Planta
DOI 10.1007/s00425-011-1458-0

ORIGINAL ARTICLE

Overexpression of the trehalose-6-phosphate synthase gene *OsTPS1* enhances abiotic stress tolerance in rice

Hao-Wen Li · Bai-Sheng Zang · Xing-Wang Deng · Xi-Ping Wang

Received: 29 March 2011 / Accepted: 6 June 2011

Abstract Trehalose plays an important role in metabolic regulation and abiotic stress tolerance in a variety of organisms. In plants, its biosynthesis is catalyzed by two key enzymes: trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). The genome of rice (*Oryza sativa*) contains 11 *OsTPS* genes, and only OsTPS1 shows TPS activity. To demonstrate the physiological function of OsTPS1, we introduced it into rice and found that *OsTPS1* overexpression improved the tolerance of rice seedling to cold, high salinity and drought treatments without other significant phenotypic changes. In transgenic lines overexpressing *OsTPS1*, trehalose and proline concentrations were higher than in the wild type and some stress-related genes were up-regulated, including *WSI18*, *RAB16C*, *HSP70,* and *ELIP*. These results demonstrate that OsTPS1 may enhance the abiotic stress tolerance of plants by increasing the amount of trehalose and proline, and regulating the expression of stress-related genes. Furthermore, we found that overexpression of some Class II *TPS*s also enhanced plant tolerance of abiotic stress. This work will help to clarify the role of trehalose metabolism in abiotic stress response in higher plants.

Keywords Trehalose · Trehalose-6-phosphate synthase · Rice · Abiotic stress

Abbreviations

ABA	Abscisic acid
CaMV	Cauliflower mosaic virus
FW	Fresh weight
HPIC	High-performance ion chromatography
RT-PCR	Reverse transcription polymerase chain reaction
T-6-P	Trehalose-6-phosphate
TPP	Trehalose-6-phosphate phosphatase
TPS	Trehalose-6-phosphate synthase
WT	Wild type

Introduction

Trehalose, a non-reducing disaccharide sugar, is present in a wide variety of organisms, including bacteria, fungi, plants and invertebrates (Elbein et al. 2003). There are five known trehalose biosynthetic pathways. The most widespread is the trehalose OtsA–OtsB pathway which is found in all the prokaryotic and eukaryotic organisms and is the only trehalose biosynthetic pathway found in plants (Paul et al. 2008; Iturriaga et al. 2009). This pathway begins as trehalose-6-phosphate synthase (TPS) catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate (G-6-P) to form trehalose-6-phosphate (T-6-P) and uridine diphosphate (UDP). Subsequently, the T-6-P is dephosphorylated into trehalose by trehalose-6-phosphate phosphatase (TPP) (Cabib and Leloir 1958; Goddijn and Van Dun 1999).

Electronic supplementary material The online version of this article (doi:10.1007/s00425-011-1458-0) contains supplementary material, which is available to authorized users.

H.-W. Li · X.-P. Wang (✉)
Key Laboratory of Gene Engineering Drug and Biotechnology, Key Laboratory of Cell Proliferation and Regulation of Ministry of Education, College of Life Sciences, Beijing Normal University, Beijing 100875, People's Republic of China
e-mail: xipingwang@bnu.edu.cn

B.-S. Zang · X.-W. Deng
Peking-Yale Joint Center of Plant Molecular Genetics and Agrobiotechnology, College of Life Sciences, Peking University, Beijing 100871, People's Republic of China

Published online: 24 June 2011

Trehalose was first identified from the ergot fungus of rye in 1832 (Almeida et al. 2007; Elbein et al. 2003). In plants, for many years trehalose was thought to be limited to the resurrection plants, such as *Selaginella lepidophylla* and *Myrothamnus flabellifolia* (Bianchi et al. 1993; Anselmino and Gilg 1913). In these plants, the concentration of trehalose can reach 10 mg (g FW)$^{-1}$, which is a detectable level (Fernandez et al. 2010). Nevertheless, the lower levels of trehalose in other higher plants render it nearly undetectable.

In 1998, several trehalose biosynthesis genes encoding TPS (Blázquez et al. 1998) and TPP (Vogel et al. 1998) were identified in *Arabidopsis thaliana*. Recently, additional TPS and TPP homologues have been detected in variety of plant species (Pramanik and Imai 2005; Zentella et al. 1999; Kosmas et al. 2006; Wang et al. 2005; Shima et al. 2007). Currently, researchers have isolated 11 *TPS* genes from both *A. thaliana* (Leyman et al. 2001) and rice (Zang et al. 2011). The TPS proteins have been classified into two distinct groups based on their homology with the yeast TPS1. However, only TPS1 (belongs to Class I) has demonstrated TPS activity (Vandesteene et al. 2010; Zang et al. 2011). The role of Class II TPS proteins which do not have TPS activity is still ambiguous.

In yeast and microorganisms, trehalose serves not only as a carbohydrate storage molecule but also as a metabolic regulator and protective agent against the various abiotic stresses (Van Laere 1989; Wiemken 1990). In plants, the role of trehalose is not yet thoroughly understood. In recent years, studies have confirmed that the expression of trehalose biosynthesis genes confers abiotic stress resistance in several species. Transgenic introduction of the yeast *TPS1* into tomato resulted in higher chlorophyll and starch content, and enhanced tolerance against drought, salt and oxidative stress (Cortina and Culiáñez-Macià 2005). Rice plants overexpressing *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB*) as a fusion gene exhibited less photo-oxidative damage and a more favorable mineral balance under salt, drought, and low-temperature stress conditions (Garg et al. 2002). In tobacco, heterologous expression of *AtTPS1* gene from *Arabidopsis* increased tolerance to several abiotic stresses, such as drought, desiccation, and temperature stresses (Almeida et al. 2005). However, trehalose gene overexpression can produce aberrations in plant growth, such as dwarfism, delayed flowering, abnormal root development, and lancet-shaped leaves (Avonce et al. 2004; Romero et al. 1997; Cortina and Culiáñez-Macià 2005).

Removal of the N-terminal extension region just before the TPS domain has been shown to increase TPS activity compared to the full length protein. Overexpression of truncated AtTPS1 (deletion of 86 aa of the N-terminal) and SlTPS1 (deletion of 99 aa of the N-terminal) in yeast resulted in higher TPS activity and more trehalose accumulation. This result indicates that plant TPS enzymes possess a high potential catalytic activity and the N-terminal region may act as an inhibitory domain which modulates TPS activity (Van Dijck et al. 2002).

In this study, we obtained transgenic rice overexpressing the N-terminal truncated *OsTPS1* and some full-length Class II *OsTPS* genes, and reported the effect of OsTPSs on abiotic stress tolerance. In addition, we discuss the possible mechanisms of OsTPS1-conferred stress tolerance.

Materials and methods

Vector construction and rice transformation

We previously isolated *OsTPS1*, *OsTPS2*, *OsTPS4*, *OsTPS5*, *OsTPS8*, and *OsTPS9* from the *indica* rice variety 'Guangluai 4' (Zang et al. 2011) using RT-PCR and inserted the genes into the pGEM®-T vector (Promega, Madison, WI, USA). An *OsTPS1*(*131-985*) fragment and full length genes for *OsTPS2*, *OsTPS4*, *OsTPS5*, *OsTPS8* and *OsTPS9* were cloned into the pENTR™ D-TOPO® cloning vector (Invitrogen, Carlsbad, CA, USA) and recombined into the pHAC vector following the LR recombination reaction. The pHAC vector is a modified pH2GW7 vector (Karimi et al. 2002) which contains the rice *Actin1* promoter instead of the *CaMV 35S* promoter (Zang et al. 2011).

The complete constructs were introduced into *Oryza sativa* L. ssp. *Japonica* cv. ZH11 or Nipponbare using the *Agrobacterium*-mediated co-cultivation method. Seeds sterilization, callus induction, co-cultivation with *Agrobacterium* AGL0, transformed calli selection and regeneration were carried out following established protocols (Hiei et al. 1994).

Yeast complementation assay

The yeast (*Saccharomyces cerevisiae*) wild-type strain W303-1A (*MATα ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 can1–100 GAL mal SUC2*) and the *tps1* mutant strain YSH6.127.-17C (*MATα tps1Δ::TRP1*) (Bell et al. 1998) were used for complementation assays.

The full-length *OsTPS1* gene and the N-terminal truncated *OsTPS1* gene were digested from the pENTR™ D-TOPO® vector and inserted into the pGADT7 vector. As a TPS positive control, the *ScTPS1* gene was isolated from yeast genomic DNA by PCR using the following primers: 5′-CACCCATATGACTACGGATAACGCTAA-3′ (forward) and 5′-TCGAGGATCCGTTTTTGGTGGCAGAGGA-3′ (reverse). The reactions were performed at 94°C for 5 min; followed by 28 cycles of 94°C for 40 s, 58°C for

40 s, and 72°C for 90 s; plus an elongation step at 72°C for 10 min.

Yeast transformation was performed following the Yeast Protocols Handbook (Clontech, Mountain View, CA, USA). For drop assays, cultures of the transformed *tps1* and wild-type (WT) control strains were grown at 30°C on synthetic medium without leucine and tryptophan (SD-Leu–Trp) containing 2% galactose, and drop assays were performed on synthetic medium without leucine and tryptophan with 2% galactose (control) or 2% glucose. Growth was analyzed after 2–3 days at 30°C.

Southern blot

Genomic DNA was isolated from mature leaves of rice using the CTAB DNA extraction method (Jeong et al. 2007). 5 µg of total DNA was digested overnight with *Hin*dIII. The digested product was separated through 0.8% agarose gel and transferred onto a Hybond-N+ membrane (Amersham Pharmacia, Piscataway, NJ, USA). The blot was hybridized with the DIG-labeled 615-bp hygromycin probe and exposed to X-ray film for signal detection. The hygromycin probe was obtained by PCR using the following primers: 5′-GCTGTTATGCGGCCATTGTC-3′ (forward) and 5′-GACGTCTGTCGAGAAGTTTC-3′ (reverse). PCR program conditions were as follows: 3 min at 94°C; 28 cycles of 30 s at 94°C, 30 s at 50°C, and 40 s at 72°C; and followed by 7 min at 72°C. DNA probe preparation, hybridization and membrane washing were performed using DIG High Prime DNA Labeling and Detection Starter Kit II according to the manufacturer's instructions (Roche, Grenzacherstrasse, Basel, Switzerland).

Stress tolerance experiments

Three independent T3 transgenic lines and a non-transformed line were used for all the stress tolerance experiments. Before stress treatments, seedlings were grown hydroponically in IRRI nutrient solution (International Rice Research Institute protocol, http://irri.org/) in a growth chamber for 2 weeks at 28°C with 16 h-light/8 h-dark cycles. For the salinity and PEG tolerance tests, 2-week-old seedlings were treated with nutrient solutions containing 150 mM NaCl or 20% PEG 6000 for 3 days and then transferred to fresh nutrient solution. For the cold tolerance test, 2-week-old seedlings were exposed to 4°C for 5 days and then allowed to resume growth at 28°C. For the acute drought tolerance test, whole plants were air dried for 4–5 h followed by re-watering. The survival rates were recorded after plants resumed growth under normal conditions for 7 days. About 120 seedlings of each line were used in each experiment, and these experiments were replicated at least three times. Tests for statistical differences between transgenic lines and the WT control were performed using the SAS program (http://www.sas.com/).

Sample preparation, RNA extraction and gene expression analysis

RT-PCR analysis was used to determine the expression profile of *OsTPS1*. For the stress-induced assay, 2-week-old rice seedlings were treated with 150 mM NaCl, 20% PEG 6000 or incubated at 4°C under continuous light and then collected at different time points (0, 1, 2, 4, 6, 12 and 24 h). Subsequently, samples were frozen in liquid nitrogen immediately after each treatment. For organ-specific assays, samples were harvested from the following tissues: root, stem, leaf, spikelets (during flowering, after fertilization, and milk grain stage), seed and germinating seed. Total RNA was extracted using the TRIZOL Reagent (TaKaRa, Otsu, Shiga, Japan) and treated with RQ1 RNase free DNase (Promega). 2 µg total RNA was reverse transcribed using the Revert Aid First-Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). The RT-PCR was carried out using the following *OsTPS1* gene-specific primers: 5′-TTGAAGTTCGGTCTGTCG-3′ (forward) and 5′-CTGCCTATCCAAGAACATG-3′ (reverse). Templates were normalized using the following *Actin1* primers: 5′-CGTCCTCTCTCTGTATGCC-3′ (forward) and 5′-TCAGCAATGCCAGGGAAC-3′ (reverse). Thermo-cycling conditions were as follows: one step at 94°C for 3 min; followed by 28 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; plus a final extension at 72°C for 7 min. The RT-PCR assay was replicated at least three times.

To assay the expression of stress-related genes in *OsTPS1* overexpression lines, total RNA was isolated from the shoots of 2-week-old transgenic seedlings. Total RNA extraction and reverse transcription were performed as described above. The first strand cDNA was used as the template for RT-PCR or Realtime PCR. The gene-specific primers for RT-PCR and Realtime PCR are shown in Online Resources 1 and 2, respectively. Realtime PCR to assay stress-related gene expression was performed on ABI7500FAST (ABI, Carlsbad, CA, USA) using SYBR Premix Ex Taq II (TaKaRa) according to the manufacturer's instructions. PCR amplifications were performed under the following conditions: 95°C for 30 s, followed by 40 cycles of amplification (95°C for 5 s, 60°C for 30 s, 72°C for 30 s). The experiments were replicated at least three times.

Trehalose extraction and quantification

Trehalose was extracted and quantified as described by Lunn et al. (2006). This method is for the extraction of soluble carbohydrates, not only T6P but also trehalose. Two

independent T3 transgenic lines and a wild type were used in the experiment. 2-week-old seedlings before and after cold stress (4 days at 4°C) were snap-frozen and ground in liquid nitrogen. The sample (0.1 g fresh weight) was dispersed in 1 mL of chloroform/acetonitrile (3:7, v/v) and shaken at −20°C for 2 h. Soluble carbohydrates, including trehalose, were extracted twice from the organic phase with 200 µL of water at 4°C by vigorous shaking for 5 min, and followed by 5 min of centrifugation at 12,000 rpm. The aqueous supernatants were pooled and then evaporated to dryness using a centrifugal vacuum dryer. The dried samples were reconstituted in 1 mL of water and filtered with a 0.45 µm aperture filter. Trehalose was quantified using the ICS3000 HPIC system (Dionex, Sunnyvale, CA, USA) with a Carbo-Pak PA10 column (4 × 250 mm) following the manufacturer's instructions. Carbohydrates were eluted at a flow rate of 0.8 mL/min with 100 mM NaOH for 30 min and monitored with an ED40 electrochemical detector (Dionex DC Amperometry).

Proline determination

Proline content was estimated using a color comparison method (Bates et al. 1973). Two-week-old seedlings of two independent T3 transgenic lines and a wild type with and without cold stress (4 days at 4°C) were used in the experiment. 0.5 g of plant material was homogenized in 5 mL of 3% aqueous sulfosalicylic acid. The homogenate was placed in a boiling water bath for 10 min and centrifuged at 12,000 rpm. The supernatant was used for the estimation of proline content. The reaction mixture consists of 2 mL proline extraction solution, 2 mL of 2.5% acid ninhydrin, and 2 mL of glacial acetic acid, which was boiled at 100°C for 30 min. The solutions were cooled to room temperature and then extracted with 4 mL of toluene by vigorous shaking. The phases were allowed to separate, the organic phase was transferred to a colorimetric tube, and the optical density was determined at 520 nm.

Microarray analysis

Gene expression analysis using the GeneChip® Rice Genome Array (Affymetrix, Santa Clara, CA, USA) was performed using 2-week-old seedlings from two transgenic lines and a non-transformed line. 25 µg of total RNA was used for each labeling reaction. cDNA synthesis, RNA probe labeling, probe annealing to the chip, signal scanning and analysis were completed by Gene Tech Company, Ltd. (Shanghai, China) in accordance with the Affymetrix Gene Chip Expression Analysis Technical Manual (Affymetrix). Scanning employed a GeneArray scanner 3000 7G (Affymetrix). Results were normalized, quantified, and analyzed using Expression Console software (Affymetrix). Data comparison analysis was carried out with Partek Genomic Suite 6.4 software (Partek, St. Louis, MO, USA).

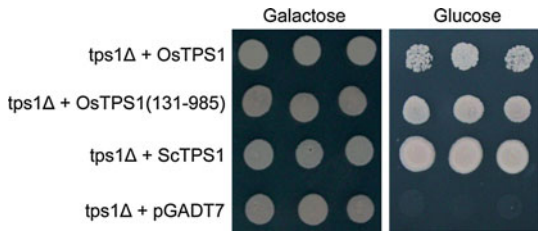

Fig. 1 Complementation of yeast tps1 mutant growth defect with expression of the truncated *OsTPS1* gene. As a control, strains were grown on galactose. The yeast tps1 mutant transformed with pGADT7-ScTPS1 and pGADT7 vector were used as positive and negative controls, respectively

Results

OsTPS1 with a Truncation at the N-terminus retains TPS activity

In yeast, overexpression of truncated *AtTPS1* and *SlTPS1* homologues lacking the N-terminal extension resulted in higher TPS activity and more trehalose accumulation (Van Dijck et al. 2002). Because of the similarity of OsTPS1 to AtTPS1 and SlTPS1 (Online Resource 3), we hypothesized that the N-terminal extension of OsTPS1 may also act as an inhibitor. To test this, we obtained an N-terminal truncation (1–130) deleted construct, and demonstrated that the truncated OsTPS1 (131–985) had higher TPS activity than the full length OsTPS1 in a yeast *tps1* mutant using yeast complementation assay (Fig. 1).

OsTPS1 expression is not induced by abiotic stress and is not organ-specific

The expression pattern of *OsTPS1* was analyzed by semi-quantitative RT-PCR using gene specific primers. For stress-induced expression assays, RNA was isolated from 2-week-old rice seedlings subjected to low temperature (4°C), osmotic stress (20% PEG), and high salinity (150 mM NaCl) at a series of time points (0, 1, 2, 4, 6, 12, and 24 h). No obvious inducement of *OsTPS1* was detected in these experiments (Fig. 2b). For organ-specific expression assays, samples were harvested from the different plant organs. Because there was no significant difference in *OsTPS1* expression levels in any of the tissues samples (Fig. 2a), we conclude that *OsTPS1* is expressed constitutively throughout the plant.

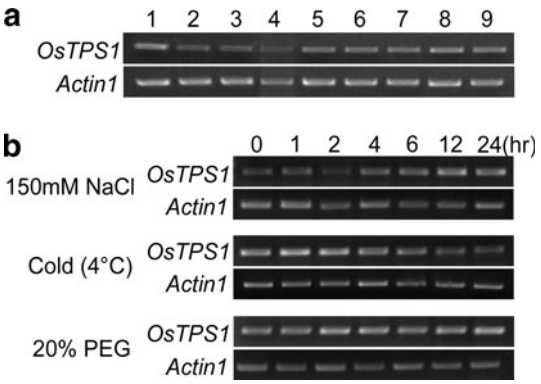

Fig. 2 Expression patterns of the *OsTPS1* gene. **a** OsTPS1 expression in various tissues. Lanes 1–9 contain samples from the following tissues: seedling, root, leaf, stem, spikelets (flowering stage), spikelets (after fertilization), spikelets (milk grain stage), seed, and seed after germination. **b** OsTPS1 expression in response to abiotic stress. Seedlings of rice ZH11 were treated with 150 mM NaCl, 20% PEG or incubated at 4°C, and then harvested at 0, 1, 2, 4, 6, 12, and 24 h. The rice Actin1 gene was used as an endogenous control

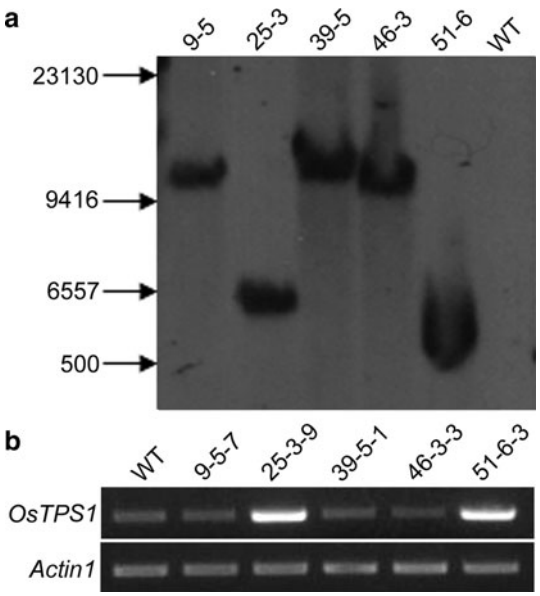

Fig. 3 Molecular characterization of OsTPS1 overexpression lines. **a** Southern-blot hybridization of transgenic rice plants. Five T2 OsTPS1 transgenic lines were identified. WT (ZH11) was included as a negative control. **b** RT-PCR analysis for OsTPS1 expression level in five independent T3 transgenic lines

Molecular analysis of *OsTPS1* in transgenic rice

Transgenic plants containing active *TPS* have shown enhanced abiotic stress tolerance (Goddijn et al. 1997; Avonce et al. 2004; Karim et al. 2007; Romero et al. 1997; Garg et al. 2002). To assess the function of *OsTPS1* in stress tolerance, we transformed the rice ZH11 (*O. sativa* L. ssp. *Japonica* cv.) with a truncated gene that encodes OsTPS1 (131-985). The *CaMV 35S* promoter was used to drive the constitutive expression of the transgene. Fifty-three independent transgenic lines harboring the *35S::OsTPS1* (131-985) construct were obtained through transformation. Using genetic segregation ratio analysis and Southern blot assay, we selected five T2 transgenic lines containing single gene insertion, which were designated 9-5, 25-3, 39-5, 46-3, and 51-6 (Fig. 3a). The expression of *OsTPS1* in the T3 transgenic plants was analyzed by RT-PCR. In lines 25-3-9 and 51-6-3, the levels of *OsTPS1* expression were moderately higher than that in the WT, whereas in the other three lines expression was equal to WT level (Fig. 3b).

Overexpression of the truncated *OsTPS1* enhances tolerance to drought, salt and cold in transgenic rice

OsTPS1 transgenic rice had no significant phenotypic effects on seedling growth besides the slight dwarfing and shorter leaf length under normal conditions (data not shown), but this was not sufficient to impact the normal growth of the transgenic lines.

To evaluate the tolerance of transgenic plants to abiotic stress, three independent homozygous T3 transgenic lines, including two high expression lines (25-3-9, 51-6-3) and a low expression line (39-5-1), were exposed to low temperature (4°C for 5 days), drought (air dried for 4–5 h), high salinity (150 mM NaCl for 3 days) or osmotic stress (20% PEG for 3 days), and then transferred to normal conditions to restore growth. After challenging with abiotic stress, the overexpression lines remained green and alive, and were more vigorous than the control (Fig. 4a). After 7 days of growth recovery, the survival rates of seedlings were counted to evaluate the stress tolerance.

In the drought tolerance test, the *OsTPS1* transgenic lines had higher survival rates than WT plants. The survival rate of *OsTPS1* transgenic plants (lines 39-5-1, 25-3-9, and 51-6-3) were 6.25, 39.58, and 35.42%, respectively, whereas WT survival was only 2.08%. Using PEG to simulate drought produced similar results. In the cold tolerance test, *OsTPS1* transgenic plants (lines 39-5-1, 25-3-9, and 51-6-3) had survival rates of 12.5, 79.17, and 89.58%, respectively, whereas the WT plants had a survival rate of 4.17%. In the salt stress test, the survival rates of lines 25-3-9 and 51-6-3 were twofold higher than the WT. However, the low expression line (39-5-1) did not show improved salt tolerance (Fig. 4b).

In summary, the transgenic lines overexpressing *OsTPS1* showed significantly increased tolerance to salinity, drought and cold, and the survival rates were associated with elevated *OsTPS1* expression levels.

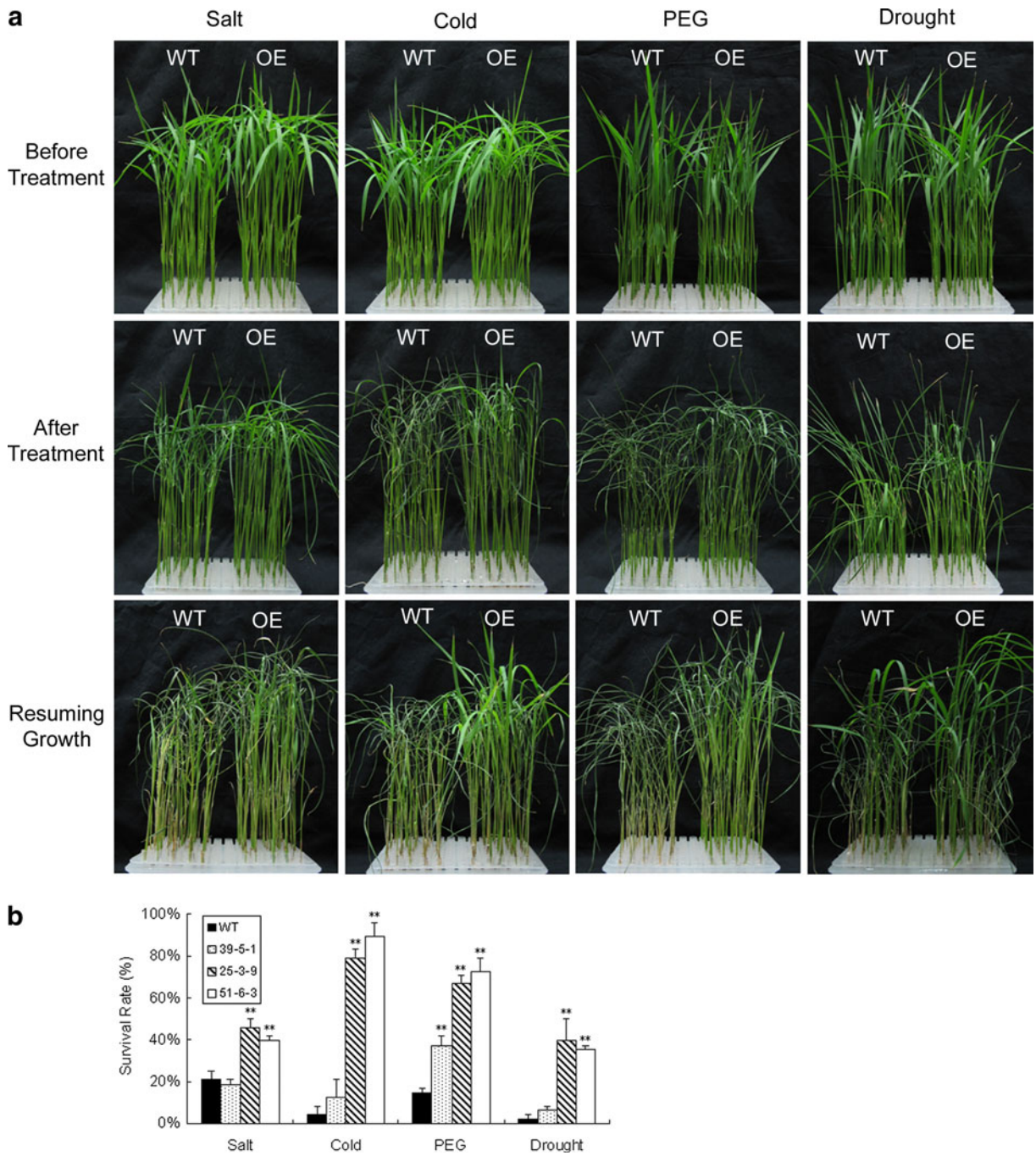

Fig. 4 Overexpression of OsTPS1 enhanced rice tolerance against abiotic stresses. **a** In the salt and PEG tolerance tests, seedlings were treated with nutrient solution containing 150 mM NaCl or 20% PEG for 3 days; for the cold tolerance test, seedlings were exposed to 4°C for 5 days; for the acute drought tolerance test, whole plants were air dried for 4–5 h. In each plate, transgenic lines overexpressing OsTPS1 (OE) are seen on the right side and wild type (WT) on the left. Three OsTPS1 transgenic lines and a WT control were used in these experiments. The photographs were taken before treatment, after treatment and after 7 days of restored growth under normal conditions. **b** The survival rates were calculated after these tolerance tests in three independent experiments. The statistically significant differences between transgenic lines as compared to the WT line are marked with *double asterisks* ($P < 0.01$)

OsTPS1 overexpression increases trehalose and proline levels in plant tissues

We measured the concentration of trehalose in the two transgenic lines with high *OsTPS1* expression. These transgenic plants exhibited higher trehalose accumulation when compared with the WT under normal conditions. The trehalose content in *OsTPS1* transgenic plants (lines 25-3-9 and 51-6-3) were 34.84 and 46.96 μg (g FW)$^{-1}$, whereas in WT the trehalose content was 23.37 μg (g FW)$^{-1}$ (Fig. 5). The

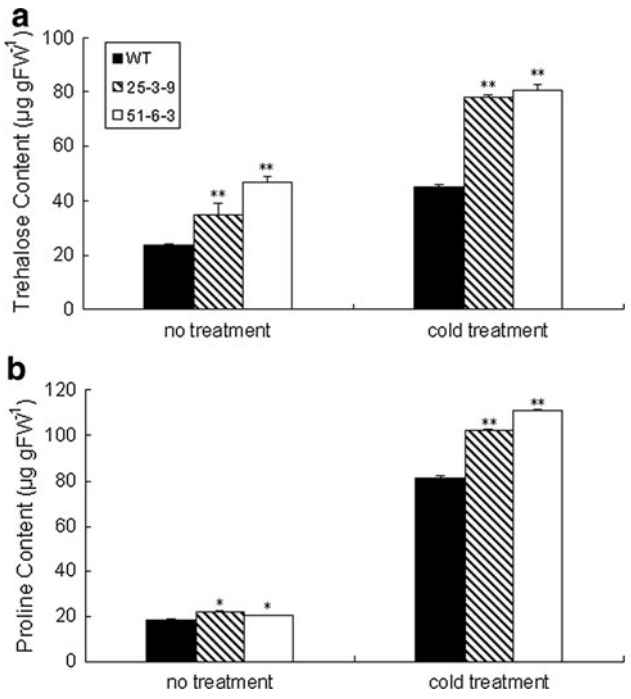

Fig. 5 Trehalose (**a**) and proline (**b**) content in OsTPS1 transgenic and nontransgenic plants with and without stress. Trehalose and proline were extracted from 2-week-old seedlings before and after 4 days cold stress. Two transgenic lines with high OsTPS1 expression and a wild type (WT) control were used in the experiment. The results were based on three independent experiments. The *asterisk* ($P < 0.05$) and *double asterisks* ($P < 0.01$) indicate statistically significant differences between the transgenic lines and the WT line

changes were similar to the levels found after cold stress treatment. The concentration of trehalose in lines 25-3-9 and 51-6-3 were 78.35 and 80.68 μg $(g\ FW)^{-1}$, respectively, whereas the WT had 45.16 μg $(g\ FW)^{-1}$ trehalose content (Fig. 5a). It is possible that the increased amount of trehalose contributes to the observed enhancement of abiotic stress tolerance.

Acidity ninhydrin analysis was performed to analyze proline accumulation in transgenic plants. Under normal conditions, proline was detected in two different *OsTPS1* transgenic lines at levels of 21.76 and 20.37 μg $(g\ FW)^{-1}$ as compared to 18.29 μg $(g\ FW)^{-1}$ in the WT. After 4 days of cold stress treatment, proline levels increased four to five times than those without the treatment. The proline content in lines 25-3-9 and 51-6-3 were 101.85, and 110.88 μg $(g\ FW)^{-1}$, respectively, whereas in the WT proline content was 81.02 μg $(g\ FW)^{-1}$ (Fig. 5b). In conclusion, proline accumulated in transgenic rice with or without stress treatment.

OsTPS1 overexpression alters the expression of stress related genes

To further investigate the molecular mechanism of stress tolerance via OsTPS1, we used the Affymetrix GeneChip® Rice Genome Array to analyze the expression of downstream genes in transgenic rice. Some stress-related genes were up-regulated more than twofold in *OsTPS1* transgenetic rice as compared to the WT including *ELIP* (Early Light-Inducible Protein, Os01g0246400), *HSP70* (Heat Shock Protein, Os03g0277300), *RAB16C* (Responsive to ABA, Os11g0454000), *CRP* (Cold Regulated Protein, Os05g0468800), *DHN6* (Dehydrin, Os11g0451700), *LEA14A* (Late Embryogenesis Abundant Protein, Os01g0225600) and *WSI18* (Water Stress Inducible Protein, Os01g0705200) (Table 1). Some of these genes have been reported to be induced by abiotic stresses, such as cold, desiccation, salinity, wounding, and ABA and have been used as marker genes for stress (Joshee et al. 1998; Yi et al. 2010; Puhakainen et al. 2004; Sun et al. 2002; Neelam et al. 2009).

We used semi-quantitative RT-PCR (Fig. 6a) and Real-time PCR (Fig. 6b) to confirm the array results. *WSI18*, *RAB16C*, *HSP70,* and *ELIP* were significantly induced in *OsTPS1* overexpressing transgenic lines. Moreover, *OsTPP1* and *OsTPP2* which transform T-6-P to trehalose, and *TRE1* which is involved in the catabolism of trehalose were also up-regulated.

The Rice Expression Chip, RT-PCR and Realtime PCR analysis results demonstrate that *OsTPS1* overexpression activates some stress-related genes, which may be responsible for the improvement in abiotic stress tolerance in *OsTPS1* transgenic rice.

Overexpressing Class II *OsTPS* genes also improves rice tolerance to abiotic stress

There are 11 *OsTPS* genes in rice which can be categorized into two groups. Because of their similarity to yeast TPS, only OsTPS1 belongs to Class I, and the others belong to Class II (Zang et al. 2011). To investigate the function of Class II *TPS* genes, we introduced *OsTPS2*, *OsTPS4*, *OsTPS5*, *OsTPS8*, and *OsTPS9* into *O. sativa* L. ssp. *Japonica* cv. Nipponbare. All of these Class II OsTPSs improved the tolerance of rice to cold and salinity stress (Fig. 7), and some of them improved the drought tolerance of transgenic rice seedlings (data not shown).

Discussion

The TPS/TPP family in higher plants

TPS and TPP proteins constitute a large protein family in higher plants. There are 11 *TPS* genes in the *Arabidopsis* and rice genomes (Leyman et al. 2001; Zang et al. 2011) and at least ten *TPP* genes in *Arabidopsis* and nine *TPP* genes in rice (Ramon and Rolland 2007; Pramanik and Imai

Table 1 Gene chip assay of stress and trehalose metabolism-related genes

Gene			L25 versus WT	L51 versus WT
Abbreviation	Full name	Locus		
ELIP	Early light inducible protein	Os01g0246400	5.5847	4.0935
HSP70	Heat shock 70KDa protein	Os03g0277300	5.0439	2.0993
RAB16C	Responsive to ABA	Os11g0454000	4.5787	1.9124
CRP	Cold regulated protein	Os05g0468800	4.2626	2.1824
DHN6	Dehydrin	Os11g0451700	3.1839	1.5533
LEA14	Late embryogenesis abundant protein	Os01g0225600	2.3010	1.5001
WSI18	Water stress inducible protein	Os01g0705200	2.0774	1.1076
TPP1	Trehalose-6-phosphate phosphatase	Os02g0661100	1.0797	1.4837
TPP2	Trehalose-6-phosphate phosphatase	Os10g0553300	1.1527	1.0829
TRE1	Trehalase	Os10g0521000	3.4775	1.8483

Stress-related genes induced more than twofold at least in one overexpression line and some trehalose metabolism genes are shown. Fold changes of gene expression from transgenic line 25-3-9 versus WT are shown as L25 versus WT, and fold changes of gene expression from transgenic line 51-6-3 versus WT are shown as L51 versus WT

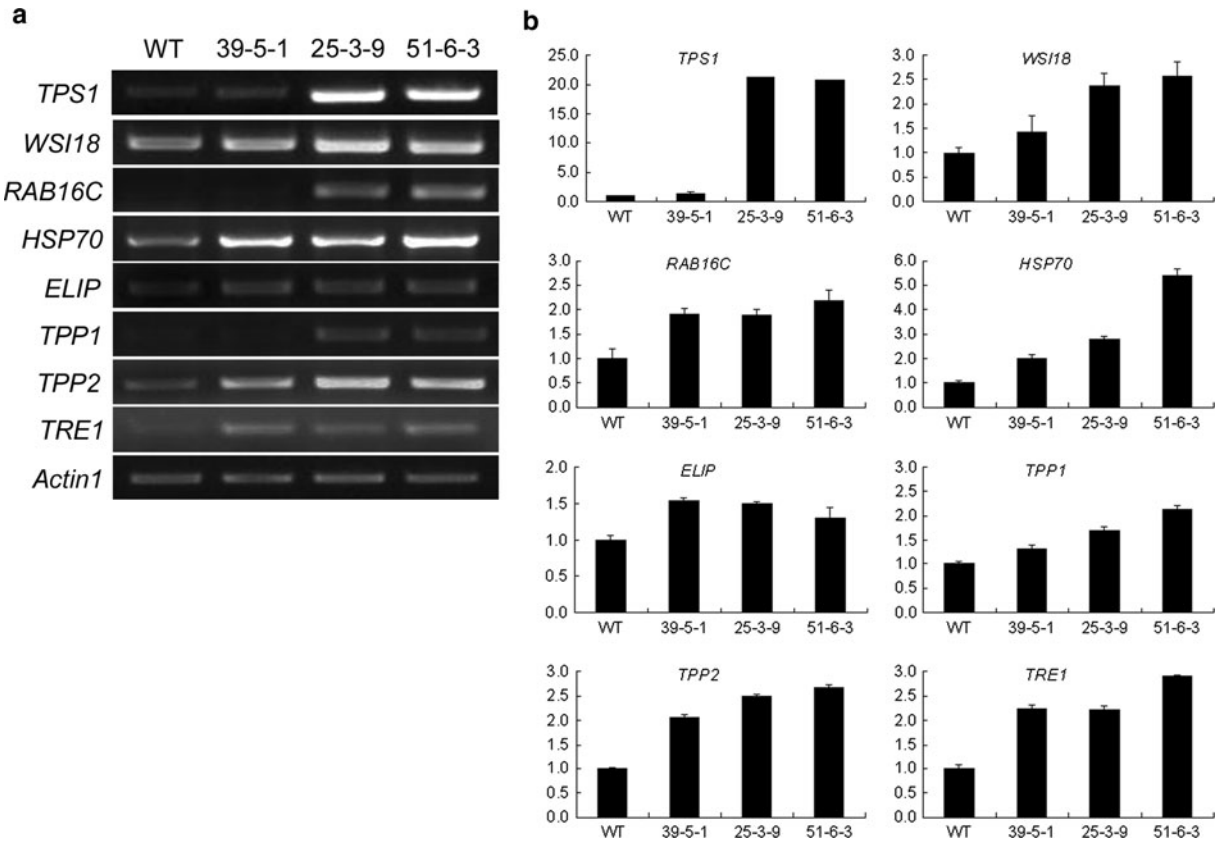

Fig. 6 Transcript levels of OsTPS1 and stress-related genes in transgenic and control plants assayed by semi-quantitative RT-PCR (**a**) and Real-time PCR (**b**). The rice Actin1 gene was used as an endogenous control. The experiment was replicated at least three times

2005). However, only a few have been shown to possess activity. In rice, OsTPS1 (Zang et al. 2011), OsTPP1, and OsTPP2 (Pramanik and Imai 2005; Shima et al. 2007) proteins have established TPS and TPP activity, respectively. However, the functions of the other TPS and TPP proteins in higher plants remain elusive.

Recent studies have shown that the *AtTPS*s have distinctive organ-specific expression profiles (Avonce et al. 2006), and many of these genes appear to be regulated by light, hormones, stress, and nutrient availability (Paul et al. 2008). It suggests that members of this large gene family may have diverse roles in the regulation of metabolism.

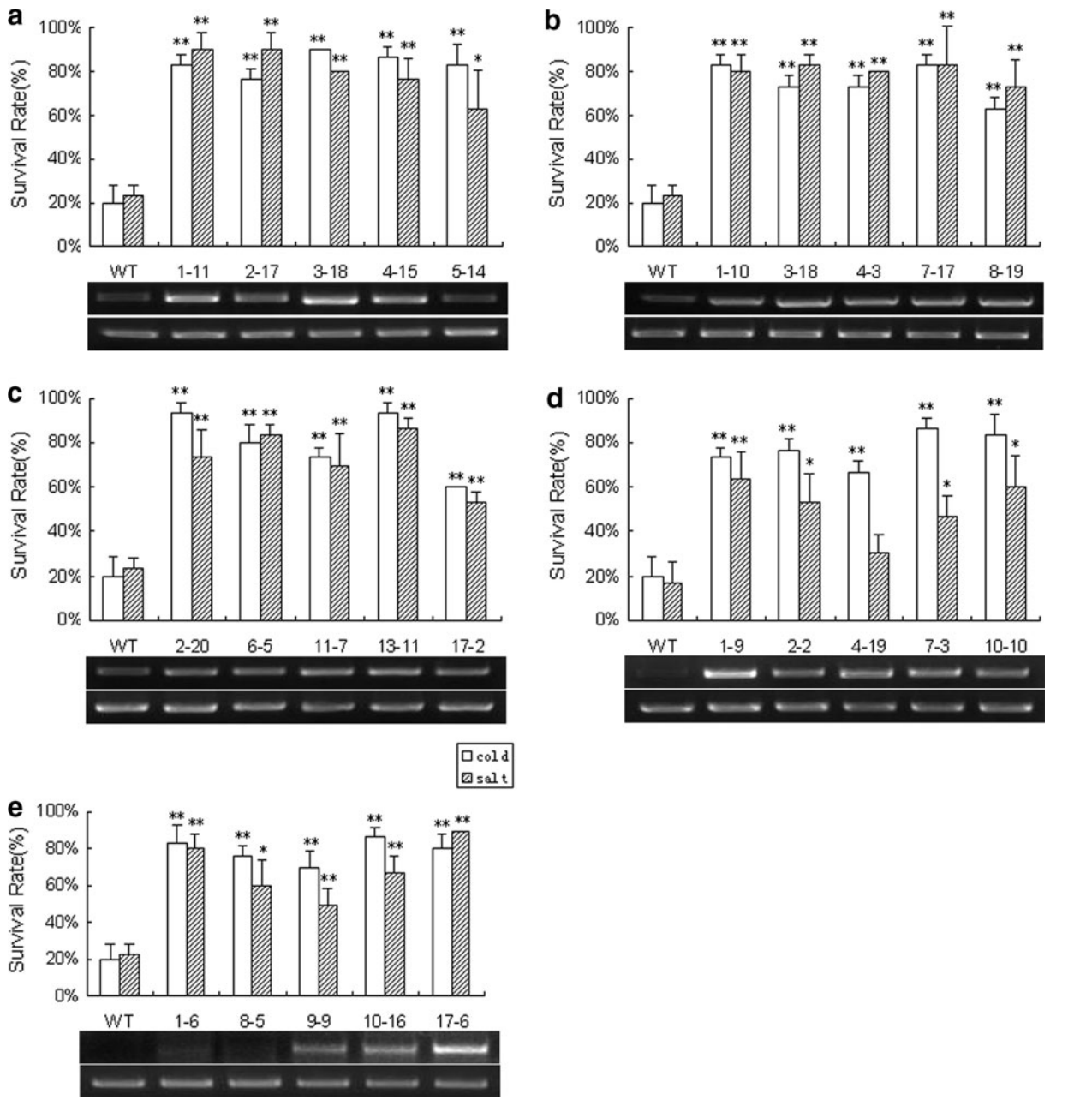

Fig. 7 Overexpression of Class II OsTPS genes enhanced rice tolerance against cold and salt. Survival rates of OsTPS2, OsTPS4, OsTPS5, OsTPS8 and OsTPS9 transgenic rice under cold and salinity are shown as **a**–**e**, respectively. The independent transgenic lines are shown on the *x*-axis in each figure. For the cold tolerance test, seedlings were exposed to 4°C for 5 days (*white bars*); for the salt tolerance test, seedlings were treated with nutrient solution containing 150 mM NaCl for 3 days (*shaded bars*). The survival rates were calculated after resumption of growth under normal conditions for 7 days. The *asterisk* ($P < 0.05$) and *double asterisks* ($P < 0.01$) indicate statistically significant differences between transgenic lines as compared to the WT line. The expression level of each gene is shown under graph. These assays are based on more than three replicates

Overexpression of *OsTPS*s enhances the tolerance of transgenic rice to several types of abiotic stress

A gene's expression pattern is closely interrelated with its function in the multicellular organisms. Our results indicated that *OsTPS1* is expressed throughout the rice plant and under a variety of abiotic stresses. Therefore, rather than playing a role in specific plant organs or stress response, *OsTPS1* may instead be involved in general regulation, and have a broader role in plant development and stress signaling. This result was similar to *AtTPS1* which is expressed ubiquitously in *Arabidopsis* (Van Dijken et al. 2004).

Abiotic stress conditions such as low temperature, drought, and high salinity have adverse effects on plant

growth and crop production. It is important to determine the molecular mechanisms of abiotic stress signal transduction and to identify methods to improve the tolerance of plants. Some researchers have reported that heterologous expression of the *E. coli* or yeast *TPS1* genes improves the abiotic stress tolerance in several plant species. However, it can also lead to a series of abnormalities in plant growth and development, such as dwarfish growth, delayed flowering, aberrant root development, and lancet-shaped leaves (Goddijn et al. 1997; Romero et al. 1997; Cortina and Culiáñez-Macià 2005). In this study, we found that homologous overexpression of the rice *OsTPS1* gene enhanced rice tolerance of salinity, low temperature, and drought (Fig. 4) without severely detrimental plant growth phenotypes. Our results confirm that OsTPS1 plays an important role in abiotic stress response and is an effective option for biotechnology breeding, the creation of novel germplasm, and scientific research.

Some Class II *TPS* genes (*OsTPS2*, *OsTPS4*, *OsTPS5*, *OsTPS8* and *OsTPS9*) improved the tolerance of rice to abiotic stress either (Fig. 7). The function and regulatory mechanism of these Class II OsTPSs were unclear. Our previous research showed that Class II TPS proteins could interact with OsTPS1 and the OsTPS family members might be incorporated into protein complexes. They potentially modify T-6-P and trehalose levels, and further regulate plant development and stress responses (Zang et al. 2011). In *Arabidopsis*, AtTPS5, AtTPS6, and AtTPS7 were phosphorylated by SnRK1 and bound to 14-3-3 proteins (Harthill et al. 2006). We speculated that Class II OsTPS proteins might regulate OsTPS1 activity in protein complexes depending on their phosphorylation status.

Possible mechanisms for OsTPS1 in plant abiotic stress tolerance

Although a great number of researches have focused on trehalose biosynthesis genes, the physiological and molecular mechanism of the enhanced stress tolerance conferred by OsTPS1 is still ambiguous. Experimental data shows that trehalose makes important contributions to abiotic stress tolerance. Based on this, we first analyzed the trehalose concentration in *OsTPS1*-overexpressing transgenic rice lines and found the level of trehalose was twofold higher in the transgenic lines (Fig. 5a), which could enhance the stress tolerance of plants. Despite a significant difference between the transgenic and nontransgenic lines, the trehalose concentration was not greatly increased in the transgenic plants because of inducement of the TPPs and trehalase. According to the Rice Expression Chip, RT-PCR and Realtime PCR analysis, *OsTPP1* and *OsTPP2*, which transform T-6-P to trehalose, and *TRE1* which is involved in the catabolism of trehalose, were up-regulated (Fig. 6). This indicates that plants might break down excessive T-6-P or trehalose to maintain a metabolic balance and avoid the toxicity of excessive accumulation of T-6-P and trehalose (Fernandez et al. 2010; Veluthambi et al. 1982).

Proline is the primary osmotic regulation substance. Plants accumulating more proline have higher stress tolence. Based on this, we measured the proline content and found that proline accumulated in transgenic lines with or without abiotic stress (cold treatment for 4 days) (Fig. 5b). The increase in proline content may contribute to the improved stress tolerance of plants.

We observed that overpression of *OsTPS1* activated several stress-related genes, such as *WSI18*, *RAB16C*, *HSP70,* and *ELIP* (Fig. 6). These genes are reported to be activated by cold, desiccation, salinity, wounding, ABA, and some other abiotic stresses (Joshee et al. 1998; Yi et al. 2010; Puhakainen et al. 2004; Sun et al. 2002; Neelam et al. 2009). Their overexpression plays a vital role in the enhancement of tolerance to various stresses (Puhakainen et al. 2004; Yi et al. 2010). Although the signal transduction pathway of these genes is not clear, we can also speculate that OsTPS1 participates in abiotic stress pathways indirectly through alteration of the expression of some stress-related genes.

In conclusion, our work demonstrates that overexpressing *OsTPS1* enhanced the tolerance of rice seedlings to multiple abiotic stresses by increasing trehalose and proline levels and activating some abiotic stress-related genes. In addition, overexpressing some Class II *OsTPS* genes also improved plant tolerance against the abiotic stress. These results are helpful in improving the plant tolerance to environmental stress through trehalose metabolism.

Acknowledgments This research was supported by grants from the Agricultural Ministry of China (2008ZX08009-003), the 863 High-tech Project from the Ministry of Science and Technology of China (2007AA10Z185), and the National Natural Science Foundation of China (2010 No. 31071378).

References

Almeida AM, Villalobos E, Araújo SS, Leyman B, Van Dijck P, Alfaro-Cardoso L, Fevereiro PS, Torné JM, Santos DM (2005) Transformation of tobacco with an *Arabidopsis thaliana* gene involved in trehalose biosynthesis increases tolerance to several abiotic stresses. Euphytica 146(1–2):165–176

Almeida AM, Cardoso LA, Santos DM, Torné JM, Fevereiro PS (2007) Trehalose and its applications in plant biotechnology. In Vitro Cell Dev Biol 43(3):167–177

Anselmino O, Gilg E (1913) Ueber das Vorkommen von trehalose in *Selaginella lepidophylla*. Ber Deut Pharm Ges 23:326–330

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G (2004) The Arabidopsis trehalose-6-p synthase AtTPS1 gene is a regulator of glucose, abscisic acid and stress signaling. Plant Physiol 136(3):3649–3659

Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G (2006) Insights on the evolution of trehalose biosynthesis. BMC Evol Biol 6(1):109–123

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39(1):205–207

Bell W, Sun W, Hohmann S, Wera S, Reinders A, De Virgilio C, Wiemken A, Thevelein JM (1998) Composition and functional analysis of the *Saccharomyces cerevisiae* trehalose synthase complex. J Biol Chem 273(50):33311–33319

Bianchi G, Gamba A, Limiroli R, Pozzi N, Elster R, Salamini F, Bartels D (1993) The unusual sugar composition in leaves of the resurrection plant *Myrothamnus flabellifolia*. Physiol Plant 87(2):223–226

Blázquez MA, Santos E, Flores C, Martínez-Zapater JM, Salinas J, Gancedo C (1998) Isolation and molecular characterisation of the Arabidopsis TPS1 gene, encoding trehalose 6-phosphate synthase. Plant J 13(5):685–689

Cabib E, Leloir LF (1958) The biosynthesis of trehalose phosphate. J Biol Chem 231:259–275

Cortina C, Culiáñez-Macià FA (2005) Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. Plant Sci 169(1):75–82

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. Glycobiol 13(4):17R–27R

Fernandez O, Béthencourt L, Quero A, Sangwan RS, Clément C (2010) Trehalose and plant stress responses: friend or foe? Trends Plant Sci 15(7):409–417

Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA 99(25):15898–15903

Goddijn OJM, Van Dun K (1999) Trehalose metabolism in plants. Trends Plant Sci 4(8):315–319

Goddijn OJM, Verwoerd TC, Voogd E, Krutwagen RWHH, De Graff PTHM, Poels J, Van Dun K, Ponstein AS, Damm B, Pen J (1997) Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant Physiol 113(1):181–190

Harthill JE, Meek SEM, Morrice N, Peggie MW, Borch J, Wong BHC, MacKintosh C (2006) Phosphorylation and 14-3-3 binding of Arabidopsis trehalose-phosphate synthase 5 in response to 2-deoxyglucose. Plant J 47(2):211–223

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J 6(2):271–282

Iturriaga G, Suárez R, Nova-Franco B (2009) Trehalose metabolism: from osmoprotection to signaling. Int J Mol Sci 10(9):3793–3810

Jeong SC, Pack IS, Cho EY, Youk ES, Park S, Yoon WK, Kim CG, Choi YD, Kim JK, Kim HM (2007) Molecular analysis and quantitative detection of a transgenic rice line expressing a bifunctional fusion TPSP. Food Control 18(11):1434–1442

Joshee N, Kisaka H, Kitagawa Y (1998) Isolation and characterization of a water stress-specific genomic gene, pwsi18, from rice. Plant Cell Physiol 39(1):64–72

Karim S, Aronsson H, Ericson H, Pirhonen M, Leyman B, Welin B, Mantyla E, Palva ET, Van Dijck P, Holmstrom KO (2007) Improved drought tolerance without undesired side effects in transgenic plants producing trehalose. Plant Mol Biol 64(4):371–386

Karimi M, Inze D, Depicker A (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7(5):193–195

Kosmas SA, Argyrokastritis A, Loukas MG, Eliopoulos E, Tsakas S, Kaltsikes PJ (2006) Isolation and characterization of drought-related trehalose 6-phosphate-synthase gene from cultivated cotton (*Gossypium hirsutum* L.). Planta 223(2):329–339

Leyman B, Van Dijck P, Thevelein JM (2001) An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. Trends Plant Sci 6(11):510–513

Lunn JE, Feil R, Hendriks JHM, Gibon Y, Morcuende R, Osuna D, Scheible WR, Carillo P, Hajirezaei MR, Stitt M (2006) Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. Biochem J 397:139–148

Neelam S, Yeon-Ki K, Anil G (2009) Rice sHsp genes: genomic organization and expression profiling under stress and development. BMC Genomics 10(1):393–410

Paul MJ, Primavesi LF, Jhurreea D, Zhang YH (2008) Trehalose metabolism and signaling. Annu Rev Plant Biol 59:417–441

Pramanik MHR, Imai R (2005) Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. Plant Mol Biol 58(6):751–762

Puhakainen T, Hess MW, Mäkelä P, Svensson J, Heino P, Palva ET (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in *Arabidopsis*. Plant Mol Biol 54(5):743–753

Ramon M, Rolland F (2007) Plant development: introducing trehalose metabolism. Trends Plant Sci 12(5):185–188

Romero C, Bellés JM, Vayá JL, Serrano R, Culiáñez-Macià FA (1997) Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. Planta 201(3):293–297

Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci USA 95(11):5857–5864

Shima S, Matsui H, Tahara S, Imai R (2007) Biochemical characterization of rice trehalose-6-phosphate phosphatases supports distinctive functions of these plant enzymes. FEBS J 274(5):1192–1201

Sun W, Van Montagu M, Verbruggen N (2002) Small heat shock proteins and stress tolerance in plants. Biochim Biophys Acta 1577(1):1–9

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 unlocks high catalytic activity and supports high trehalose levels on expression in yeast. Biochem J 366(Pt 1):63–71

Van Dijken AJH, Schluepmann H, Smeekens SCM (2004) Arabidopsis trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. Plant Physiol 135(2):969–977

Van Laere A (1989) Trehalose, reserve and/or stress metabolite? FEMS Microbiol Rev 63(3):201–210

Vandesteene L, Ramon M, Le Roy K, Van Dijck P, Rolland F (2010) A single active trehalose-6-p synthase (TPS) and a family of putative regulatory TPS-like proteins in arabidopsis. Mol Plant 3(2):406–419

Veluthambi K, Mahadevan S, Maheshwari R (1982) Trehalose toxicity in *Cuscuta reflexa*. Plant Physiol 69(6):1247–1251

Vogel G, Aeschbacher RA, Müller J, Boller T, Wiemken A (1998) Trehalose 6-phosphate phosphatases from *Arabidopsis thaliana*: identification by functional complementation of the yeast tps2 mutant. Plant J 13(5):673–683

Wang YJ, Hao YJ, Zhang ZG, Chen T, Zhang JS, Chen SY (2005) Isolation of trehalose-6-phosphate phosphatase gene from tobacco and its functional analysis in yeast cells. J Plant Physiol 162(2):215–223

Wiemken A (1990) Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie Van Leeuwenhoek 58(3):209–217

Yi N, Kim YS, Jeong MH, Oh SJ, Jeong JS, Park SH, Jung H, Choi YD, Kim JK (2010) Functional analysis of six drought-inducible

promoters in transgenic rice plants throughout all stages of plant growth. Planta 232(3):743–754

Zang BS, Li HW, Li WJ, Deng XW, Wang XP (2011) Analysis of trehalose-6-phosphate synthase (TPS) gene family suggests the formation of TPS complexes in rice. Plant Mol Biol. doi:10.1007/s11103-011-9781-1

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-thosphate synthase complements growth and stress-tolerance defects in a yeast tps1 mutant. Plant Physiol 119(4):1473–1482