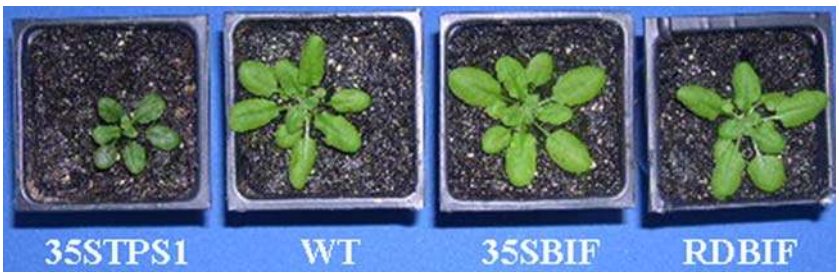

Fig. 5 Morphology of transgenic plants with different gene constructs. Four-week-old representative plants transformed with *35S::TPS1* (35STPS1), *35S::TPS1–TPS2* (35SBIF) or *rd29A::TPS1–TPS2* (RDBIF) construct and wild type (WT). All plants were homozygous lines (T_3 generation) except for the 35STPS1 plants (T_2 generation)

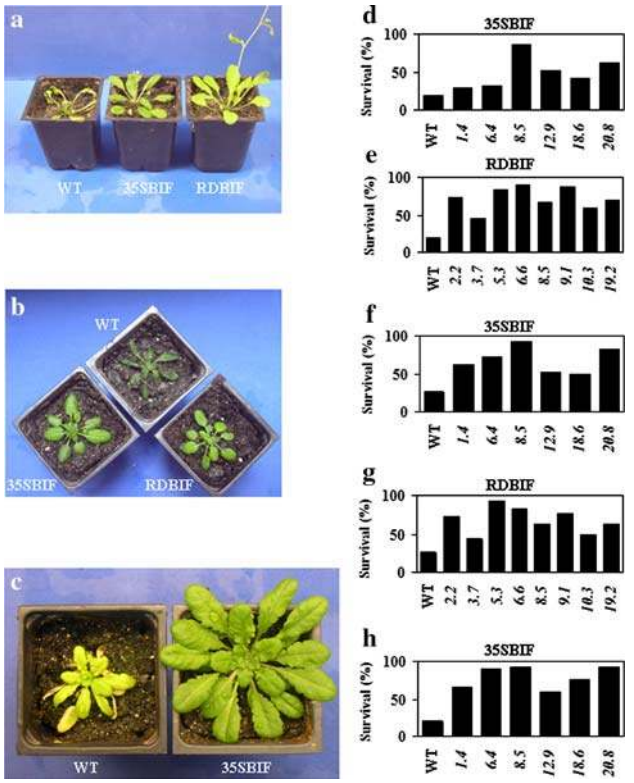

Fig. 6 **a**–**h** Tolerance of transgenic Arabidopsis to multiple abiotic stresses. Appearance of 4-week-old representative lines transformed with either *35S::TPS1–TPS2* (35SBIF, line *8.5*) or *rd29A::TPS1–TPS2* (RDBIF, line *5.3*) construct and wild type (WT), after drought-stress (**a**), freezing-stress (**b**) or heat-stress (**c**). Photograph was taken 2 weeks (recovery period) after treatment except for drought stress where it was taken 1 day after treatment. Survival rate of 35SBIF and RDBIF plants was determined in 10 individuals per transgenic line after drought-stress (**d**, **e**), freezing-stress (**f**, **g**) or heat-stress (**h**)

different freezing temperatures after pre-adaptation for 3 days at 4°C. We assayed several freezing temperatures and found that the transgenic plants could tolerate 2 days at −15°C without severe wilting or visible necrotic damage, recovering completely afterwards and continuing normal growth, whereas the wild type plants wilted within a few hours after stress application and did not revive at all after the 2-week recovery period (Fig. 6b). Survival of transgenic lines after freezing stress was higher than wild type plants (Fig. 6f, g).

The capacity to tolerate heat stress of transgenic lines expressing *TPS1–TPS2* gene fusion with the *35S* promoter was also assayed. Plants expressing *TPS1–TPS2* under the control of the *rd29A* promoter were not assayed because this promoter is not heat-inducible (Yamaguchi-Shinozaki and Shinozaki 1994). Several plants from each line were heat-shocked in a temperature range from 40 to 60°C, with increments of 4°C in each independent experiment until a temperature was found that transgenic but not wild type plants could tolerate, so as to maximize the phenotypic difference between them. The transgenic *35S::TPS1-TPS2* plants were able to withstand up to 56°C for 3 h, without prior heat pre-adaptation, compared to wild type plants which had a visible loss of greening and showed slow growth after a 2-week recovery period (Fig. 6c). There was a higher survival rate for transgenic plants after heat stress (Fig. 6h).

To determine whether transgenic lines accumulating trehalose were also salt-tolerant, plants transformed with either *35S::TPS1–TPS2* and *rd29A::TPS1–TPS2* constructs were subjected to increasing concentrations of NaCl. Although salt stress retarded growth in both transgenic and wild type plants compared with untreated individuals, it was notorious that plants overexpressing the *TPS1–TPS2* gene fusion could tolerate higher salt concentrations than their untransformed counterparts, as judged by the extent of growth retardation and loss of greening after 2 weeks of recovery (Fig. 7a). The *35S::TPS1–TPS2* plants could tolerate much better than *rd29A::TPS1–TPS2* plants, up to 350 mM NaCl, presumably because the former lines accumulate more trehalose. In fact, the *35S::TPS1–TPS2* representative line *8.5* contained 38.4 µg/g fresh weight, whereas *rd29A::TPS1–TPS2* representative line *5.3* had only 16.7 µg/g fresh weight of trehalose (Fig. 3). We also estimated the survival for the different transgenic lines subjected to salt stress. The different lines had a significantly higher survival rate than wild type plants in all the different NaCl concentration treatments (Fig. 7b, c).

Discussion

Trehalose is a small molecule that can be made by five different biosynthesis routes, is present in many different organisms and functions as an efficient protectant against different abiotic stresses (Elbein et al. 2003; Avonce et al. 2006). Heterologous expression of the *E. coli* or yeast *TPS1* gene in plants leads to pronounced abnormalities in growth, development and metabolism (Holmström et al. 1996; Goddijn et al. 1997; Romero et al. 1997; Yeo et al. 2000; Cortina and Culiáñez-Macià 2005). Overexpression of the Arabidopsis *AtTPS1* gene in its homologous background does not produce such drastic effects but still there is a delay in flowering (Avonce et al. 2004). The accumulation of higher levels of T6P are probably causing these defects since T6P has turned out to be a fine-tuned signaling molecule that has a regulatory role in carbohydrate utilization during plant growth, embryo development and flowering (Eastmond et al. 2002; Schluepmann et al. 2003, 2004; van Dijken et al. 2004). Therefore, it was decided to construct a bifunctional TPS–TPP enzyme, which while maintaining trehalose biosynthesis capacity would not lead to excess accumulation of free T6P in the cytosol avoiding undesired secondary effects in the

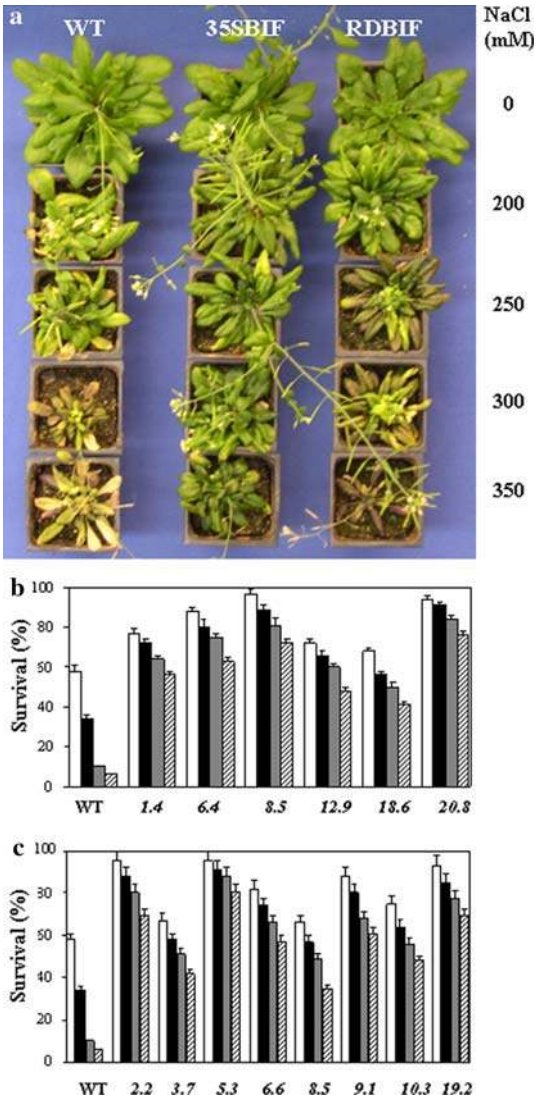

Fig. 7 **a–c** Salt tolerance of Arabidopsis plants. Effect of different concentrations of NaCl on plant growth, flowering and greening of 4-week-old representative lines (**a**) transformed with either *35S::TPS1–TPS2* (35SBIF, line *8.5*) or *rd29A::TPS1–TPS2* (RDBIF, line *5.3*) construct and wild type (WT). Survival rate of 35SBIF (**b**) and RDBIF (**c**) plants was determined in 10 individuals per transgenic line after salt-stress. *White* (200), *black* (250), *grey* (300) and *dashed* (350) *bars* indicate NaCl concentration in mM

transgenic plants. The TPS–TPP fusion was derived from an in-frame fusion of yeast *TPS1* and the 3′-end of the *TPS2* gene that corresponded to the phosphatase domain. This fusion protein yielded an active bifunctional TPS and TPP enzyme with trehalose biosynthetic capacity and restored stress tolerance when expressed in yeast. A similar construct has been reported for the fusion of the *E. coli otsA* and *otsB* genes encoding TPS and TPP, respectively, which has both enzyme activities and synthesizes trehalose when expressed in bacteria (Seo et al. 2000).

The yeast *TPS1–TPS2* gene fusion was used to transform *Arabidopsis thaliana* to test its potential to generate stress-tolerant plants. As previously reported (Holmström et al. 1996; Goddijn et al. 1997; Romero et al. 1997; Yeo et al. 2000; Cortina and Culiáñez-Macià 2005), plants transformed with *TPS1* alone showed slow growth, sterility and dark-green leaves. On the other hand, the expression of yeast *TPS1–TPS2* in Arabidopsis did not provoke growth or morphological alterations, even if the strong and constitutive *35S* promoter was used. Similarly, when the bifunctional *E. coli* TPS–TPP enzyme was overexpressed in plants it resulted in rice plants with a normal morphology and growth (Garg et al. 2002; Jang et al. 2003).

The role of trehalose as a stress protectant has been shown in different microorganisms by genetic analysis and by means of transgene expression in plants. For example, the overexpression of the *TPS1* gene in tobacco, potato and tomato improved drought tolerance (Holmström et al. 1996; Romero et al. 1997; Yeo et al. 2000; Cortina and Culiáñez-Macià 2005). Transgenic rice expressing the *E. coli* TPS-TPP were tolerant to drought, chilling and salt stress (Garg et al. 2002; Jang et al. 2003). Similarly, in the present study we showed that Arabidopsis plants overexpresing the *TPS1–TPS2* gene displayed improved tolerance to several abiotic stresses. Firstly, we showed that upon drought stress, plants displayed significant drought tolerance compared to the wild type.

Previous work have shown that accumulation of trehalose in transgenic rice allowed recovery of plants after 6 h of 4°C treatment (Jang et al. 2003) or 3 days at 10°C (Garg et al. 2002). Recently, it has been shown that a TPP coding gene from rice is induced by chilling-stress (Pramanik and Imai 2005). In yeast trehalose protects against freeze stress (Kim et al. 1996). We found that *35S::TPS1-TPS2* and *rd29A::TPS1–TPS2* lines could tolerate 2 days at −15°C, after pre-adaptation for 3 days at 4°C, without severe wilting or visible necrotic damage and showed complete recovery and continued growth.

Another harsh condition that plants suffer is heat stress. In yeast it is well known that trehalose is involved in heat tolerance (De Virgilio et al. 1994; Zentella et al. 1999). Here we found that transgenic lines expressing the *TPS1–TPS2* gene fusion had a better capacity to tolerate heat stress. They were able to withstand up to 56°C during 3 h, without prior heat pre-adaptation. As far as we know, this is the first report where trehalose in shown to protect against freeze and heat stress in plants.

Trehalose has been shown to protect against salinity in several organisms. *Saccharomyces cerevisiae* can grow in the presence of 0.9 M NaCl when a plant or yeast *TPS1* gene is overexpressed (Zentella et al. 1999). Rice plants can tolerate 100 mM NaCl after overexpressing an *E. coli* TPS–TPP gene fusion (Garg et al. 2002; Jang et al. 2003). We showed that plants expressing the yeast TPS–TPP bifunctional enzyme could tolerate significantly higher

concentrations of NaCl (200–350 mM). However, the *35S::TPS1–TPS2* plants could tolerate higher salt concentrations than *rd29A::TPS1–TPS2* plants, presumably because the former lines accumulate more trehalose.

It is well established that sugars are signaling molecules that modulate growth, development and stress responses (Rolland et al. 2006). It has been shown that T6P has a regulatory role in carbohydrate utilization during plant growth and development (Eastmond et al. 2002; Schluepmann et al. 2003, 2004; van Dijken et al. 2004). We have reported before that overexpression in Arabidopsis of its *AtTPS1* homolog leads to a glucose and abscisic-acid insensitive phenotype and to stress tolerance, suggesting that trehalose metabolism plays an important role in signaling gene regulation to integrate environmental and metabolic cues during vegetative development (Avonce et al. 2004). A maize *Ramosa3* mutant encoding a TPP enzyme has abnormal inflorescence branching but it was not yet determined whether T6P or trehalose is the signaling molecule (Satoh-Nagasawa et al. 2006). Recently, it was reported that exogenously applied trehalose enhanced *ABI4* transcription in *Arabidopsis*, affected starch metabolism and caused growth inhibition (Ramon, et al. 2007).

In this work we found that plants expressing the *35S::TPS1–TPS2* fusion construct also showed the glucose insensitive phenotype, although it was less pronounced than that of the plants overexpressing *AtTPS1*. This suggests a lower accumulation of T6P in the *35S::TPS1–TPS2* plants than in *35S::AtTPS1* plants, supporting the idea that this metabolite, rather than trehalose, is responsible for the glucose-insensitive phenotype. However, the expression of *TPS1–TPS2* from the *35S* promoter in *Arabidopsis* led to a higher accumulation of trehalose (1.5-fold increase) compared to plants overexpressing its *AtTPS1* homolog from the same promoter (Avonce et al. 2004). The latter plants are drought tolerant but do not tolerate salt stress (data not shown). Therefore, trehalose rather than T6P seems to support stress tolerance.

Our results clearly show that engineering trehalose metabolism in plants can substantially increase their capacity to withstand extreme environmental stresses, and they demonstrate that the *TPS1–TPS2* gene fusion is an excellent tool to produce such stress-tolerant plants without negative side-effects. The yeast TPS–TPP fusion protein thus represents a great potential for generating stress-tolerant crop plants for agriculture.

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