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ORIGINAL RESEARCH

Cloning and Characterization of Functional Trehalose-6-Phosphate Synthase Gene in Maize

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Abstract Trehalose is a non-reducing disaccharide of glucose that functions as a compatible solute in the stabilization of biological structures under heat and desiccation stress in bacteria, fungi, and some "resurrection plants". In the plant kingdom, trehalose is biosynthesized by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). Over-expression of exogenous and endogenous genes encoding TPS and TPP is reported to be effective for improving abiotic stress tolerance in tobacco, potato, tomato, rice, and *Arabidopsis*. On the basis of bioinformatics prediction, we cloned a fragment containing an open reading frame of 2,820 bp from maize, which encodes a protein of 939 amino acids. Phylogenetic analysis showed that this gene belongs to the class I subfamily of the *TPS* gene family. Analysis of conserved domains revealed the presence of a TPS domain and a TPP domain. Yeast complementation with *TPS* and *TPP* mutants demonstrated that this protein has the activity of trehalose-6-phosphate synthase. Semi-quantitative RT-PCR and real-time quantitative PCR indicated that the expression of this gene is upregulated in response to both salt and cold stress.

Keywords Maize · Trehalose-6-phosphate synthase · Salt stress · Cold stress

Introduction

Trehalose, an α-linked non-reducing disaccharide composed of two glucose moieties (α-D-glucopyranosyl-1, 1-α-D-glucopyranoside), is widely distributed in bacteria, fungi, invertebrates, and plants. In micro-organisms, trehalose serves as a protectant against heat and desiccation stress (van Laere 1989), through the stabilization of protein structures and biomembranes (Crowe et al. 1987; Hottiger et al. 1994). Trehalose is the major blood sugar in insects and serves as an energy storage molecule enabling flight (Becker et al. 1996). In the plant kingdom, trehalose was thought to exist only in some "resurrection plants" such as *Myrothamnus flabellifolia* and *Selaginella lepidophylla* (Adams et al. 1990; Müller et al. 1995a). In recent years, it has been found that higher vascular plants have actively transcribed genes encoding for the corresponding biosynthetic enzymes and accumulate trace levels of trehalose (Garcia et al. 1997; Blázquez et al. 1998; Garg et al. 2002; Chary et al. 2008). Trehalose synthesized only in the TPS/TPP pathway in plants, (Avonce et al. 2006). The first step of this pathway is the linkage of uridine diphosphoglucose and glucose 6-phosphate by trehalose-6-phosphate synthase (TPS) to form trehalose 6-phosphate (T6P). Then, the phosphate group is removed by trehalose-6-phosphate phosphatase (TPP), resulting in trehalose.

Exogenous and endogenous trehalose synthesis genes have been used to transform tobacco, potato, tomato, *Arabidopsis*, and rice to take advantage of the protective role played by trehalose against abiotic stress (Holmström et al. 1996; Goddijn et al. 1997; Romero et al. 1997; Yeo et al. 2000; Garg et al. 2002; Jang et al. 2003; Avonce et al. 2004; Cortina and Culiáñez-Macià 2005; Karim et al. 2007; Miranda et al. 2007; Ge et al. 2008). The abiotic stress tolerance of these transgenic plants was improved dramatically. However, in some of these transgenic attempts, the expression of exogenous trehalose synthesis genes caused physiological disorder, resulting in undesired side effects such as stunt growth, dark-green, and lanceolate leaf

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(Holmström et al. 1996; Goddijn et al. 1997; Romero et al. 1997; Yeo et al. 2000; Cortina and Culiáñez-Macià 2005).

Maize is one of the most important crops worldwide. Sources of abiotic stress, such as drought, excessive salinity, and temperature extremes are serious threats to its successful production. Traditional breeding for the improvement of abiotic stress tolerances, which are usually complex traits that are influenced by coordinated and differential expression of gene networks, is particularly challenging. The successful bioengineering of trehalose synthesis genes encouraged us to transform and overexpress endogenous trehalose synthesis genes in maize. On the other hand, developmental and morphological disorders were found in the mutant of the gene relative to trehalose metabolism in maize (Satoh-Nagasawa et al. 2006). Therefore, it is necessary to explore the function and expression of endogenous genes relative to trehalose metabolism in maize.

Materials and Methods

Plant materials, Growth Conditions, Stress Treatments, and First Strand Synthesis

Seeds of the maize inbred line 18-599 were sterilized and germinated in vermiculite. Seedlings at the two-leaf stage were transplanted into a plastic mesh grid for aquaculture and grown hydroponically at 28°C with 12 h light/12 h dark (illumination of 20,000 lux) and a relative humidity of 60–80% with modest aeration. The nutrient solution was replaced every 3 days. At the three-leaf stage, identical seedlings were subjected to salt stress or cold stress. For the salt stress, NaCl was added to the nutrient solution to a final concentration of 150 mmol/L, and for the cold stress, the nutrient solution was chilled to 12°C; all other conditions were kept constant. At time zero (control) and at 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h after the stress treatment, leaves and roots were sampled separately from three seedlings and frozen immediately in liquid nitrogen. RNA was isolated using TRIzol® reagent (Invitrogen, USA) and reverse-transcribed with a PrimeScript™ RT reagent kit (TakaRa, China) according to the manufacturer's instructions.

Database Searches, Data Acquisition

The maize protein-coding_genes database and genomic survey sequence bac_contigs databases were downloaded from http://ftp.maize-sequence.org. *Arabidopsis* AtTPS1 (NP_177979.1) was used as the query sequence in a BLAST search against maize deduced protein sequences in the protein-coding_genes database using the TBLASTN program. The object sequence with the highest level of homology with AtTPS1 was used as the query in a BLAST search of the bac_contigs.fasta database. The completely matched sequence together with its sequences within 5,000 bp upstream and downstream, a total of 15,000 bp, was used to analyze the gene structure using GeneFinder software (http://linux1.softberry.com).

Open Reading Frame Cloning

Primers were designed on the basis of the predicted maize *TPS* gene using primer 5.0. To facilitate plasmid construction in the following steps, the XhoI (CTCGAG) and SphI (GCATGC) recognition sites were introduced into the forward primer: 5′TCCCTCGAGCACCGCTCGCGTCCGCCTAAT3′ and into the reverse primer 5′GGATCCGGGTGTAGCTCTGTCGCGCATAC3′, respectively. PCR amplification was done using LA Taq polymerase (TakaRa, China) with proofreading activity. The temperature cycle was: 20 s at 95°C, 20 s at 60°C, and 3 min at 72°C for six cycles; 20 s at 95°C, and 3 min at 68°C for 30 cycles; and 5 min at 68°C. The amplified product was purified using a DNA purification kit (Tiangen, China), cloned into the pMD18-T vector (TakaRa, China) and sequenced by Invitrogen Biotechnology Co. Ltd (China). The sequence was checked for open reading frames (ORFs) by the ORF finder program available at http://www.ncbi.nl-m.nih.gov.

Phylogenetic Analysis and Conserved Domain Prediction

Multiple sequence alignment was done with the deduced protein sequence and the deposited functional TPS protein sequences of four other species in the NCBI protein database, using CLUSTAL X software (Thompson et al. 1997). Phylogenetic analysis of these sequences was done with the MEGA4.0 program (Tamura et al. 2007). The conserved domains of the deduced protein were analyzed using the NCBI on-line server at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Plasmid Construction and Yeast Complementation Assay

Plasmid pMD-18T containing the cloned fragment was digested by restriction endonucleases XhoI and SphI and inserted into plasmid pRS6 (Zentella et al. 1999) to form the yeast expression vector. This was used to transform the yeast *TPS1* deletion mutant (*tps1*Δ) strain YSH290 and the *TPS2* deletion mutant (*tps2*Δ) strain YSH450 (Hohmann et al. 1993; Neves et al. 1995). In yeast, *TPS2* encoding for a functional TPP protein and transformation was done as described (Elble, 1992). The transformants were screened on minimal medium without histidine plus 2% (*w*/*v*) galactose (Gal). For each transformed mutant strain, eight independent transformants were selected to test their ability to restore the growth defect on minimal medium without

histidine plus 2% (*w*/*v*) glucose (Glc). For the TPS complementation assay, the transformant of the *TPS1* deletion mutant, the control W3O3-1A, and the *TPS1* deletion mutant were streaked on agar plates made with minimal medium supplemented with 2% Gal or 2% Glc and incubated at 30°C. For the TPP complementation assay, the transformant of the *TPS2* deletion mutant, the untransformed *TPS2* deletion mutant, and the wild-type strain W3O3-1A were streaked on minimal medium plus 2% Glc and incubated at 38°C.

Semi-quantitative RT-PCR

For semi-quantitative reverse-transcribed PCR (RT-PCR), a pair of primers (5′GGTTGCAGCGTTTCCTATTG3′/5′ AATCAAGAGATC-GGTCCAGATG3′) was designed to amplify a 368-bp fragment of maize *TPS1* gene. A 250-bp fragment of the *18S* ribosomal RNA gene (5′CTGA GAAACGGCTACCACA3′/5′CCCAAGGTCCAACTAC GAG 3′) was used as endogenous control for template standardization. After optimization of the parameters used for exponential amplification, the temperature cycle was designed as 32 cycles for *TPS1* and 23 cycles for the *18S* ribosomal RNA gene. In each cycle, the temperature protocol was 10 s at 95°C, 20 s at 56°C, and 20 s at 72°C. RT-PCR amplification was done at least three times. The *18S* primers were used for the genomic DNA contamination assay. The RNA samples for each treatment were treated with RNase A and used as templates, and the temperature protocol was one cycle of 5 min at 95°C, 23 cycles of 10 s at 95°C, 20 s at 56°C, and 20 s at 72°C.

Real-Time Quantitative PCR

Primers of *TPS1* and *18S* used for semi-quantitative RT-PCR were used in real-time quantitative PCR analysis.*18S* ribosomal RNA gene and another 358-bp fragment of *GAPDH* gene (5′ACTTCGGCATTGTTGAGG3′/5′AAGT-CGGTAGAAACCAGAT3′) were used as endogenous controls to normalize the input RNA and efficiency of reverse transcription between the samples. Real-time quantitative PCR were performed on iQ™ 5 thermal cycler (Bio-Rad, USA) using SYBR® Premix Ex Taq™ ‖ reagent kit (TakaRa, China) according to the manufacturer's instruction. The temperature protocol was one cycle of 20 s at 95°C, 40cycles of 10 s at 95°C, 20 s at 56°C, and 20 s at 72°C, fluorescence was detected at 72°C. All products were subjected to the melting curve analysis between 55°C and 95°C to determine the specificity of the PCR reaction. Experiments included a non-template control. All reactions were performed on three independently reverse-transcribed series of RNA samples. All figures show one series, with the error bars based on one technical repeat.

Results and Discussion

Cloning of Maize *TPS1* Gene

In a search of the protein-coding genes database, a protein sequence deduced from the maize encoding gene AC187243.3_FG026 was found to have the highest level of homology (68%) to *Arabidopsis* AtTPS1. With the specific primers designed on the basis of the identified encoding gene sequence, a fragment of 3,025 bp was amplified from the cDNA sample of maize inbred line 18-599, which contains a 2,820 bp ORF from positions 111 to 2930 in this fragment. This ORF sequence was registered at GenBank with accession number EU659122.2 and named *ZmTPS1*. The amplified fragment was inserted into the yeast expression vector pRS6 to construct *ZmTPS1*-pRS6, which was used to transform *TPS1* mutant (*tps1*Δ) strain YSH290 and *TPS2* mutant (*tps2*Δ) strain YSH450 of yeast. The *tps1*Δ mutant is unable to grow on medium containing Glc as the sole carbon source because of its inability to regulate the flow of Glc into glycolysis (Van Aelst et al. 1993; Thevelein and Hohmann. 1995), the *tps2*Δ mutant is sensitive to a temperature of 38°C, probably for the accumulation of T6P (De Virgilio et al. 1993). After transformation, the *tps1*Δ mutant is able to grow with Glc as the sole carbon source (Fig. 1); however, the *tps2*Δ mutant is still unable to grow at 38°C (Fig. 2). These results

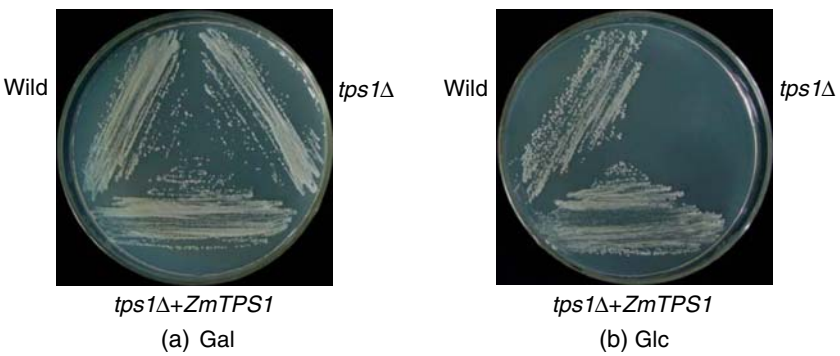


Fig. 1 Complementation of the *tps1*△ mutant by *ZmTPS1*. Yeast *TPS1* mutant strain transformed by gene *ZmTPS1*, the untransformed control and the wild strain were spread on minimal medium plate plus 2% Gal (**a**) or 2% Glc (**b**)

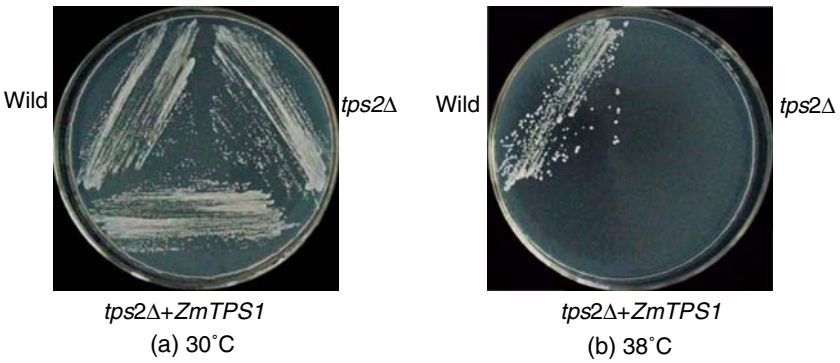


Fig. 2 *ZmTPS1* did not rescue the heat-sensitive phenotype of yeast *tps2* deletion mutation. Yeast *TPS2* mutant strain transformed by gene *ZmTPS1*, the untransformed control and the wild strain were spread on minimal medium plate plus 2% Glc and grown at 30°C (**a**) or 38°C (**b**)

indicated that *ZmTPS1* encodes a protein that functions as TPS but has no TPP activity. A maize gene (*RA*3) encoding for the active TPP protein has been identified (Satoh-Nagasawa et al. 2006). The cloning of gene *ZmTPS1* confirms that maize has the ability to synthesize trehalose.

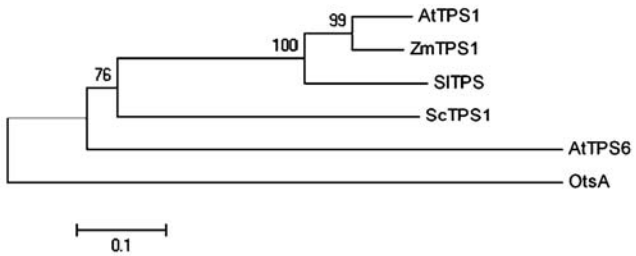
Deduced Amino Acid Sequence and Phylogenetic Relationship of Maize TPS1

The polypeptide encoded by maize *TPS1* is 939 amino acids long. Multiple sequence alignment showed that maize TPS1 has an identity of 74% with *Arabidopsis thaliana* functional protein TPS1 (NP_177979.1), 35% with *A. thaliana* functional protein TPS6 (NP_974105.1), 65% with *S. lepidophylla* (AAD00829.1), 50% with *Saccharomyces cerevisiae* (CAA48296.1), and 34% with *Escherichia coli* (1GZ5_A). Plant *TPS* genes can be grouped into two subfamilies, depending on whether they display most similarity to yeast *TPS1* or *TPS2*. In *Arabidopsis*, *AtTPS1–4* belong to the class I subfamily and *AtTPS5–11* belong to the class II subfamily. *Zm*TPS1 has a close phylogenetic relationship with AtTPS1 (Fig. 3a) and belongs to the class I subfamily. Most of the highly conserved residues involved in substrate binding and catalysis of trehalose 6-phosphate synthesis were found in the invariance center: such as Arg9, Trp40, Tyr76, Trp85, and Arg300 for the binding of glucose 6-phosphate and Gly22, Asp130, His154, Arg262, Asp361, and Glu369 for the binding of glucose (Fig. 3b; Gibson et al. 2002). All of the known plant functional TPS proteins are conserved in these residues, except AtTPS6, which varies at Arg9, Gly22, Trp40, and Arg262 and belongs to the class II subfamily. An analysis of conserved domains revealed a TPS domain at residues 93–558 and a possible TPP domain at residues 598–822 in the maize TPS1 protein. Nevertheless, transformation of the yeast mutants proved maize TPS1 had only TPS activity. Like *SlTPS1*and other *TPS* genes that belong to the class I subfamily, *ZmTPS1* has no TPP catalytic activity because of the lack of phosphatase boxes (Zentella et al. 1999; Leyman et al. 2001). There are two conserved regions in the amino acid sequence (TPS and TPP) of all plant TPS proteins, whereas the two enzymes are separate entities in *E. coli.* In yeast, possible fusion of *TPS* and *TPP* genes have been found in *TPS*2, *TPS*3, and *TSL*1, which are components of the trehalose-6-phosphate synthase/phosphatase complex. It is possible that fusion of plant *TPS* genes has occurred. The maize polypeptide TPS1 has a 92 residue N-terminal extension compared with bacterial and fungal TPS proteins. Actually, all plant TPS proteins contain a specific N-terminal extension not found in bacterial or fungal TPS proteins. Truncation of the N-terminal extension of *Arabidopsis* and *S. lepidophylla* trehalose-6-phosphate synthase AtTPS1 and SlTPS1 unlocks their high-level catalytic activity (Van Dijck et al. 2002). The specific N-terminal extension was proved to interact with KCA1, which is closely related to mitosis (Geelen et al. 2007). This suggests that trehalose metabolism impinges on regulation of the cell cycle.

Expression of the Maize *TPS1* Gene

Under salt stress, the expression of *ZmTPS1* gene in leaf was upregulated at 0.5 h and decreased to normal at 2 h after salt stress and then, was upregulated again at 48 h; the expression in root was upregulated at 1 h, decreased at 2 h, and then was downregulated from 4 to 12 h. Under cold stress, the expression in leaf was constantly upregulated, and value peaked at 0.5 h after stress; in root, the expression was also upregulated and value peaked at 0.5 h after stress *t*, however, decreased to normal at 1 h, and then was upregulated from 4 to 12 h, finally down-regulated at 48 h (Figs. 4 and 5). These results suggest that maize *TPS1* participates in the response to both salt and cold stress. The originally upregulated expression of maize *TPS1* is likely to promote the content of T6P that induces expression of the stress signal transduction-related genes. Over-expression of *AtTPS1* in *Arabidopsis* caused the transgenic plant insensitive to exogenous ABA, suggesting an interaction between *AtTPS1* gene expression and ABA metabolism, further supporting its possible role as a second messenger (Avonce et al. 2004).In *Arabidopsis*, a correlation between the level of T6P and the induction of several

a



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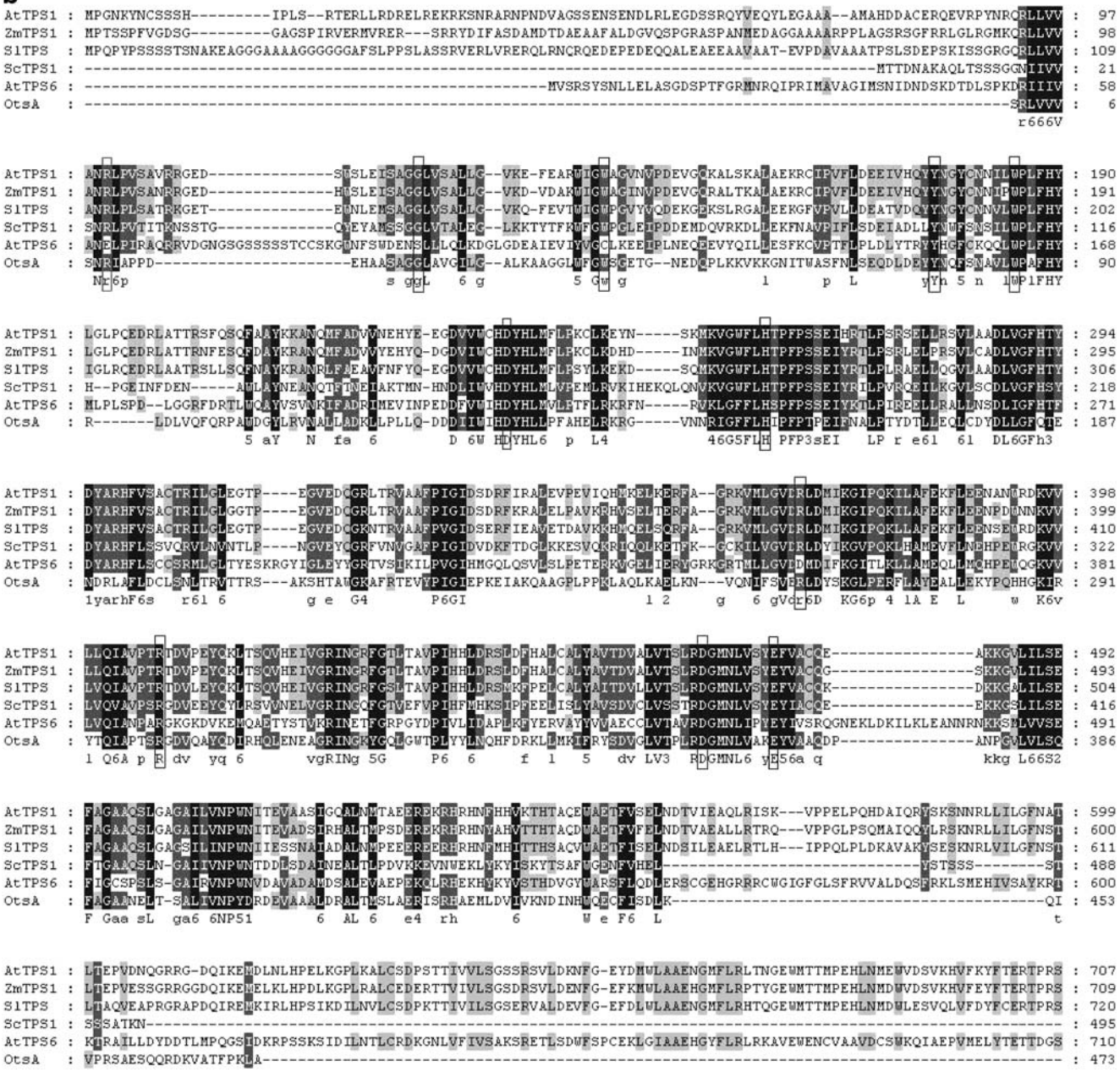

Fig. 3 **a** Neighbor-joining tree among the deduced protein and functional TPS protein sequences of four other species. A bootstrap analysis (1,000 replicates) was performed. OtsA of *E. coli* was used as an out group. **b** Multiple alignment of TPS domain among deduced the amino acid sequences of protein ZmTPS1 and deposited functional TPS proteins of four other species. Gaps that were introduced to optimize the alignment are indicated by *dashes*; identical residues are *shaded*. The *framed* amino acids are conserved residues involved in substrate binding and catalysis

Fig. 4 Semi-quantitative RT-PCR analysis of *ZmTPS1* under salt and cold stresses 20 μl reaction volume for each sample, 6 μl RT-PCR product for each sample was separated on a 2% (*w*/*v*) agarose gel. Possible genomic DNA contamination did not affect the template standardization (data not shown)

genes known to be involved in plant response to abiotic stress had been revealed by microarray data (Schluepmann et al. 2004). T6P is indispensable for carbohydrate utilization in *Arabidopsis*; its level determines the capacity to use the sugar supplied and determines the accumulation of respiratory intermediates in seedlings (Schluepmann et al. 2003). Immunogold localization of TPS protein in leaf segments of wild-type and transgenic tobacco plants expressing the *AtTPS1* gene revealed the presence of TPS protein in the vacuoles and in the cell wall and, to a lesser extent, in the cytosol. This confirms the important role of TPS in sugar metabolism and within the plant, which could explain its role in plant stress tolerance (Almeida et al. 2007). The re-upregulation of maize *TPS1* appears to lead to an accumulation of trehalose and to maintain the expression of genes that produce osmoprotectants because the content of trehalose is too low to serve as a protectant (Garg et al. 2002; Schluepmann et al. 2004; Ge et al. 2008). Rice seedlings treated with 1% (*w*/*v*) NaCl accumulated trehalose at a rate of 7 μg per 100 mg of fresh weight at the third day, but were undetectable on the second day (Garcia et al. 1997). Earlier reports indicated that *Arabidopsis*, and many other higher plants, accumulate trehalose at only trace levels (Blázquez et al. 1998; Garg et al. 2002; Chary et al. 2008). This is probably due to the low-level activity of synthesis enzymes and relatively high level of trehalase activity (hydrolytic enzyme) (Vogel et al. 1998; van Dijck et al. 2002). The accumulation of trehalose was increased dramatically in soybean, cowpea, tobacco, and *Arabidopsis* after trehalase activity was inhibited by validamycin A (Müller et al. 1995b; Goddijn et al. 1997; Müller et al. 2001). Over-expression of exogenous or endogenous TPS and TPP encoding genes in transgenic plants led to an increase of abiotic tolerance, but the trehalose content was still very low (Garg et al. 2002; Jang et al. 2003; Avonce et al. 2004; Karim et al. 2007; Miranda et al. 2007). We suggest that the major role of trehalose in higher plants is not osmotic protection, but signal transduction. Therefore, the mechanism of signal transduction involving trehalose in higher plants needs to be explored.

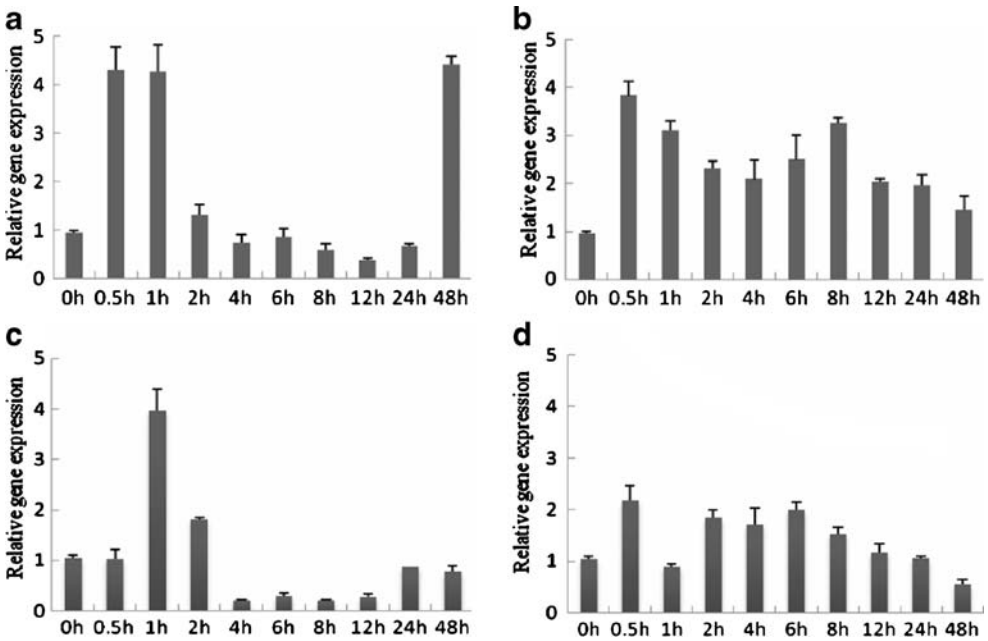

Fig. 5 Real-time quantitative RT-PCR analysis of *ZmTPS1* under salt and cold stresses. Expression data were normalized using *18S* rRNA and *GAPDH* as endogenous controls. **a** Relative expression in leaf under salt stress, **b** relative expression in leaf under cold stress, **c** relative expression in root under salt stress, **d** relative expression in root under cold stress

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