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ORIGINAL ARTICLE

Overexpression of the trehalose-6-phosphate phosphatase gene *OsTPP1* confers stress tolerance in rice and results in the activation of stress responsive genes

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Abstract Trehalose plays a protective role in yeast and microorganisms under abiotic stresses. However, little is known about its role in higher plants when subjected to environmental challenges. A systematic search of rice databases discovered a large TPS/TPP gene family in the rice genome, which is similar to that found in *Arabidopsis thaliana*, especially in the gene family structure. Expression analysis demonstrated that *OsTPP1* was initially and transiently up-regulated after salt, osmotic and abscisic acid (ABA) treatments but slowly up-regulated under cold stress. *OsTPP1* overexpression in rice enhanced tolerance to salt and cold stress. Analysis of the overexpression lines revealed that *OsTPP1* triggered abiotic stress response genes, which suggests a possible transcriptional regulation pathway in stress induced reprogramming initiated by *OsTPP1*. The current study revealed the mechanism of an *OsTPP* gene involved in stress tolerance in rice and also suggested the use of *OsTPP1* in abiotic stress engineering of crops.

Keywords Abiotic stress tolerance · *OsTPP1* · Rice · Trehalose

Abbreviations

ABA	Abscisic acid
CaMV	Cauliflower mosaic virus
HPIC	High-performance ion chromatography
MCS	Multiple-clone-site
TPP	Trehalose-6-phosphate phosphatase
TPS	Trehalose-6-phosphate synthase
T-6-P	Trehalose-6-photophate

Introduction

Trehalose is a disaccharide sugar widely distributed in bacteria, fungi, insects, plants and invertebrate animals. In microbes and yeast, trehalose is produced from glucose by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP), and serves as a sugar storage, metabolic regulator and protects against abiotic stress (Wiemken 1990; Strom and Kaasen 1993). However, its role in plants is not yet fully elucidated. Under osmotic stress, trehalose was shown to accumulate at high levels in resurrection plants (such as *Selaginalla lepidophylla*) and was therefore proposed to function in stress protection (Wingler 2002). Whereas in many other higher plants such as *Arabidopsis thaliana*, trehalose is barely detectable, which precludes the role as a sugar reserve or stress protectant (Goddijn and Smeekens 1998; Vogel et al. 2001; Schluepmann et al. 2003). Interestingly, TPP and TPS genes are broadly found in the genomes of higher

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plants and have formed large gene families (Leyman et al. 2001; Schluepmann et al. 2004) implying the importance of these genes. Mellor (1992) considered trehalose as a symbiotic determinant between higher plants and microorganisms. However, there is no direct evidence supporting this hypothesis so far. In yeast, the precursor of trehalose, trehalose-6-photophate (T-6-P) is a key regulator in the glycolytic pathway (Blazquez et al. 1993). Recent studies also suggest a role of trehalose in the regulation of sugar metabolism (Vogel et al. 1998; Matthew Paul 2001; Wingler 2002; Eastmond and Graham 2003). In higher plants, genetic and reverse genetic analysis demonstrated that trehalose and T-6-P are essential for carbohydrate metabolism and plant development. For instance, loss of function of *AtTPS1* resulted in an embryo-lethal phenotype in Arabidopsis (Eastmond et al. 2002; Schluepmann et al. 2003; Gomez et al. 2006); and a mutation in *RA3*, a maize TPP gene, resulted in abnormalities of the inflorescence architecture (Satoh-Nagasawa et al. 2006).

There is also evidence suggesting that trehalose and T-6-P may contribute to plant abiotic stress acclimation. Overexpression of hetero-TPS and TPP genes in plants improved plant tolerance to abiotic stresses, but most transgenic plants exhibit growth inhibition (Goddijn et al. 1997; Romero et al. 1997; Yeo et al. 2000). In rice overexpression of bi-functional fusion of bacteria TPS and TPP (named TPSP) increased trehalose content and enhanced rice tolerance to abiotic stresses without alteration of growth (Garg et al. 2002; Jang et al. 2003). Further analysis showed that photosystem II damage was alleviated by TPSP overexpression under abiotic stress (Garg et al. 2002; Jang et al. 2003). From these data, the increased resistance of rice to abiotic stress was often considered to result from the increase of trehalose content.

To better understand the function of trehalose in plants, the regulation mechanisms of native TPS/TPP genes coping with abiotic stresses should be elucidated. In the work of searching the rice genome database, we found a large TPS/TPP gene family including the TPP genes found by Pramanik and Imai (2005). Among the gene family, multiple members are regulated by environmental stresses (Rabbani et al. 2003; Wang et al. 2003; Chao et al. 2005; Pramanik and Imai 2005), implicating these genes function in plant-environment interactions. A member of the *TPP* gene family, *OsTPP1* is of particular interest due to its initial transiently up-regulated pattern and different responses to salt stress (Chao et al. 2005). To further elucidate the role of *OsTPP1* in circumventing stress and the mechanism of action of trehalose and T-6-P function in rice acclimation to environmental stress, we generated the transgenic rice overexpressing *OsTPP1* and characterizaed the function of *OsTPP1* in response to abiotic stresses.

Materials and methods

Database searches, data acquisition, sequence alignment and phylogenetic analysis

The putative functions of trehalose synthase or phosphatase were used as key words in searches of the TIGR Rice Database (http://www.tigr.org/tdb/e2k1/osa1/). Nucleotide and protein sequences were downloaded and adjusted to correct prediction errors by comparison with the KOME database (http://cdna01.dna.affrc.go.jp/cDNA/) and the available expressed sequence tags. Protein sequences were aligned using ClustalX (Thompson et al. 1997) and further adjusted manually in BioEdit. Sequence similarity was assessed using BioEdit. Phylogenetic Tree reconstruction was completed by applying the Maximum Likelihood method with Tree-puzzle-5.2 for Windows (Schmidt et al. 2002) and bootstrapped for 1,000 times to provide overall levels of support for each lineage within gene families. TPP domains from all TPS and TPP homologues were included in the analysis and ScTPS2 was chosen as the outgroup.

Plant materials and stress treatments

For expression pattern analysis of *OsTPP1*, Nona Bokra (*Oryza sativa L. ssp. indica* pv. Nona) and Nipponbare (*Oryza sativa L. ssp. japonica*) (obtained from International Rice Research Institute, Metro Manila, Philippines) were grown in hydroponic culture under controlled conditions (13 h light, 24°C/11 h dark, 22°C cycle) after germination. The two-week-old seedlings were subjected to salt (150 mM NaCl), osmotic (20% PEG4000), cold (6∼8°C) stresses or abscisic acid (ABA) (10 μM) (only Nona Bokra). Seedlings were harvested after 0, 20 min, 1, 3, 6, 12, 24 and 72 h treatment and immediately frozen in liquid nitrogen for further RNA isolation and expression analysis. Similar procedures were utilized for treatments of *OsTPP1* overexpression lines and controls.

RNA extraction and expression analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) following manufacturer's instructions. The first strand cDNA was synthesized using Access RT-PCR system (Promega) and subsequently used as template for RT-PCR or Real Time PCR. Primers for RT-PCR and Real Time PCR (supplemental data 1) were designed to flank introns.

RT-PCR was replicated at least three times. Real Time PCR was performed on RoterGene 3000 using Taqman Probe for *OsTPP1* expression and SYBR Green for stress related genes expression. The subsequent data analysis was conducted following the method reported by Pfaffl (2001).

Seed-germination assay

Fifty seeds of two independent overexpression lines, OX5, OX8 and empty vector line were chosen for seed germination tests. The seeds were placed in a 9 cm diameter plate and immersed in water with or without 60 or 100 mM NaCl. Each test was replicated three times. Germination assay was performed at 30°C in the dark environment. After 5 days, only seeds with a shoot longer than half of the seed length were counted and the corresponding germination rate was calculated in Microsoft Office Excel.

Overexpression construct, plant transformation and screening

The full-length cDNA of *OsTPP1* (1.48 kb) was isolated from the cDNA library of Nona Bokra (Ren et al. 2005) by high fidelity PCR. Forward and reverse primers (Supplemental data 1) were introduced with a *Bam*HI and a *Pst*I site respectively. The fragment was recombined into pBluescript SK using *Bam*HI and *Pst*I. Three independent clones were sequenced. The transition vector was digested with *Bam*HI and *Pst*I, and the *OsTPP1* fragment was inserted into the multiple-clone-site (MCS) of the pHB expression vector controlled by the cauliflower mosaic virus (CaMV) 35S promoter (Mao et al. 2005). The complete construct and empty pHB vector were introduced into Nipponbare by an *Agrobacterium*-mediated transformation, as previously reported (Hiei et al. 1994). Positive transgenic lines were screened by 50 mg l^{-1} hygromycin and re-affirmed by Basta. Stable inherited homozygous transgenic lines at T3 or T4 generations were identified for further analysis.

Trehalose extraction and determination

Trehalose was extracted and determined as described by Jang et al. (2003). Shoot (1 g) from two-week-old transgenic seedlings before or after 4 days salt stress (125 mM NaCl) was ground in liquid nitrogen and extracted in 10 ml boiling water for 10 min. After that, the extract was centrifuged at 12,300*g* for 10 min and then the supernatant was filtered with a 0.45 μm aperture filter. Trehalose content was determined by high-performance ion chromatography (HPIC) with a Carbo-Pak PA1 column (4×250 nm) using the DX500 HPIC system (Dionex 500, Dionex, Sunnyvale, CA, USA). Trehalose (Sigma) was used as the standard.

Results

Analysis of TPS and TPP genes in rice

By searching for genes coding putative TPS or TPP in the TIGR rice genome (http://www.tigr.org) and KOME (http://cdna01.dna.affrc.go.jp/cDNA) databases, 14 TPS loci and 13 TPP loci in total were found (Table 1). Similar to that seen in Arabidopsis, OsTPSs contain both a TPS and a TPP domain, while OsTPPs only contain an independent TPP domain (Table 1). Phylogenetic analysis of the TPP domains demonstrated that the whole TPS/TPP gene family in rice was grouped into three classes (Class I/II/III) (Fig. 1). Class I consisted of OsTPS1 which displayed high identity to AtTPS1 (Blazquez et al. 1998) and SlTPS1 (Zentella et al. 1999); Class II consisted of all the remaining OsTPSs; and Class III consisted of all independent TPP genes. The phylogenetic tree displayed a similar structure to that in Arabidopsis (Leyman et al. 2001), suggesting the gene family was highly conserved and formed before the divergence of dicotyledonous and monocotyledonous angiosperms. Notably, in Class I, rice only contained one member (OsTPS1), whereas Arabidopsis contained four members (AtTPS1-AtTPS4). Previous studies have shown that AtTPS1 contains an additional N-terminal extension and is indispensable in embryo development (Leyman et al. 2001; Eastmond et al. 2002). The additional N-terminal extension distinguished AtTPS1 from other members in Class I. OsTPS1 displayed the highest identity and similar primary structure to AtTPS1 and was the only member of Class I in rice. It suggested that OsTPS1 possibly plays a similar role in rice as AtTPS1 in Arabidopsis.

Isolation of *OsTPP1* from Nona Bokra

Previous research has revealed that one of the *TPP* homologues, *OsTPP1* was quickly induced under salt stress (Chao et al. 2005). In order to further assess the function of *OsTPP1* in stress tolerance, the full-length cDNA was isolated from the cDNA library of the highly salt-tolerant rice variety Nona Bokra (*O. sativa, var. indica*; Ren et al. 2005). Compared with the full length sequence of the corresponding Nipponbare cDNA in KOME (Clone name: J033127L03), no differences exist between the cDNA sequence from Nona Bokra and Nipponbare (data not shown), except for two base substitutions, which do not result in amino acid changes.

OsTPP1 was induced by abiotic stresses and exogenous ABA

The result of the phylogeny reconstruction revealed that *AtTPPA* and *OsTPP1* were closely allied. In the previous

Table 1 Putative genes involved in trehalose metabolism in rice

Class	Gene name	Full length (aa)	Locus	Accession no.	EST	TPS domain	TPP domain	Phosphatase boxes
I	OsTPS1	980	LOC_Os05g44210	AK067066	Yes	Yes	Yes	–
II	OsTPS2	913	LOC_Os01g54560	AK064990	Yes	Yes	Yes	+
	OsTPS3	899	LOC_Os05g44100	AK072066	Yes	Yes	Yes	+
	OsTPS4A	886	LOC_Os02g54820.1		Yes	Yes	Yes	+
	OsTPS4B	847	LOC_Os02g54820.2		Yes	Yes	Yes	+
	OsTPS4C	750	LOC_Os02g54820.3		Yes	Yes	Yes	Only one
	OsTPS4D	547	LOC_Os02g54820.4		Yes	Yes	Yes	+
	OsTPS5	886	LOC_Os09g25890		No	Yes	Yes	+
	OsTPS6	866	LOC_Os09g23350	AK105382	Yes	Yes	Yes	+
	OsTPS7A	878	LOC_Os01g53000.1	AK101207	Yes	Yes	Yes	+
	OsTPS7B	786	LOC_Os01g53000.2		Yes	Yes	Yes	+
	OsTPS8	863	LOC_Os09g20990		Yes	Yes	Yes	+
	OsTPS9	862	LOC_Os08g31980	AK099398	Yes	Yes	Yes	+
	OsTPS10A	824	LOC_Os08g34580.1	AK072132	Yes	Yes	Yes	+
	OsTPS10B	629	LOC_Os08g34580.2		Yes	Yes	Yes	Only one
	OsTPS11	860	LOC_Os03g12360	AK100541	Yes	Yes	Yes	+
	OsTPS12[a]	272	LOC_Os05g03810		Yes	Yes	Yes	Only one
	OsTPS13[a]	221	LOC_Os12g32130		Yes	Yes	Yes	Only one
	OsTPS14[a]	201	LOC_Os05g06160		Yes	Yes	Yes	–
III	OsTPP1	371	LOC_Os02g44230	AK103391	Yes	No	Yes	+
	OsTPP2	500	LOC_Os06g11840	AP004727	Yes	No	Yes	+
	OsTPP3	382	LOC_Os10g40550	AK069361	Yes	No	Yes	+
	OsTPP4	261	LOC_Os12g09060		No	No	Yes	+
	OsTPP5	310	LOC_Os03g26910	AK106316	Yes	No	Yes	+
	OsTPP6A	370	LOC_Os08g31630.1	XM_482349	Yes	No	Yes	+
	OsTPP6B[a]	297	LOC_Os08g31630.2		No	No	Yes	Only one
	OsTPP7	367	LOC_Os02g51680	AK121015	Yes	No	Yes	+
	OsTPP8	366	LOC_Os07g43160	AK109902	Yes	No	Yes	+
	OsTPP9	349	LOC_Os04g46760	XM_473444	Yes	No	Yes	+
	OsTPP10	359	LOC_Os07g30160	AK070085	Yes	No	Yes	+
	OsTPP11A	375	LOC_Os09g20390.1	XM_450681	Yes	No	Yes	+
	OsTPP11B	330	LOC_Os09g20390.2		Yes	No	Yes	+
	OsTPP11C[a]	260	LOC_Os09g20390.3		Yes	No	Yes	Only one
	OsTPP12[a]	257	LOC_Os08g25410		No	No	Yes	Only one
	OsTPP13[a]	177	LOC_Os06g23010		No	No	Yes	–
Trehalase	Trehalase	563	LOC_Os10g37660					

Genes functioning as putative TPS or TPP or trehalase were searched in the rice sequence database and listed. The classification was based on the features of the primary structure and identity of proteins

[a] Putative pseudogene

report, *AtTPPA* has been identified as a functional T-6-P phosphatase (Vogel et al. 1998), and *OsTPP1* was also strongly supported as a T-6-P phosphatase in rice (Pramanik and Imai 2005). Expression analysis suggested that *OsTPP1* was up-regulated in rice by abiotic stress (Pramanik and Imai 2005), especially in Nona Bokra in which it was initially and transiently up-regulated in response to high salt stress (Chao et al. 2005). This prompted us to further investigate the time-course expression pattern of *OsTPP1* under salt (150 mM NaCl), osmotic (20% PEG4, 000), and cold (6∼8°C) stresses. The results suggested that *OsTPP1* was induced by salt, osmotic and cold stress (Fig. 2). Detailed observation showed that the expression pattern under abiotic stress in Nona Bokra was approximately similar with that in Nipponbare except for some small differences in the induction time (Fig. 2). In Nona

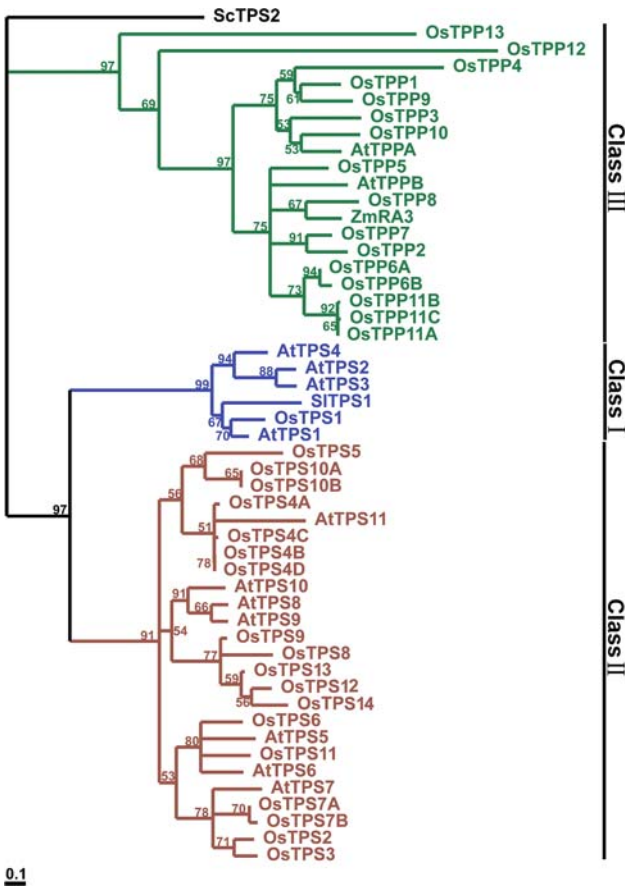

Fig. 1 Phylogenetic analysis of TPP domains. The C-terminal TPP domains of TPS genes and the whole TPP genes were aligned using ClustalX. The most likelihood phylogenetic tree was constructed based on the aligned sequences file using tree-puzzle 5.2 for windows. ScTPS2 was used as the outgroup. *Numbers* above branches represent >50% bootstrap support for 1,000 replicates

Bokra, *OsTPP1* was significantly induced after only a 20 min salt treatment, with peak transcript level noted after 1 h, and declining thereafter (Fig. 2a). In Nipponbare, the peak transcript level appeared at 20 min, and then declined (Fig. 2b) indicating that *OsTPP1* was up-regulated a little bit earlier in Nipponbare under salt stress. Under osmotic stress, the expression pattern of *OsTPP1* in Nona Bokra was similar to that under salt stress (Fig. 2a), while in Nipponbare, *OsTPP1* was transiently and strongly induced to peak levels at 20 min, and then declined continuously, but still remained at a high level even after 24 h (Fig. 2b). Under cold stress, the expression of *OsTPP1* in Nona Bokra continuously increased for 24 h, and remained at high levels during the entire 72 h study period (Fig. 2a). Quantitative Real-Time PCR data from Nona Bokra indicated up to a 20-fold increase in transcript levels following cold stress treatment (Fig. 2c), while in Nipponbare, the induction was earlier, and the peak level appeared in 3 h. However the induction level was lower than that in Nona Bokra (Fig. 2a). We also analyzed the expression of *OsTPP1* when exposed to exogenous ABA (10 μM) in Nona Bokra (Fig. 2a). The result indicated that the expression pattern was similar to that under salt stress. These combined data demonstrated that *OsTPP1* was induced by salt, osmotic and cold stress, as well as exogenous ABA.

OsTPP1 overexpression in rice enhanced tolerance to salt and cold stresses

To date, little is known regarding the role of *OsTPP1* in rice under abiotic stress. To elucidate the function of *OsTPP1* under these conditions, we overexpressed *OsTPP1* in Nipponbare under the control of the CaMV 35S promoter using a pHB expression vector (Mao et al. 2005). In total, 28 positive transgenic lines were screened by hygromycin and re-affirmed by Basta. Most of them showed a normal phenotype during the whole life cycle under normal growing conditions, no alterations in the traits such as plant height and seed yield were observed. Stable inherited homozygous transgenic lines were obtained at the T2 generation. Their descendants (T3 or T4) were used for further phenotypic analysis.

We selected three transgenic lines to examine the expression levels of *OsTPP1* using RT-PCR. As expected, we found that *OsTPP1* was overexpressed in transgenic plants (Fig. 3a). To further investigate whether trehalose accumulated in transgenetic rice, HPIC analysis was performed to quantify trehalose levels in the shoots of transgenetic rice. Before salt stress, the trehalose content in the overexpression lines and vector line was about 70 μg gFW^{-1}, and after salt stress for 4 days, the content was increased to about 115 μg gFW^{-1} (Fig. 3b). Though the content before or after stress in the overexpression lines seemed higher than that in vector line, the difference was not significant (Fig. 3b).

To examine the tolerance of transgenic plants to abiotic stress, two-week-old seedlings were exposed to salt (125 mM NaCl). Following 1 week of exposure, seedlings exhibited salt induced damage symptoms such as wilted leaves (data not shown). However, after prolonged salt treatment, overexpression lines were more vigorous, and more leaves of them remained green and alive compared with the control (Fig. 4b). No differences between overexpression lines and the control were noted following osmotic stress treatment, with wilting and death of all seedlings (data not shown). Seedlings (two-week-old) were grown at 6–8°C for 18 days to investigate the effects of cold tolerance. Subsequently, the plants were transferred to normal conditions to restore growth. Cold treatment resulted in dehydration and wilting of control plants, however *OsTPP1* overexpression lines were less damaged and hydrated (Fig. 4c). Additionally, overexpression lines were mostly able to recuperate and grow during the restorative post-cold

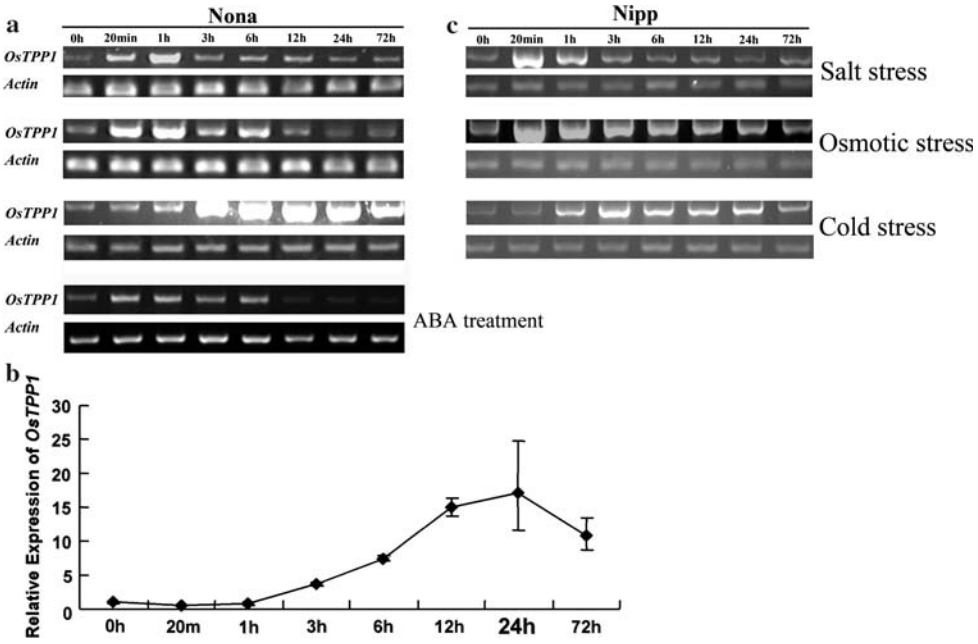

Fig. 2 Expression of *OsTPP1* under salt, osmotic and cold stresses and exogenous ABA treatment. **a** Semi-quantitative RT-PCR analysis of *OsTPP1* expression under salt, osmotic and cold stress and exogenous ABA treatment in Nona Bokra. **b** Real-Time PCR analysis of expression of *OsTPP1* under cold stress in Nona Bokra. Error bars mean ± SE ($n = 3$). **c** Semi-quantitative RT-PCR analysis of *OsTPP1* expression under salt, osmotic and cold stress in Nipponbare. Total RNA from the shoots of the two-week-old seedlings was used for RT-PCR analysis. Salt stress: 150 mM NaCl; Osmotic stress: 20% PEG4,000; Cold stress: 6°C; ABA treatment: 10 μM ABA

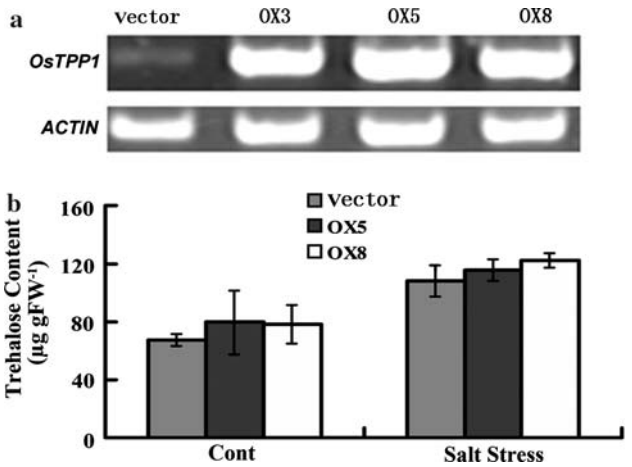

Fig. 3 *OsTPP1* was overexpressed in independent transgenic lines. **a** Semi-quantitative RT-PCR confirmed *OsTPP1* was overexpressed in transgenic lines. **b** Trehalose content in shoots of transgenetic rice before and after salt treatment (125 mM NaCl). Vector line, a line transformed with an empty vector; OX5, OX8, two independent overexpression lines. *Cont*, control (before salt treatment). *Error bars* mean ± SD ($n = 3$)

period while control lines almost died (Fig. 4d). These results indicated that *OsTPP1* plays an important role in salt or cold stress.

OsTPP1 overexpression in rice enhanced the seed germination rate under salt stress

In order to further analyze salt tolerance of *OsTPP1* overexpression lines, we examined the seed germination ability of the transgenic plants under salt stress. Seeds were immersed in water with or without salt (60 or 100 mM NaCl) at 30°C for 5 days, the germinated seeds were counted and the germination rate was calculated. When immersed in water, the differences between the vector line and overexpression lines were not significant (Fig. 5). While in salt solution, the seed germination rate was obviously decreased (even at a low concentration of 60 mM). However, compared with the vector line, the germination rate of overexpression lines OX5 and OX8 remained relative high at both 60 and 100 mM salt environments. In contrast, the empty vector line exhibited almost the same germination rate with the overexpression lines when immersed in water, but when in a salt environment, the germination rate was significantly lower than the overexpression lines (Fig. 5). This result indicated that overexpression of *OsTPP1* in rice enhanced seed germination rate under salt stress.

OsTPP1 overexpression altered the expression of stress responsive genes

Although overexpression of *OsTPP1* improved salt and cold tolerance (Figs. 4, 5), the trehalose content was not significantly increased compared with the vector line (Fig. 3b). Therefore, we asked if *OsTPP1* conferred abiotic stresses via other pathways rather than accumulating trehalose as a protective mechanism, for example activating stress tolerance related genes. To examine this hypothesis, we investigated the expression levels of ten stress responsive genes, *Lip5* (Aguan 1991), *Lip9* (Aguan 1991), *OsAsr1* (Vaidyanathan 1999), *SKC1* (Ren et al. 2005), *WSI18* (Saijo et al. 2000), *DREB1B* (Dubouzet et al. 2003), *OsMPK3* (Agrawal et al. 2002; Garg et al. 2002), *OsMPK4* (NM_001071691), *OsMPK6* (Agrawal et al. 2002; Garg et al. 2002) and *OsMKK1* (AJ507154) under normal

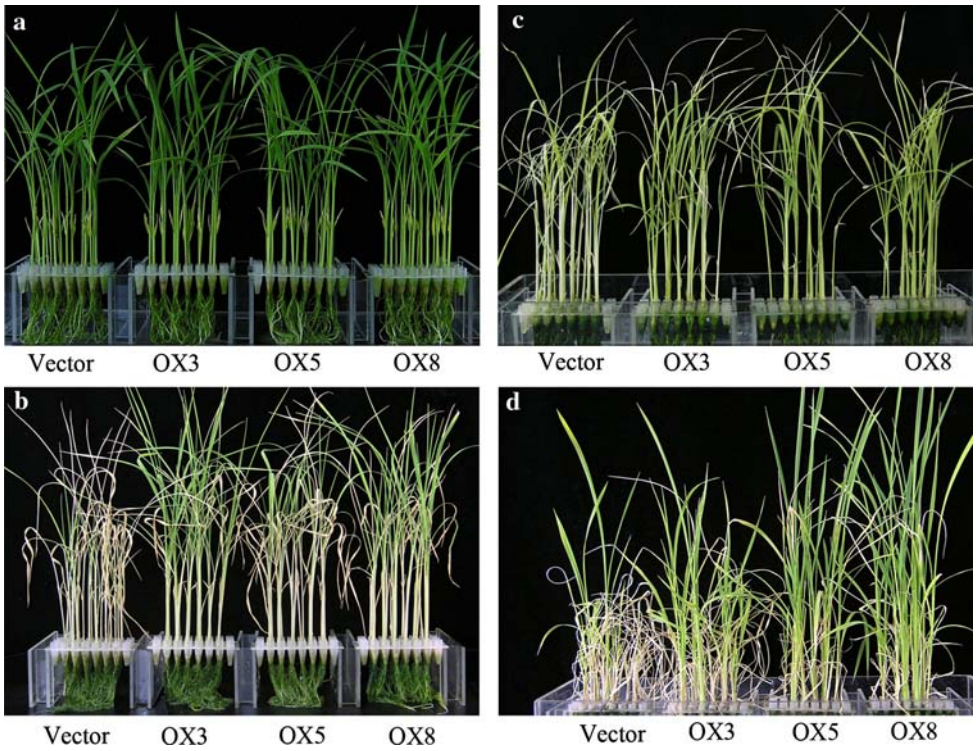

Fig. 4 Overexpression of *OsTPP1* enhanced rice tolerance to salt and cold stresses. **a** Two-week-old seedlings were grown under normal conditions before stresses. **b** Seedlings after salt stress for 11 days. **c** Seedlings after cold stress for 18 days. **d** Seedlings were grown under normal conditions for 21 days for restored growth, following by 18 days of cold stress

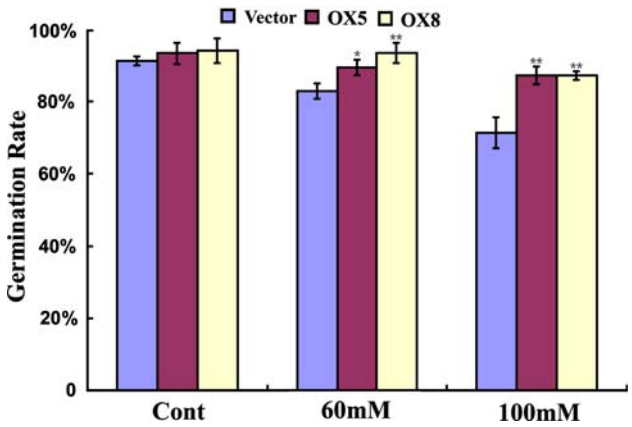

Fig. 5 Seed germination rate of the transgenic plants under salt stress. The *asterisk* ($P < 0.05$) and *double asterisks* ($P < 0.01$) indicate statistically significant differences of OX5 and OX8 compared to Vector line. *Cont* control (without salt treatment). *Error bars* indicate ± SD ($n = 3$)

conditions in *OsTPP1* overexpression lines by RT-PCR and Real Time PCR. Among these genes, all but *OsMPK4* and *OsMKK1* have been reported to be induced by abiotic stress and consequently used as marker genes. *SKC1* and *WSI18* displayed no difference in expression levels between the overexpression and vector lines (Fig. 6a). The cold responsive genes *Lip5*, *Lip9* and the osmotic responsive gene *OsAsr1* were slightly up-regulated in the overexpression lines (Fig. 6a, b). *DREB1B* which was notably up-regulated by cold stress (Dubouzet et al. 2003) was also slightly up-regulated in the overexpression lines (Fig. 6a). Recently, many Mitogen-Activated Protein Kinase (MAPK) genes in rice were found to be involved in abiotic stress response (Agrawal et al. 2002; Wen et al. 2002). Therefore, we investigated four of these genes including *OsMPK3*, *OsMPK4*, *OsMPK6* and *OsMKK1*. Among these genes, *OsMPK4*, *OsMPK6* and *OsMKK1* were the closest homologues of *AtMPK4*, *AtMPK6* and *AtMKK2* respectively, which were well characterized in the signaling of abiotic stress in Arabidopsis (Ichimura et al. 2000; Droillard et al. 2004; Teige et al. 2004). *OsMPK6* and *OsMKK1* displayed no expression difference between the vector and overexpression lines. However, *OsMPK3* and *OsMPK4* were up-regulated in the overexpression lines (Fig. 6a, b), especially *OsMPK3*. The expression of *OsTPS1* in the overexpression lines was also lightly up-regulated (Fig. 6a, b). These results suggested that *OsTPS1* was possibly activated under a positive feedback mechanism regulated by *OsTTP1*, and in vivo, *OsTPS1* might function as trehalose-6-phosphate synthase. Our data demonstrated that *OsTPP1* plays a role in the activation of stress response genes, which might be the main reason for the enhanced tolerance to abiotic stress of *OsTPP1* overexpression lines.

Discussion

Abiotic stresses are the main factors responsible for the reduction of yield and quality of crops. The goal of much recent research has been to elucidate the mechanisms of plant acclimation to abiotic stresses and thus improve crop

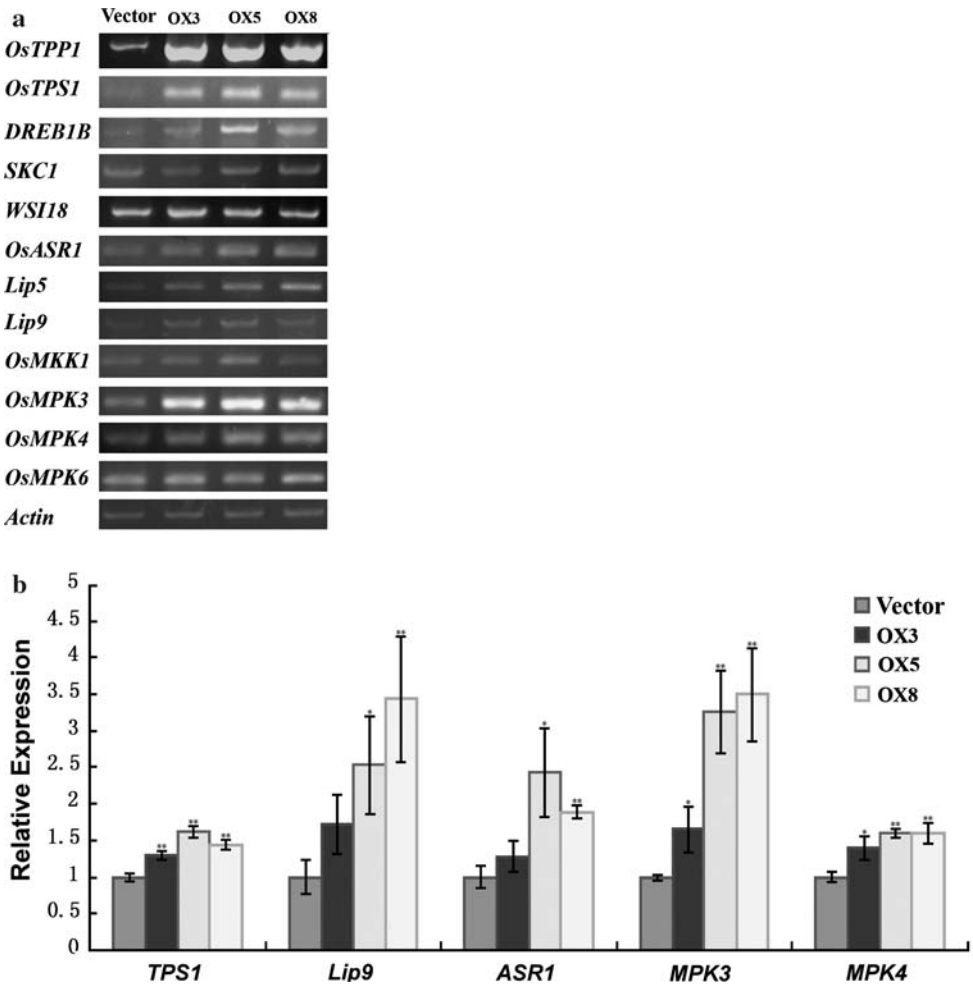

Fig. 6 Expression of stress related genes in *OsTPP1* overexpression lines under normal conditions. Total RNA for expression analysis was isolated from shoots of two-week-old transgenetic seedlings. Expression of stress responsive genes were determined by semi-quantitative RT-PCR (**a**) or Real Time PCR (**b**). The *asterisk* ($P < 0.05$) and *double asterisk* ($P < 0.01$) indicate statistically significant differences of overexpression lines compared to vector line. *Error bars* indicate ± SD ($n = 3$)

tolerance. While overexpression of *E. coli.* TPS and TPP fusion proteins have been shown to increase abiotic stress tolerance in rice (Garg et al. 2002; Jang et al. 2003), little is known regarding the role of endogenous TPP and TPS in plants. Previous research suggested that many *TPS* and *TPP* homologues, especially *OsTPP1* in rice were induced by salt stress (Chao et al. 2005; Pramanik and Imai 2005), leading us to further investigate their roles in abiotic stress.

A large TPS and TPP family present in rice genome suggests trehalose metabolism plays an important role in plant life

To date, 11 TPS and 10 TPP genes have been identified in Arabidopsis (Leyman et al. 2001; Schluepmann et al. 2004), and many TPP homologues were also found in maize (Satoh-Nagasawa et al. 2006). Some of these genes have been demonstrated to encode functional TPSs or TPPs (Blazquez et al. 1998; Vogel et al. 1998, 2001; Eastmond et al. 2002; Pramanik and Imai 2005; Satoh-Nagasawa et al. 2006). The present study identified a larger gene family in the rice genome, containing at least 14 TPS loci and 13 TPP loci. According to the phylogenetic analysis, they could be grouped into three classes, similar as the gene family structure of Arabidopsis (Leyman et al. 2001). Our data suggested that the gene family is conserved in plants, and formed prior to the divergence of dicotyledonous and monocotyledonous angiosperms.

The large TPS/TPP family led us to question whether they are functional redundancies or serve different roles in the regulation of trehalose levels. Although functional redundancies may exist, diverse functions in regulatory properties of plant life are more likely. We noticed that expression patterns of many TPPs are different, for example, TPP homologues *RA3* and *SAR* in maize (Satoh-Nagasawa et al. 2006), *SAR* (*OsTPP8* named in this study) (Satoh-Nagasawa et al. 2006) and *OsTPP1* in rice. These results indicated that genes in this family expressed with the spatio-temporal difference, and therefore exerted different functions to the plant. Secondly, the mutation of a single TPS or TPP gene often resulted in obvious plant defects (Eastmond et al. 2002; Satoh-Nagasawa et al. 2006). These

observations demonstrated that other genes could not complement the mutation, therefore their functions were obviously redundant.

OsTPP1 is fine tuned under abiotic stress

Pramanik and Imai (2005) reported that *OsTPP1* was strongly induced by low temperature in *Japonica* rice cultivar Yukihikari. In addition, the induction under moderate or severe cold stress was quite different not only in root but also in shoot. Under other abiotic stress such as salt and drought or ABA treatment, the transcription of *OsTPP1* was also quickly activated. In our study, a similar induction of *OsTPP1* was also observed in both the *Japonica* rice Nipponbare and *Indica* rice Nona Bokra. These results indicated that *OsTPP1* was fine tuned under abiotic stress. Considering that *OsTPP1* was induced differently under different temperature regimes (Pramanik and Imai 2005), the expression difference under cold stress found in the present work and previously (Pramanik and Imai 2005) was possibly caused by chilling temperature.

Overexpression of *OsTPP1* enhanced salt and cold tolerance of rice via constructively activating abiotic stress-induced genes

We generated *OsTPP1* overexpression lines to investigate *OsTPP1* involvement in rice abiotic stress tolerance. When challenged with high salt and low temperature, overexpression lines exhibited greater tolerance compared to those transformed with an empty vector. Testing the seed germination rate under salt treatments showed that overexpression lines were also more tolerant to salt than vector lines. These results confirmed that *OsTPP1* plays a role in abiotic stress response, and further proposes its use in abiotic stress engineering.

The significant increase in trehalose levels in overexpression lines was not observed in this study. This may have been due to the fact that trehalose is synthesized in two steps and in the current study only the second step was affected. It was difficult to accept that the enhanced salt and cold tolerance of rice by *OsTPP1* was mainly due to the protective role of trehalose. We thus investigated the transcript levels of some well-known abiotic stress-induced genes in *OsTPP1* overexpression lines and observed that *Lip5*, *Lip9* and *OsAsr1* were constitutively and slightly activated in these lines, as well as *DREB1B*, which was demonstrated to be induced only by cold. These data revealed that *OsTPP1* triggers, at least in part, stress inducible genes and suggested a novel regulation pathway under abiotic stress. Many studies have indicated that MAPK signal transduction modules play crucial roles in regulating plant biological processes including responses to abiotic stress. In our research, two Mitogen-Activated Protein Kinases, *OsMPK3* and *OsMPK4*, were also up-regulated in the overexpression lines. *OsMPK4* is the closest homologue of *AtMAPK4* which is up-regulated by abiotic stress (Ichimura et al. 2000; Droillard et al. 2004). In our study, *OsMPK4* was also induced by salt stress, as well as *OsMPK3* (data not shown). In previous reports, *OsMPK3* was induced by various abiotic stresses and also involved in a moderate low-temperature signaling pathway in rice (Wen et al. 2002). Hence, we speculated that constitutively activation of *OsMPK3* possibly contributed greatly to the enhanced tolerance of the overexression lines. In the present study, *OsTPS1* was also up-regulated in the overexpression lines. Possibly it was the feedback of decreased T-6-P content caused by overexpressed *OsTPP1* that in vivo indirectly activated the TPS activity of OsTPS1.

Although we cannot exclude the possibility that OsTPP1 might activate its downstream genes by directly regulating one or more transcription factors, it is more likely that the regulatory role of OsTPP1 is mediated by its substrate (T-6-P) or production (trehalose). Recently, T-6-P has been widely accepted to be a regulating molecule in plant sugar metabolism and development (Eastmond and Graham 2003; Eastmond et al. 2003; Avonce et al. 2004). Though there is still no direct evidence, it is reasonable to propose that T-6-P plays a regulating role in the response to abiotic stress.

In conclusion, we demonstrated that *OsTPP1* functions as a T-6-P phosphatase and is regulated by abiotic stresses. Overexpression of *OsTPP1* enhanced rice tolerance to salt and cold. Additionally, *OsTPP1* triggered a series of abiotic response genes contributing to rice stress tolerance. Our work provides a novel insight into the functional mechanism of trehalose metabolism, and also presents potential applications in abiotic stress engineering.

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