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# Analysis of trehalose-6-phosphate synthase (TPS) gene family suggests the formation of TPS complexes in rice

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**Abstract** Trehalose-6-phosphate (T6P), an intermediate in the trehalose biosynthesis pathway, is emerging as an important regulator of plant metabolism and development. T6P levels are potentially modulated by a group of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) homologues. In this study, we have isolated 11 *TPS* genes encoding proteins with both TPS and TPP domains, from rice. Functional complement assays performed in yeast *tps1* and *tps2* mutants, revealed that only *OsTPS1* encodes an active TPS enzyme and no OsTPS protein possesses TPP activity. By using a yeast two-hybrid analysis, a complicated interaction network occurred among OsTPS proteins, and the TPS domain might be essential for this interaction to occur. The interaction between OsTPS1 and OsTPS8 in vivo was confirmed by bimolecular fluorescence complementation and coimmunoprecipitation assays. Furthermore, our gel filtration assay showed that there may exist two forms of OsTPS1 (OsTPS1a and OsTPS1b) with different elution profiles in rice. OsTPS1b was particularly cofractionated with OsTPS5 and OsTPS8 in the 360 kDa complex, while OsTPS1a was predominantly incorporated into the complexes larger than 360 kDa. Collectively, these results suggest that O*sTPS* family members may form trehalose-6-phosphate synthase complexes and therefore potentially modify T6P levels to regulate plant development.

**Keywords** Trehalose-6-phosphate synthase · Interaction · Complex · Trehalose-6-phosphate · Rice

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## Introduction

Trehalose is a ubiquitous disaccharide and is usually synthesized by the classic two-step process in a variety of organisms including bacteria, fungi, plants, and invertebrates (Elbein et al. 2003). Firstly, trehalose-6-phosphate synthase (TPS) catalyzes the synthesis of trehalose-6-phosphate (T6P) from UDP-glucose and glucose-6-phosphate. Secondly, T6P is dephosphorylated to trehalose by trehalose-6-phosphate phosphatase (TPP). Trehalose is abundant in certain microorganisms and insects, where it functions as a carbon source as well as an osmoprotectant

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(Elbein 1974; Elbein et al. 2003). In few resurrection plants, such as *Selaginella lepidophylla* and *Myrothammus flabellifolius*, trehalose can also accumulate to high levels and act as a stress protectant during desiccation (Adams et al. 1990; Drennan et al. 1993). Although only trace amounts of trehalose are detected in the majority of higher plants (Muller et al. 1995), trehalose may play an important role in the protection of plants from osmotic stress. Trehalose in rice can accumulate in low levels after NaCl treatment and exogenously applied trehalose can decrease NaCl-induced growth inhibition (Garcia et al. 1997). Transgenic plants that expressed TPS and/or TPP genes usually showed increased tolerance to abiotic stress (Garg et al. 2002; Ge et al. 2008; Goddijn et al. 1997; Holmstrom et al. 1996; Jang et al. 2003; Karim et al. 2007; Miranda et al. 2007; Romero et al. 1997). However in some cases, even if the increased amount of trehalose was limited, transgenic plants exhibited phenotypic alterations (Goddijn and van Dun 1999; Goddijn et al. 1997; Romero et al. 1997). Several studies using mutant plants have revealed the importance of trehalose metabolism in the control of plant development (Avonce et al. 2004; Chary et al. 2007; Eastmond et al. 2002; Gomez et al. 2006; Satoh-Nagasawa et al. 2006; van Dijken et al. 2004).

Although the role of trehalose is ill-defined, additional evidence highlights the specific functions of the biosynthetic precursor of trehalose, T6P, suggesting that T6P plays a crucial role in controlling plant metabolism and development. Remarkably, accompanied by upward versus downward alterations of T6P levels, transgenic plants expressing heterogenous *E. coli otsA* (*TPS*) showed opposite effects to those expressing *E. coli otsB* (*TPP*) on growth and photosynthetic capacities (Pellny et al. 2004; Schluepmann et al. 2003). T6P was initially found to regulate carbohydrate utilization in *Arabidopsis* (Schluepmann et al. 2003), and it seemed to modulate two pivotal signaling routes in plants. Firstly, T6P plays a major role in controlling the entry into starch synthesis by modulating the activity of ADP-glucose pyrophosphorylase (Kolbe et al. 2005; Schluepmann et al. 2004; Wingler et al. 2000), which catalyzes the first rate-limiting step of starch synthesis. Secondly, a recent study showed that T6P can inhibit the activity of Snf1-related protein kinase (SnRK1) (Zhang et al. 2009) which is an important integrator in the regulatory network of plant metabolism, development and stress response (Baena-Gonzalez et al. 2007). Moreover, the expression of some *TPS* genes is also affected by the carbon status in *Arabidopsis* and rice. The expression of three *TPS* genes of *Arabidopsis* and four *TPS* genes of rice increased significantly in response to sucrose starvation in suspension culture cells (Contento et al. 2004; Wang et al. 2007). In *Arabidopsis*, sucrose feeding results in a rapid induction of *AtTPS5*, but a repression of *AtTPS8, AtTPS9, AtTPS10* and *AtTPS11* (Osuna et al. 2007; Schluepmann et al. 2004). Similarly, *AtTPS5* is induced and *AtTPS8*, *AtTPS*9 and *AtTPS*10 are repressed by glucose addition (Price et al. 2004).

Owing to the numerous effects on plant metabolism and development, T6P levels should be precisely controlled. Intriguingly, a large number of putative genes encoding enzymes of trehalose synthesis were found in the genomes of higher plants (Avonce et al. 2006; Lunn 2007). For example, in Arabidopsis, there is a multigene family with 11 TPS and 10 TPP members. Eleven TPS proteins putatively have both TPS and TPP domains and can be grouped into two classes, depending on their similarity to ScTPS1 or ScTPS2 (Leyman et al. 2001). Class I comprises AtTPS1–4, and AtTPS1 possesses TPS activity (Blazquez et al. 1998). Class II includes AtTPS5–11, and has neither TPS nor TPP activity (Ramon et al. 2009; Vogel et al. 2001). All 10 TPP proteins contain only one TPP domain, which make up Class III. Among them, TPP activities of AtTPPA and AtTPPB have been reported (Vogel et al. 1998). In *Arabidopsis*, only AtTPS1 has been demonstrated to be an active TPS enzyme and Class II TPS proteins are assumed to be regulatory proteins (Vandesteene et al. 2010). However, the direct association of these Class II TPS with active enzymes remains unclear. In this study, we report the isolation and molecular characterization of 11 *TPS* genes from rice. Among these 11 members, only *OsTPS1* encoded an active TPS, and none showed TPP activity in yeast. In addition, we confirm that there are complex interactions among OsTPS1–11 and these OsTPS proteins likely form TPS complexes in rice.

## Materials and methods

### Gene cloning and sequence analysis

To search for homologous TPS sequences of rice, the BLAST program was used with National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/), the MSU Rice Genome Annotation Resource (Ouyang et al. 2007) and the Beijing Genomics Institute Rice Information System (Zhao et al. 2004). The full length open reading frames (ORF) of these *OsTPS* genes were amplified by reverse transcription polymerase chain reaction (RT–PCR). Primers were designed based on previously published sequences of full-length cDNAs and/or genes predicted by the MSU gene models. Due to GC richness in the *OsTPS1* 5′ region, a pair of flanking primers was adopted: 5′ CATCTCTCTCTCCTCC 3′ and 5′ CT ACTGCCTATCCAAGAACATG 3′. Total RNA was extracted from rice (*Oryza sativa* L. subsp. indica cv. “Guangluai 4”) using TRIzol reagent (Invitrogen), reverse transcription was done using SuperScript II RNase $H^{-}$

reverse transcriptase (Invitrogen) to produce 1st strand cDNA. Candidate genes were amplified by *TaKaRa LA Taq*™ with GC Buffer, and primer pairs are listed in Supplementary Table 1. The following PCR parameters were used: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 56 or 58°C for 45 s, and 72°C for 3 min. The PCR products were cloned into pGEM®-T vector (Promega) and verified by DNA sequencing. The phylogenetic relationships were analyzed among these OsTPS proteins and other previously reported TPS and TPP sequences of *Arabidopsis thaliana, Selaginella lepidophylla, Oryza sativa, Saccharomyces cerevisiae* and *Escherichia coli*. The amino acid sequences of TPS and TPP domains of these proteins were retrieved by SMART software (Schultz et al. 1998) and were aligned using Clustal_X (Thompson et al. 1997). Tree reconstruction was performed by applying the neighbor joining or maximum likelihood methods with the PHYLIP package (Felsenstein 1993), with 1,000 bootstrap replicates, and the corresponding consensus trees were obtained.

### Yeast complementation assays

The following yeast strains were used: wild-type (WT) *S. cerevisiae* strain W303-1A (*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 GAL mal SUC2)* and the isogenic *tps1* mutant YSH6.127.-17C (*MAT*a *tps1*△*:: TRP1)* (Bell et al. 1998). The *ScTPS2* gene deletion strain was constructed as previously described (Wang et al. 2005). The *Trp* marker gene replaced the fragment from position 208–890 bp of the *TPS2* gene through homologous recombination mediated by PCR products (Lorenz et al. 1995). The *tps2* strains were screened on synthetic dropout (SD) medium lacking tryptophan at 30°C.

pGAL, a modified pGADT7 plasmid without the active domain of GAL4, was used as an expression vector. Plasmid pGADT7 (Clontech) was digested with *Hind* III, an 801 bp fragment containing the active domain was replaced by a 389 bp fragment without the active domain, which was amplified from pGADT7 with the primers 5′ CACCAAGCTTGAGATCTTTAATACGACTCAC 3′ (sense primer) and 5′ GAAGAAGTCCAAAGCTTC 3′ (antisense primer). Each full-length *OsTPS* ORF was digested from pGEM®-T vector and inserted into pGAL vector (restriction enzyme cutting sites are underlined in Supplementary Table 1). As a TPS positive control, the *ScTPS1* gene was isolated from yeast genomic DNA by PCR using primers 5′ CACCCAT**ATG**ACTACGGATAC GCTAA 3′ (sense primer) and 5′ TCGAGGATCCGTTT TTGGTGGCAGAGGA 3′ (antisense primer). We adopted the *OsTPP1* gene as a TPP positive control. *OsTPP1* gene was obtained by RT–PCR, the following PCR primer pair was used: 5′ CACCCAT**ATG**GATTTGAGCAATAGCTC 3′ (sense primer) and 5′ GCAGGTCGACCACTGAGTGC TTCTTCCA 3′ (antisense primer).

Yeast was grown at 30°C in rich or minimal medium with the appropriate auxotrophic requirements plus 2% galactose (Gal) or glucose (Glu) as previously described (Bell et al. 1998). Transformation was performed as previously described (Elble 1992), and transformants were selected in synthetic medium without leucine (SD-Leu).

### Yeast two-hybrid analysis

Yeast two-hybrid assays were performed by using the MATCHMAKER Two-Hybrid System 3 (Clontech) according to the manufacturer's instructions. All full-length *OsTPS* ORFs were inserted into pGBKT7 and pGADT7 individually, restriction enzyme cutting sites are shown in Supplementary Table 1. All possible combinative pairs were cotransformed into yeast strain AH109 and transformants were grown on synthetic dropout medium without the amino acids leucine and tryptophan (SD-Leu/-Trp). One millimeter diameter yeast colonies were selected and diluted in 100 μl sterile $H_2O$; 6 μl of each cell suspension was spotted onto SD minimal medium without adenine, histidine, leucine and tryptophan (SD-Ade/-His/-Leu/-Trp) to detect protein–protein interaction, and plates were incubated at 30°C until colonies appeared (2–6 days).

To identify the interacting regions of OsTPS5 and OsTPS8 with other OsTPS homologues, TPS or TPP domain deletions were constructed. The candidate gene fragments were amplified by PCR (primers listed in Supplementary Table 2). The *Eco*R I/*Xho* I fragments of *OsTPS5(1-551), OsTPS5(519-750), OsTPS8(1-601)* and *OsTPS8(498-824)* were subcloned into pGADT7. Interactions between the candidate gene fragments and all full length genes were detected by yeast two-hybrid assays.

### Protoplast isolation and bimolecular fluorescence complementation analysis

Rice protoplasts were isolated in accordance with previous reports (Bart et al. 2006). Stem tissues of etiolated 10–12 day old seedlings were cut into approximately 0.5 mm strips, the tissue pieces were immediately placed into a Petri dish with enzyme solution (0.6 M mannitol, 10 mM MES (pH 5.7), 1.5% Cellulase RS, 0.4% Macerozyme, 0.1% BSA, 1 mM $CaC1_2$, 5 mM $\beta$-mercaptoethanol and 100 μg/ml ampicillin). The chopped tissue was vacuum-infiltrated for 1 h and continuously digested for 3 h in the dark with gentle shaking (40 rpm). After incubation, the enzyme solution containing protoplasts were filtered through a 35 μm nylon mesh and centrifuged at 300×*g* for 5 min at 4°C to pellet the cells. The pelleted

protoplasts were washed once in cold W5 solution (154 mM NaCl, 125 mM $CaC1_2$, 5 mM KC1, 2 mM MES (pH 5.7)) and resuspended in the same solution to a concentration of 1–2 × $10^6$ cells/ml. Protoplasts were resuspended at the same density in MMG solution (0.6 M mannitol, 15 mM $MgC1_2$, 4 mM MES (pH 5.7)) before PEG-mediated transformation. The PEG transfection experiments was carried out as previously described (Sheen 2002), 10 μl plasmid DNA (about 10 μg DNA of each construct) were mixed with 100 μl of suspended protoplasts. After that, 110 μl 40% PEG (0.6 M mannitol, 100 mM $CaC1_2$, 40% (v/v) PEG4000) was added, and then the mixtures were incubated for 20–30 min at room temperature. After incubation, 0.44 ml W5 solution was added to the tube to dilute PEG. Cells were resuspended in 1 ml W5 medium and incubated 16 h at 28°C in the dark.

A pair of split-YFP vectors was used for BiFC assays, pUC-SPYNE and pUC-SPYCE enables the expression of proteins of interest fused either to an N-terminal YFP (amino acids 1–155) or to a C-terminal YFP (amino acids 156–239) driven by CaMV 35S promoter (Walter et al. 2004). The full length and N-terminal regions of *OsTPS1, OsTPS5* and *OsTPS8* gene were amplified by PCR (primers listed in Supplementary Tables 2 and 3). The *OsTPS1* and its N-terminal fragment digested with *Bam*H I/*Xho* I were inserted into the pUC-SPYNE and pUC-SPYCE, respectively. The *Xba* I/*Xho* I sites were used to construct in-frame fusions of *OsTPS5, OsTPS8* and their N-terminus with split-YFP.

Fluorescence microscopy was performed using an Olympus BX51 research microscope. YFP fluorescence was visualized under excitation and emission filters U-MNIB3 (Ex470-495/DM505/BA510IF); images were captured with U Series Digital camera (Apogee Instruments, Inc).

### Construction of expression plasmids and generation of transgenic rice plants

6× Myc epitope tags were PCR-amplified from pYL436 vector (accession no. AY737283) and introduced into pENTR™ D-TOPO® cloning vector (Invitrogen). *OsTPS1(41-985)*, *OsTPS1(131-985)* fragments and *OsTPS5*, *OsTPS8* full length genes were inserted before the 6× Myc epitope tags to generate the entry clone separately. Every gene fused with C-terminal Myc epitope tag was recombined into the pHAC vector by the LR recombination reaction (Invitrogen). pHAC was modified from pH2GW7 (Karimi et al. 2002), where the rice *OsAct1* promoter was used instead of the cauliflower mosaic virus (CaMV) 35S promoter.

Each of these expression constructs with Myc tags was introduced into *O. sativa L. subsp. Japonica cv. Nipponbare* by an *Agrobacterium*-mediated cocultivation method (Hiei et al. 1994). The plantlets, regenerated from calli resistant to hygromycin B, were transferred to soil and grown in a greenhouse to maturity.

### Antibodies, gel filtration chromatography and immunoprecipitation in vivo

A fragment containing nucleotides 1,651–2,952 of *OsTPS1* and the full length ORF of *OsTPS8* were individually cloned into the pET-30a expression vector. The 6 × His-tag fusion proteins were expressed in *E. coli* strain BL21(DE3). Polyclonal antibodies were raised by immunizing rabbits with the purified fusion proteins as antigens. To detect the specificity of anti-OsTPS1 antibody and anti-OsTPS8 antibody, full length OsTPS1 and OsTPS8 proteins were expressed in BL21(DE3) as His-tagged proteins. 2 μg and 500 ng purified His-OsTPS1 or His-OsTPS8 proteins were boiled in sample buffer, run on SDS–PAGE gels, and blotted onto polyvinylidene difluoride membranes (Millipore). A sample incubated with anti-OsTPS1 antibody and a parallel sample incubated with anti-OsTPS8 antibody.

Fourteen-day-old rice leaf tissues of WT and transgenic lines were ground in liquid $N_2$ and homogenized in extraction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% Nonidet P-40, freshly added 1 mM phenylmethylsulfonyl fluoride, and 1× Roche Complete Protease-Inhibitor Cocktail). The extract was centrifuged at 4°C for 15 min, the supernatant was filtered through a 0.22 μm filter and protein concentration was measured by a Bradford assay kit (Bio-Rad). About 200–300 μg total protein was injected on Superose 6 10/300 GL column (Amersham Biosciences), the gel filtration procedure has been described (Peng et al. 2001). Twenty-six fractions of 0.5 ml efflux were collected and concentrated using StrataClean™ Resin (Stratagene). Equal volumes of each fraction were loaded onto a 10% SDS–PAGE gel and blotted onto polyvinylidene difluoride membranes (Millipore). The blots were probed with anti-Myc (Sigma), anti-OsTPS1 and anti-OsTPS8.

For the coimmunoprecipitation assay, the lysis buffer was the same used for gel filtration. About 1 mg total protein was incubated with Myc affinity matrix (Covance) at 4°C for 3 h with gentle rotation. After incubation, Myc-affinity matrix was thoroughly washed in the same lysis buffer. Protein blot analyses were performed using different primary antibodies.

## Results

### Isolation and comparative analysis of rice *TPS* genes

By BLAST search analysis, 11 *TPS* gene loci were found in the rice genome. The full length ORFs of these 11 *OsTPS* genes (Table 1) were isolated by RT–PCR from *indica* rice variety Guangluai 4. OsTPS1–11 has similar domain organization, consisting of a TPS domain and a TPP domain in tandem (Fig. 1a). However, the gene structure of *OsTPS1* (Fig. 1b) is appreciably different from other *OsTPS* genes in that it involves 17 exons versus 2–3 exons. Moreover, OsTPS1 probably have a very long N-terminal extension in comparison with other OsTPS proteins (Fig. 1a). There are five ATG codons in frame in the putative first exon of *OsTPS1* gene (Fig. 1b). Although the protein OsTPS1(131-985) translated from the fifth ATG also contains the TPS domain sequence, several software programs that predicted gene models suggested that the first ATG is the putative initiation codon.

The evolutionary patterns of these rice TPS proteins revealed OsTPS1 and other OsTPS proteins might belong to two different clades (Fig. 2). The phylogenetic analysis was performed on the basis of the sequences in TPS or TPP conserved regions of the known TPSs and TPPs from rice, *Arabidopsis, S. lepidophylla, S. cerevisiae* and *E. coli.* Amino acid sequences of the TPS domain or the TPP domain were aligned using CLUSTAL_X (Thompson et al. 1997). Phylogenetic trees, which were generated by using neighbor joining and maximum likelihood methods with the PHYLIP software (Felsenstein 1993), showed similar results, and the dendrograms obtained by neighbor joining are shown (Fig. 2). Apparently, OsTPSs can also be grouped into two subfamilies, as are AtTPS1–11 (Leyman et al. 2001). Only OsTPS1 belongs to Class I, whereas this group in *Arabidopsis* contains four proteins (AtTPS1–4).

**Table 1** Trehalose-6-phosphate synthase gene family in rice

| Gene | Accession no. | Locus (MSU) | CDS length (bp) |
|---|---|---|---|
| *OsTPS1* | HM050424 | LOC_Os05g44210 | 2,958 |
| *OsTPS2* | HM050425 | LOC_Os01g54560 | 2,745 |
| *OsTPS3* | HM050426 | LOC_Os01g53000 | 2,637 |
| *OsTPS4* | HM050427 | LOC_Os03g12360 | 2,583 |
| *OsTPS5* | HM050428 | LOC_Os02g54820 | 2,253 |
| *OsTPS6* | HM050429 | LOC_Os05g44100 | 2,700 |
| *OsTPS7* | HM050430 | LOC_Os08g31980 | 2,589 |
| *OsTPS8* | HM050431 | LOC_Os08g34580 | 2,475 |
| *OsTPS9* | HM050432 | LOC_Os09g25890 | 2,661 |
| *OsTPS10* | HM050433 | LOC_Os09g23350 | 2,658 |
| *OsTPS11* | HM050434 | LOC_Os09g20990 | 2,592 |

The other OsTPS proteins are part of Class II, as are AtTPS5–11. All plant TPPs which have only a TPP domain constitute Class III (Fig. 2b).

Among plant Class I TPS proteins, AtTPS1 and SlTPS1 were proven to be plant active TPS enzymes (Blazquez et al. 1998; Zentella et al. 1999). OsTPS1 showed more similarity to them and was the sole member of this group in rice, which implies that OsTPS1 potentially has TPS activity. Moreover, further analysis of amino acid sequences supported this hypothesis. Study of the three-dimensional structure of *E. coli* TPS enzyme showed that some conserved residues were involved in substrate binding and catalysis (Gibson et al. 2002). These residues are very important for TPS enzymatic activity. Without some of them, ScTSL1, ScTPS3 and all *Arabidopsis* Class II AtTPS proteins were unable to replace the role of ScTPS1 in the yeast *tps1* mutant (Bell et al. 1998; Ramon et al. 2009; Vogel et al. 2001). OsTPS1 possesses all of these conserved residues, but other OsTPS proteins lacked some of them (Supplementary Fig. 1).

Unlike OsTPS1, all of the Class II OsTPSs have similar phosphatase boxes (Thaller et al. 1998) in the TPP domain (Supplementary Fig. 2). In *Arabidopsis*, phosphatase boxes are present in the Class II TPS proteins rather than in Class I TPS proteins (Leyman et al. 2001), which is another distinguishing feature between Class II TPSs and Class I TPSs.

### Only OsTPS1 complements *S. cerevisiae tps1* mutant

Since OsTPS1–11 showed homology to other active TPS enzymes, complementation studies in *S. cerevisiae tps1* mutant were used to determine whether they encode functional TPS proteins. The growth defect of yeast *tps1* mutant on glucose-containing medium is due to uncontrolled glycolysis, which results in the buildup of phosphorylated intermediates and Pi sequestration (Bell et al. 1992; Gonzalez et al. 1992). The full length ORF fragments of *OsTPS1–11* and *ScTPS1* were each ligated into pGAL vector which was modified from pGADT7 by deleting the GAL4 active domain region. Each of these constructs was transformed into the yeast *tps1* mutant, and the transformants expressing OsTPS proteins (Supplementary Fig. 3) were selected for functional complement experiments. Expression of *ScTPS1* could rescue *tps1* mutant cells from glucose toxicity, and the transformants grew normally when compared to WT strains transfected with the empty vector on glucose-containing medium. Driven by the strong *ADH1* promoter, expression of *OsTPS1* also recovered the growth of *tps1* mutant on the medium with glucose, whereas expression of the other 10 *OsTPS* genes did not rescue the glucose-sensitive defect of *tps1* mutant (Fig. 3a). Furthermore, the expression of OsTPS1(131-985), which lacks the N-terminal extension

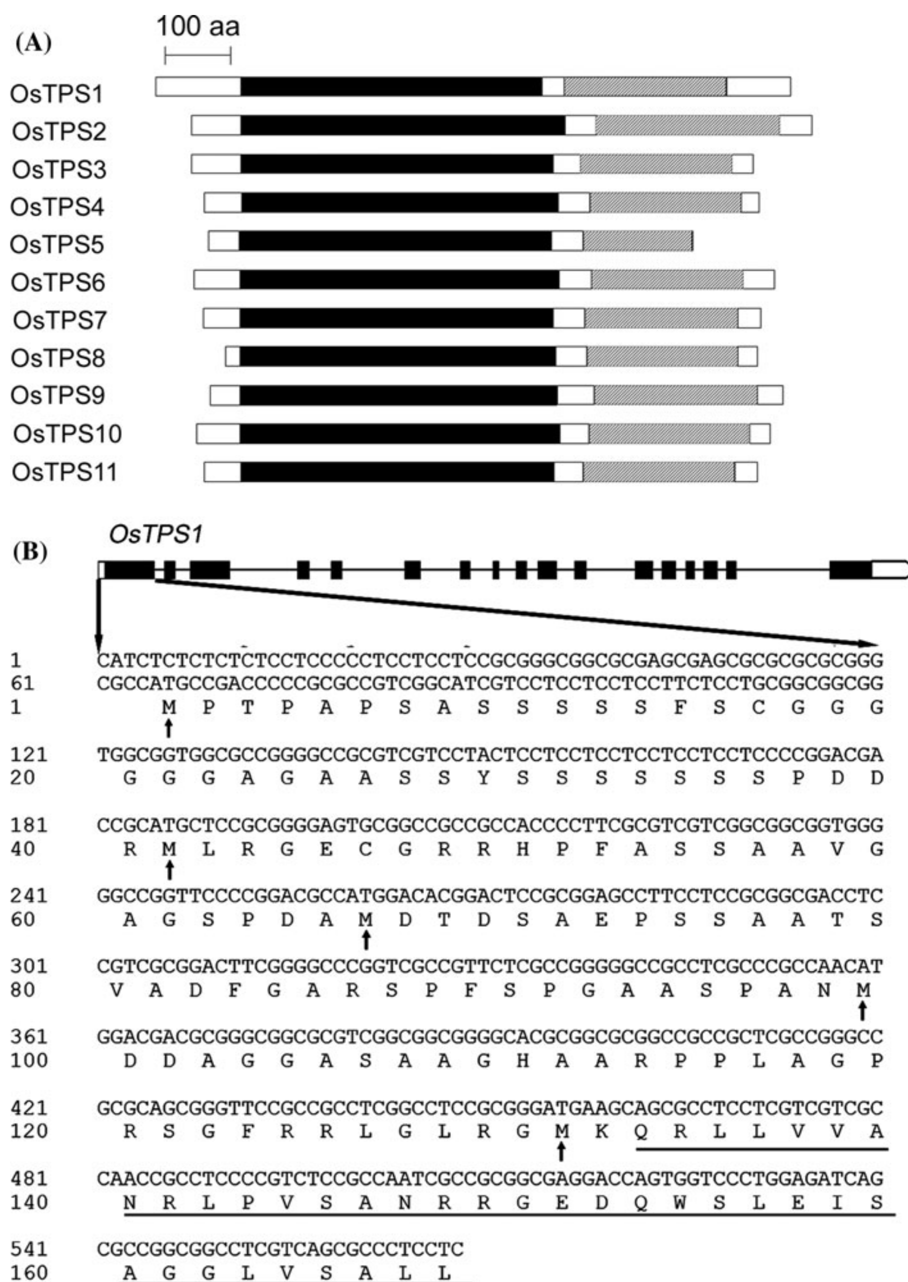


**Fig. 1** Architecture analysis of OsTPS homologues and structure of the *OsTPS1* gene. **a** Distribution of the TPS and TPP domain in OsTPS proteins. The protein features of OsTPS homologues were analyzed using SMART software. The TPS domain (Pfam: Glyco_transf_20) and the TPP domain (Pfam: Trehalose_PPase) are indicated as *gray rectangles*. **b** Structure of the *OsTPS1* gene. *Black rectangles* represent exons, *thin lines* represent introns, and *open rectangles* represent 5′ and 3′ UTRs. The leader sequence and the first exon of the *OsTPS1* gene are shown. Five ATG codons earmarked with *arrow* are in frame with the open reading frame sequence. The amino acid residues of the TPS domain were *underlined*

before the TPS domain, can also complement *tps1* mutant (data not shown).

## None of the *OsTPS* genes could complement *S. cerevisiae tps2* mutant

Class II TPSs contain phosphatase boxes which are conserved in the phosphatase superfamily (Thaller et al. 1998), which suggests that they possibly act as TPP enzymes. OsTPS proteins, with the exception of OsTPS1, belong to this subfamily. To explore whether OsTPS proteins have phosphatase activity, we expressed them individually (Supplementary Fig. 3) in a heat-sensitive *S. cerevisiae tps2* mutant (De Virgilio et al. 1993). In addition, OsTPP1, a member of Class III which was identified as an active TPP enzyme (Pramanik and Imai 2005), was adopted as a positive control. Wild type yeast cells transformed with the empty vector grew well at both 30 and 38.6°C, but the *tps2* mutant could not grow at 38.6°C (Fig. 3b). Only OsTPP1 restored the growth of *S. cerevisiae tps2* mutant under high temperature conditions. The inability of *tps2* mutant expressing *OsTPS1–11* to grow under high temperature conditions indicated that none of OsTPS proteins encoded a functional TPP enzyme in yeast.

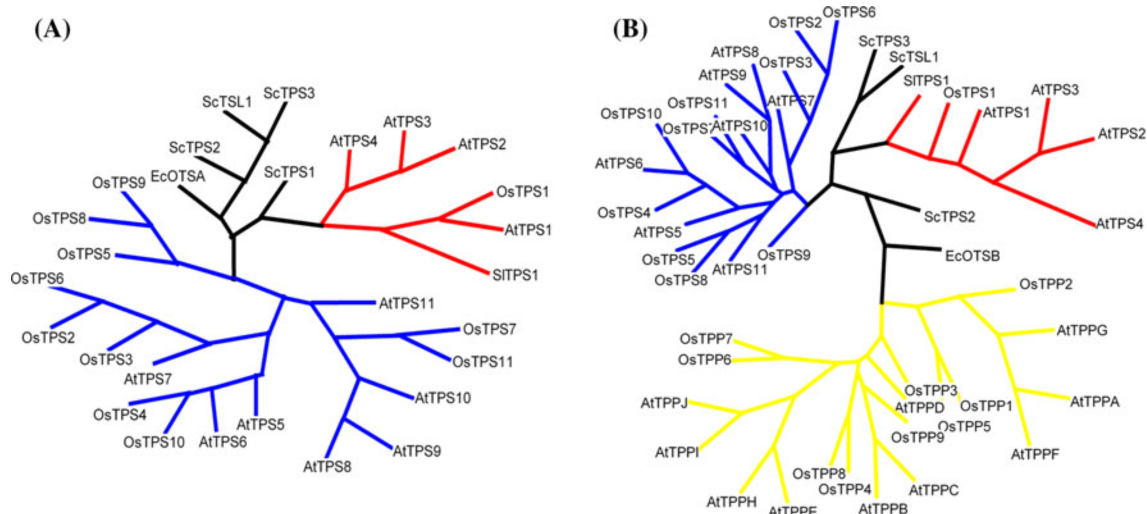


**Fig. 2** Dendrogram of the 11 OsTPS proteins. The proteins are: OsTPS1–11 (this work) and OsTPP1–9 (Pramanik and Imai 2005) from rice; AtTPS1–11 (Leyman et al. 2001) and AtTPPA-J (Avonce et al. 2006) from *A. thanlina*; SlTPS1 (accession no. AAD00829) from *S. lepidophylla*; ScTPS1 (accession no. Q00764), ScTPS2 (accession no. P31688), ScTPS3 (accession no. P38426), ScTSL1 (accession no. P38427) from *S. cerevisiae*; EcotsA (accession no. P31677) and EcotsB (accession no. P31678) from *E. coli*; the sequences of TPS domain or TPP domain were retrieved by the SMART software and were aligned using Clustal_X. The neighbor joining phylogenetic tree was constructed based on the aligned sequences by PHYLIP software package. **a** Phylogenetic analysis of TPS domain. **b** Phylogenetic analysis of TPP domain

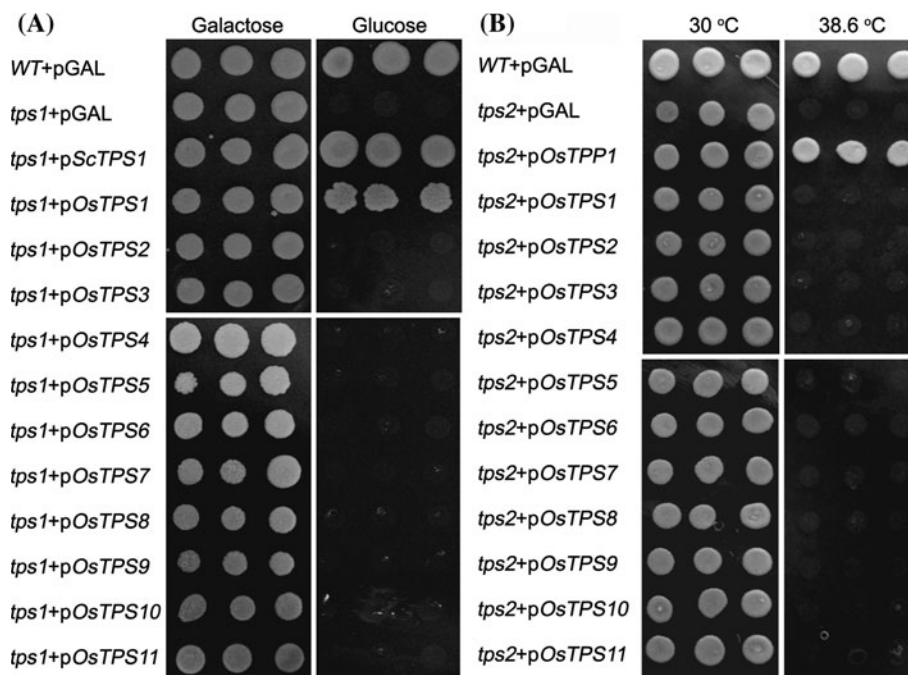


**Fig. 3** Complementation of the *tps1* or *tps2* mutants by the expression of *OsTPS1–11* under the control of the *ADH1* promoter. **a** *S. cerevisiae tps1* mutant was transformed with p*OsTPS1–11* (pGAL containing *OsTPS1–11* gene individually), p*ScTPS1* (pGAL containing *ScTPS1* gene) or pGAL vector alone, and spotted on minimal medium without leucine (SD-leu) supplemented with 2% Galactose or 2% Glucose as indicated. The wild type strain W303-1A (WT) was also transformed with pGAL vector. **b** *S. cerevisiae tps2* mutant was transformed with p*OsTPS1–11* and p*OsTPP1* (positive control).The strain W303-1A (WT) was also transformed with pGAL vector. All *S. cerevisiae* cells were grown on minimal medium without Leucine (SD-Leu) containing 2% Glucose at 30°C, and a copy was incubate at 38.6°C

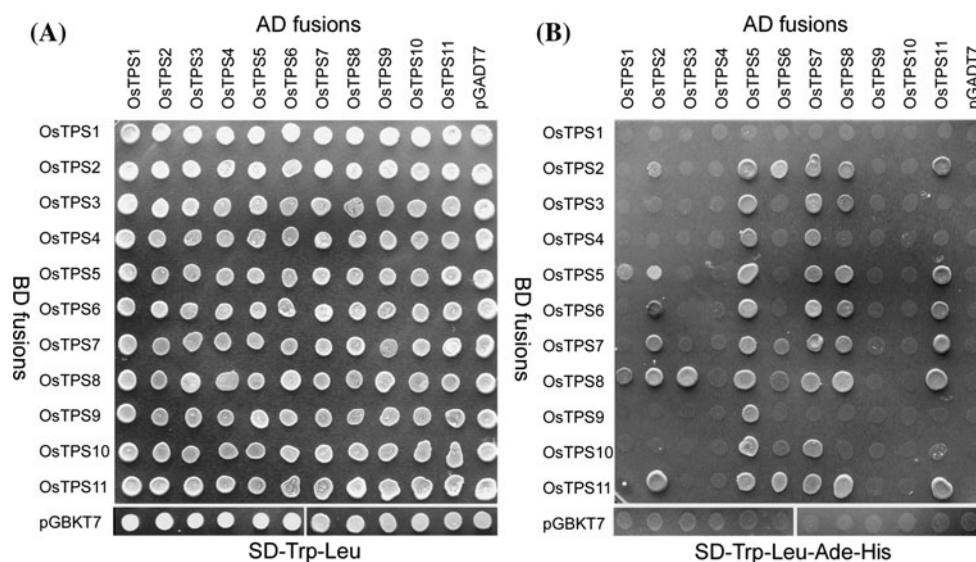


**Fig. 4** Interaction analysis of full length OsTPS homologues by the yeast-two hybrid assay. AH109 were cotransformed with the pGADT7 (AD) and pGBKT7 (BD) constructs containing OsTPS homologues. transformants grown on SD-Trp-Leu or on SD-Trp-Leu-Ade-His at 30°C for 3 days

## Comprehensive yeast two-hybrid analysis indicates that rice TPSs have complex interactions

Lacking TPS and TPP activities, ScTPS3 and ScTSL1 in the yeast trehalose synthesis complex act as regulatory subunits (Bell et al. 1998; Reinders et al. 1997). On account of the low homology among plant Class II TPS isoforms and yeast TPS3 and TSL1 subunits, the regulatory function of plant Class II TPS proteins was hypothesized but not demonstrated for a long time. To study the function of Class II OsTPSs, we firstly tested the interactions among these OsTPSs by yeast two-hybrid analysis. Each full-length OsTPS1–11 protein was fused to the activation domain (AD) or the DNA-binding domain (BD) of Gal4 transcription factor. All possible combinations were cotransformed into yeast strain AH109, and transformants were selected on SD-Leu/-Trp. Subsequently, six individual colonies were isolated from the SD-Leu/-Trp plates and were tested for the ability to grow on SD-Ade/-His/-Leu/-Trp. Figure 4 shows the growth of the transformants on SD-Leu/-Trp and SD-Ade/-His/-Leu/-Trp after 3 days incubation. When the transformants appeared on SD-Ade/-His/-Leu/-Trp in less than 6 days, we assumed that the coexpressed proteins in the transformants could interact with each other (Table 2 summarizes the results). In summary, OsTPS2, OsTPS5, OsTPS6, OsTPS7, OsTPS8 and OsTPS11 can interact not only with themselves but with most of the other OsTPSs. Moreover, OsTPS3, OsTPS4, OsTPS9 and OsTPS10 did not form a homodimer and could only interact with a few members of the rice TPS family in yeast. Significantly, OsTPS1 can interact directly with OsTPS5 and OsTPS8. AD-OsTPS1 appeared to interact with BD-OsTPS5 and BD-OsTPS8, and BD-OsTPS1 showed weak interaction with AD-OsTPS5. Although no transformants containing BD-OsTPS1 and AD-OsTPS8 were found on SD-Ade/-His/-Leu/-Trp in less than 6 days, some tiny clones emerged after lengthening the incubation time (9 days) (Supplementary Fig. 4), indicating very weak interaction between BD-OsTPS1 and AD-OsTPS8. Additionally, it is possible that OsTPS1 could interact weakly with OsTPS7, OsTPS11 and with itself (Supplementary Fig. 4), which might be due to transient interactions or the results of misfolding proteins in yeast cells.

## The TPS domains of OsTPS5 and OsTPS8 might mediate their interactions with other rice TPSs

All rice TPSs have similar protein domain structures, containing a TPS domain in the N-terminal region and a TPP domain in the C-terminal region. In order to determine which domain is responsible for protein–protein association, we amplified the N- and C-terminus of OsTPS5 and OsTPS8 which showed strong interaction with the others in previous yeast two-hybrid assays, and then fused these fragments to AD (Fig. 5a). We partnered these deletion constructs with the plasmid containing *OsTPS1–11* full-length cDNA fused with BD and transformed them into yeast separately. Evidently, coexpression of the N-terminal segment of OsTPS5 or OsTPS8 with each of the full-length OsTPS1–11 in yeast resulted in the emergence of the transformants on SD-Ade/-His/-Leu/-Trp medium after 3 days, and the deletion of the non-TPS part of TPS5 and TPS8 might result in tighter interactions than full length TPS5 and TPS8 proteins. No C-terminal fragment of OsTPS5 or OsTPS8 interacted with OsTPS1–11 full-length proteins, and the transformants containing these constructs did not grow on SD-Ade/-His/-Leu/-Trp medium (Fig. 5b). The N-terminus of OsTPS11, OsTPS11(1-574) was also subcloned into pGADT7 vector, and the protein–protein

**Table 2** Interaction pattern among all full length OsTPSs in rice obtained by yeast two-hybrid analysis

| | pGADT7 | AD-OsTPS1 | AD-OsTPS2 | AD-OsTPS3 | AD-OsTPS4 | AD-OsTPS5 | AD-OsTPS6 | AD-OsTPS7 | AD-OsTPS8 | AD-OsTPS9 | AD-OsTPS10 | AD-OsTPS11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pGBKT7 | – | – | – | – | – | – | – | – | – | – | – | – |
| BD-OsTPS1 | – | – | – | – | – | **+** | – | – | – | – | – | – |
| BD-OsTPS2 | – | – | (+++) | – | – | **+++** | **++** | **+++** | **++** | – | – | **+++** |
| BD-OsTPS3 | – | – | – | – | – | +++ | – | +++ | **++** | – | – | – |
| BD-OsTPS4 | – | – | – | – | – | **+++** | – | **+++** | – | – | – | **+** |
| BD-OsTPS5 | – | **++** | **++** | – | **+** | (+++) | **+** | **+++** | **+++** | – | **+** | **+++** |
| BD-OsTPS6 | – | – | **+++** | – | – | **+++** | (+) | **+++** | **++** | – | – | **+++** |
| BD-OsTPS7 | – | – | **+++** | – | **+** | **+++** | **++** | (+++) | **+++** | – | **+** | **+++** |
| BD-OsTPS8 | – | ++ | **+++** | ++ | + | **+++** | **+++** | **+++** | (+++) | + | + | **+++** |
| BD-OsTPS9 | – | – | – | – | – | +++ | – | – | – | – | – | – |
| BD-OsTPS10 | – | – | – | – | – | **+++** | + | **++** | – | – | – | **++** |
| BD-OsTPS11 | – | – | **+++** | – | **+** | **+++** | **+++** | **+++** | **++** | – | **+** | (+++) |

*BD-OsTPS* Bait proteins, *AD-OsTPS* prey proteins; –, no interaction (no growth after 6 days); + (about 5–6 days), ++ (about 3–4 days) and +++ (about 1–2 days) represent the interaction ability by investigating the emerging days of transformants on SD-Ade/-His/-Leu/-Trp. Homodimers were bracketed, bold entries shows heterodimers with bidirectional bait-prey identity

interactions between AD-OsTPS11(1-574) and BD-OsTPS1–11 fusion proteins were detected using yeast two-hybrid assays. OsTPS11(1-574) could interact with OsTPS2, OsTPS5, OsTPS6, OsTPS7, OsTPS8, OsTPS10 and OsTPS11 in less than 6 days, but these interactions were weaker than that of full length OsTPS11 protein (Supplementary Fig. 5).

We also investigated the possibility that the OsTPS1–11 could interact with OsTPP1, which is a Class III TPP protein and has only one TPP domain. AD-OsTPP1 did not appear to interact with any rice TPS protein fused to BD, and BD-OsTPP1 did not interact with any of AD-OsTPS fusions (data not shown).

### The interactions among OsTPS1, OsTPS5 and OsTPS8 are confirmed by bimolecular fluorescence complementation (BiFC) analysis in rice protoplasts

To test the possibility of interactions among OsTPS homologues in live rice cells, we adopted the BiFC transient assay in rice protoplasts. We generated several pairs of split-YFP constructs with either pUC-SPYNE or pUC-SPYCE vector (Walter et al. 2004). The ORF fragments of *OsTPS1, OsTPS5* and *OsTPS8* were fused to NYFP or CYFP individually, and every pairwise construct was transfected into rice protoplasts. Visible YFP fluorescence mediated by homodimerization or heterodimerization of OsTPS1, OsTPS5 and OsTPS8 was observed (Fig. 6b). In addition, the N-terminal segments of OsTPS1, OsTPS5 and OsTPS8 were cloned into pUC-SPYNE and pUC-SPYCE vectors. Then, these NYFP and CYFP fusion proteins were coexpressed in rice cells. Homodimerization or heterodimerization of OsTPS1(1-621), OsTPS5(1-551) and OsTPS8(1-601) resulted in a strong YFP signal in rice protoplasts (Fig. 6c). Only background fluorescence can be observed in the cells transfected with any combination containing empty vector. The fluorescence intensity showed high transfection efficiency of rice protoplasts (Supplementary Fig. 6).

### OsTPS1 and OsTPS8 are incorporated into the TPS complex(es)

Based on evidence showing the existence of complex interactions among members of the OsTPS family memebers, we hypothesized that the OsTPS complex should be present in rice. To further substantiate the occurrence of OsTPS complex in vivo, gel filtration fractionation analysis

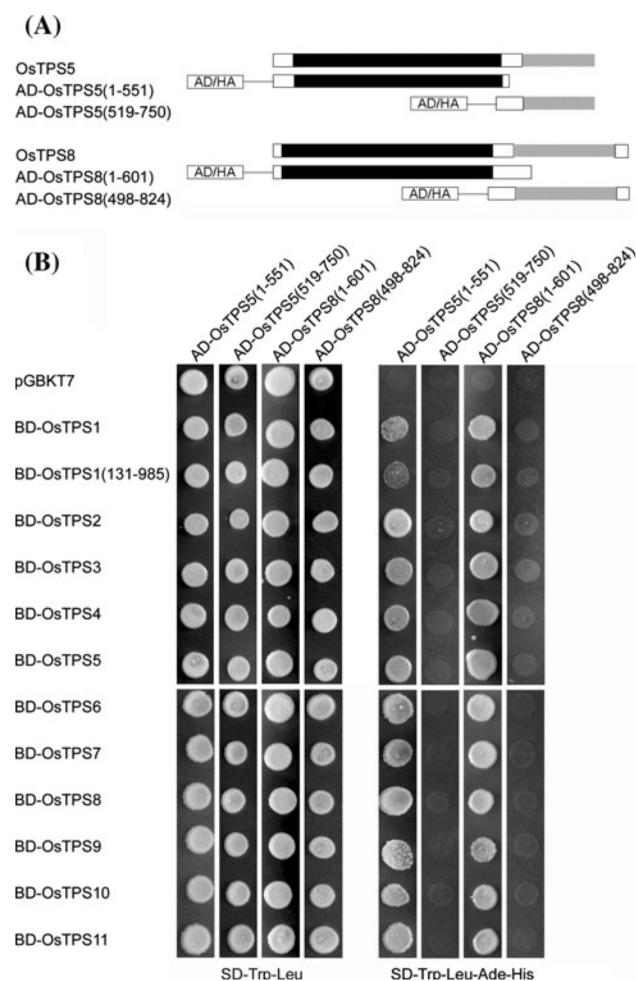


**Fig. 5** Mapping the domain of OsTPS5 and OsTPS8 that interacts with other OsTPSs. Yeast two-hybrid experiments were performed to test the interaction between the N- and C-terminal domains of OsTPS5 or OsTPS8 with all full length OsTPS1–11 proteins in all possible combinations. **a** Schematics of the fragments of OsTPS5 and OsTPS8 used in yeast two hybrid assays are shown. **b** Domain analysis OsTPS5 and OsTPS8 that interacts with other OsTPSs. Transformants grew on SD-Trp-Leu or SD-Trp-Leu-Ade-His at 30°C for 3 days. Growth of yeast cells coexpressing full length OsTPS1–11 fused with BD and fragment of OsTPS5 or OsTPS8 fused with AD. Growth on SD-Trp-Leu indicates both constructs are present in yeast. Growth on SD-Trp-Leu-Ade-His indicates protein–protein interaction

and coimmunoprecipitation assays were carried out. Anti-OsTPS1 was generated against the C-terminal 434 amino acids of OsTPS1, and anti-OsTPS8 was generated against the full length OsTPS8 protein. The specificity detection of anti-OsTPS1 and anti-OsTPS8 antibody with recombinant OsTPS1 and OsTPS8 proteins from *E. coli* are shown as Supplementary Fig. 7. There were no obvious cross-reactivities between OsTPS1 protein and anti-OsTPS8 antibody or OsTPS8 protein and anti-OsTPS1 antibody, when the expressed protein was less than 500 ng. In addition, we constructed OsTPS1(41-985)-Myc, OsTPS1(131-985)-Myc, OsTPS5-Myc and OsTPS8-Myc transgenic plants respectively, which produced OsTPS1(41-985), OsTPS1(131-985), OsTPS5 or OsTPS8 protein with a hexa MYC epitope tag fused to the C terminus driven by a constitutive *OsACT1* promoter.

Gel filtration chromatography was first used to investigate the formation of the TPS complex in rice. Total soluble proteins from wild type and the above-mentioned transgenic rice seedlings were fractionated according to their native sizes by fast protein liquid chromatography using Superose 6 column. The concentrated collections in each fraction were separated on SDS–PAGE, and then anti-Myc, anti-OsTPS1 and anti-OsTPS8 were used in subsequent western blot detection. The elution profiles of endogenous OsTPS1 and OsTPS8 proteins in WT and OsTPS8-Myc transgenic plants are shown in Fig. 7a. Immunoblot analysis of the fractions from WT and OsTPS8-Myc transgenic extracts using anti-OsTPS1 antibody showed that, two protein bands, designated OsTPS1a and OsTPS1b, were detected with approximate apparent molecular weights of 100 kDa, corresponding to the predicted size of OsTPS1. OsTPS1b is slightly larger than OsTPS1a, which is possibly due to post-transcriptional modification or by another transcript of OsTPS1. Our fractionation assay showed that OsTPS1b occurred as a complex of ~ 360 kDa, while OsTPS1a mainly occurred in complexes larger than 360 kDa (Fig. 7a). The elution profile of Myc-tagged OsTPS1(41-985) showed that the overexpressed truncated OsTPS1 protein was present not only in the 360 kDa but also in the larger complex (about 600–800 kDa) (Fig. 7b), and the elution profile of Myc-tagged OsTPS1(131-985) gave the same result. Interestingly, the elution profile of OsTPS8 protein in WT rice showed that endogenous OsTPS8 protein is likely to be cofractionated with OsTPS1b in the 360 kDa complex. The elution profile of Myc-tagged OsTPS8 was similar to that of endogenous OsTPS8 in OsTPS8-Myc transgenic plants (Fig. 7a, b). However, Myc-tagged OsTPS5 was predominately found in the 360 kDa complex, but a small portion of OsTPS5 could also be detected in higher molecular weight fractions (Fig. 7b). The putative degraded OsTPS1 proteins with apparent molecular weights of about 80 kDa perhaps were present as monomers or dimers, which mainly eluted in fractions 17–21 (Fig. 7a). The degradation products of TPS protein were previously found in *S. cerevisiae* and *S. lepidophylla* (Londesborough and Vuorio 1991; Marquez-Escalante et al. 2006).

To test the association of OsTPS1 and OsTPS8 in vivo, immunoprecipitation assays were performed using OsTPS1(41-985)-Myc and OsTPS8-Myc transgenic plants, as the two polyclonal antibodies were not recommended for immunoprecipitation experiments. Myc-tagged fusion proteins were immunoprecipitated from transgenic rice seedling extracts using an anti-Myc affinity matrix.

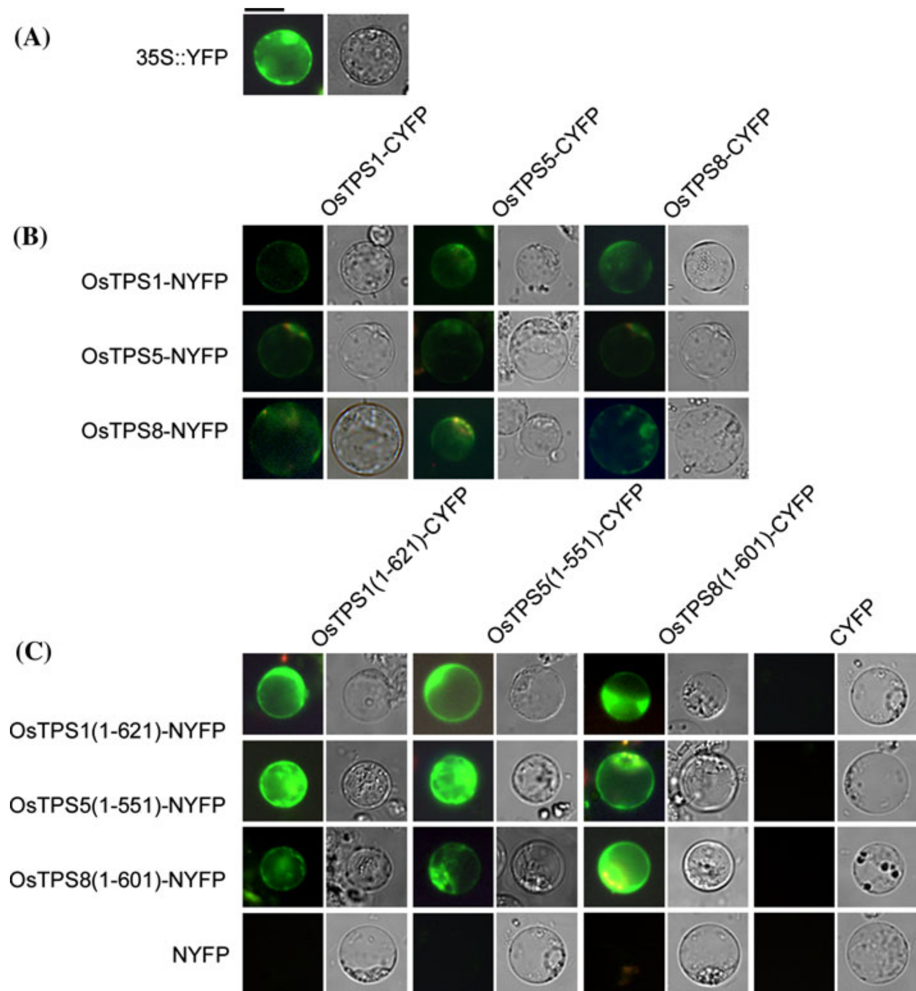


**Fig. 6** BiFC visualization of protein interactions in rice protoplast cells. Every group contains epifluorescence images (shown on the *left*) and bright field images (shown on the *right*). **a** Expression of YFP full length protein under 35S promoter control. Scale bar, 20 μm. **b** OsTPS1, OsTPS5, OsTPS8 full length proteins were fused with N terminal or C terminal of YFP separately. Every pairs of constructs encoding the indicated fusion proteins were cotransfected into rice protoplasts. **c** The N-terminal fragments containing TPS domain of OsTPS1, OsTPS5 and OsTPS8 were constructed into different split-YFP vectors. Microscopy detects the interaction of them in rice protoplasts. The empty vectors were cotransfected rice protoplasts with the fusion proteins avoiding of contingency

Endogenous OsTPS1 was pulled down with OsTPS8-Myc and endogenous OsTPS8 was pulled down with OsTPS1(41-985)-Myc by using anti-Myc antibody (Fig. 7c), indicating that OsTPS1 interacted with OsTPS8 in vivo.

## Discussion

Eleven *TPS* genes isolated from rice were grouped into two subfamilies based on their amino acid sequences, phylogenetic relationships and functional complementation analysis. Class I contained only OsTPS1 and Class II comprised the other ten OsTPSs. Functional complementation studies in *S. cerevisiae tps1* and *tps2* mutants indicated that OsTPS1 showed TPS activity and Class II OsTPSs perhaps have lost both TPS and TPP activities. The Class II OsTPS proteins lacked several conserved residues involved in the TPS domain that are known to play a role in substrate binding and catalysis (Gibson et al. 2002), which might explain the absence of TPS activity. Moreover, Lunn (2007) proposed that the deficient substitution of the Lys 149 residue involved in binding the T6P molecule might explain the lack of TPP activity in the class II TPS proteins (Lunn 2007). Without TPS and TPP activities (Ramon et al. 2009; Vogel et al. 2001), the Class II *TPS* genes in *Arabidopsis* were retained under evolutionary selection pressure and expressed in a temporal and tissue-specific manner (Avonce et al. 2006; Ramon et al. 2009), implying they possess a particular function.

In this study, we confirmed that Class II OsTPS5 and OsTPS8 can interact with OsTPS1 by yeast two-hybrid and BIFC analysis, and we showed that complicated interactions occur among Class II OsTPS proteins. In view of the limitations of the yeast two-hybrid system, such as three-dimensional structure alterations or lack of post-translational modifications such as phosphorylation and 14-3-3 binding (Glinski and Weckwerth 2005; Harthill et al. 2006) in yeast, whether additional Class II OsTPS proteins can interact with OsTPS1 cannot be ruled out. Consequently, we hypothesize Class II TPS proteins in rice may regulate the activity of OsTPS1 by direct or indirect interactions.

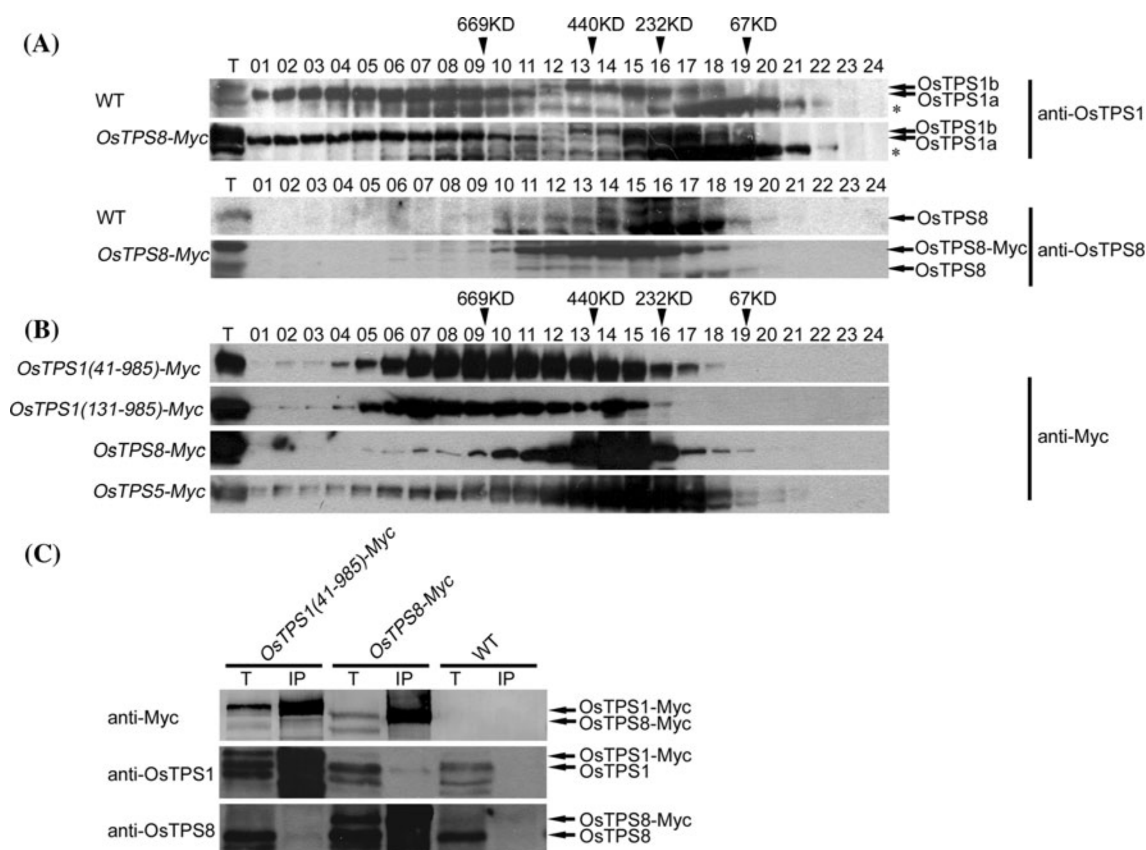


**Fig. 7** OsTPS1 and OsTPS8 are presented in the TPS complexes. **a** Gel filtration patterns of OsTPS1 and OsTPS8 proteins in WT and OsTPS8-Myc transgenic plants. Cofractionation assays were performed on a Superose 6 10/300 gel filtration column. Total soluble proteins were extracted from the young leaves of WT and OsTPS8-Myc transgenic plants. After separation by 10% SDS–PAGE, the protein blot analysis was carried out using anti-OsTPS1 and anti-OsTPS8 antibodies. Arrowheads indicate protein positions, and asterisk indicates putative degraded OsTPS1 protein form. Protein size markers are indicated at the top in kilodaltons (KD). T, total soluble proteins; *Lanes* 1–24 indicate the corresponding fractions eluted from the gel filtration column (started at 8 ml, 0.5 ml per fraction). **b** Gel filtration patterns of Myc-tagged OsTPS1(41-985), OsTPS1(131-985), OsTPS5 and OsTPS8 proteins in corresponding transgenic plants. The crude extract of OsTPS1(41-985)-Myc, OsTPS1(131-985)-Myc, OsTPS5-Myc and OsTPS8-Myc transgenic lines were fractionated using Superose 6 10/300 column. Western blot was performed using anti-Myc antibody. **c** Coimmunoprecipitation between OsTPS1 and OsTPS8. Total protein extracts prepared from OsTPS1(41-985)-Myc and OsTPS8-Myc transgenic lines were incubated with anti-c-Myc affinity matrix. The precipitates and total extracts were subjected to immunoblot analysis with antibodies against Myc, OsTPS1 and OsTPS8. The antibodies used for protein blots are indicated at *left*

Similar to the relationships among the subunits of the yeast trehalose synthesis complex (Reinders et al. 1997), the interactions among OsTPS proteins found by yeast two-hybrid analysis suggest that OsTPS proteins could be incorporated into the same protein complex in rice. Using the BiFC approach in live rice cells, we verified that two of the Class II members, OsTPS5 and OsTPS8, could interact directly with the only member of Class I, OsTPS1. The association of OsTPS1 and OsTPS8 was confirmed by coimmunoprecipitation experiments (Fig. 7c), indicating that they can act as the subunits of the TPS complex.

To elucidate the formation of the TPS complex in rice, we investigated the gel filtration profiles of OsTPS1, OsTPS5 and OsTPS8 in WT and the transgenic rice seedlings. Gel filtration analysis showed that OsTPS1a and OsTPS1b, two isoforms of OsTPS1, were mainly present in the 360 kDa complex and the larger complexes, respectively (Fig. 7a). Intriguingly, OsTPS5 and OsTPS8 proteins were mainly present in the 360 kDa complex, which suggests that these two Class II TPS proteins are likely to engage with OsTPS1b. It is unknown why OsTPS1b particularly occurred in the 360 kDa complex cofractionating with OsTPS5 and OsTPS8; OsTPS1b might be generated from a different transcript or modified from OsTPS1a. A shorter transcript of *OsTPS1* lacking the last exon was predicted in the MSU Rice Genome Annotation Resource (Ouyang et al. 2007), but we did not isolate this transcript and other alternatively spliced transcripts. It is possible to produce different OsTPS1 variants by translation from different start codons, and the N-terminal extension of OsTPS1 before the TPS domain probably plays a role in the formation of a larger complex containing OsTPS1a

proteins. Even though it was translated from the fifth initiation codon, OsTPS1(131-985) still possessed TPS activity and could interact with OsTPS5 and OsTPS8 in the yeast two-hybrid experiments (data not shown). Moreover, a portion of Myc-tagged OsTPS1(41-985) and OsTPS1(131-985) overexpressed in rice were found in the 360 kDa complex, which indicated that the truncated OsTPS1 proteins might engage with OsTPS5 and OsTPS8 in the same complex. However, compared to the elution profiles of OsTPS1a in high molecular weight fractions, Myc-tagged truncated OsTPS1 proteins were mainly incorporated into the 600–800 kDa complex. We supposed that the N-terminal extension of OsTPS1 might take part in the formation of the larger complexes, similar to a previous report about the N-terminal extension of AtTPS1 in *Arabidopsis* (Geelen et al. 2007). Geelen et al. (2007) pointed out that the N-terminal extension of AtTPS1 can interact with cell cycle kinase CDKA;1 and kinesin KCA1, and that CDKA;1 and tubulin might be cofractionated with AtTPS1 complex by gel filtration analysis (Geelen et al. 2007). Consequently, we presumed that OsTPS1a has the N-terminal extension, which might make accessible of some non-TPS proteins to the larger complexes. In this case, another bold assumption is that OsTPS1b may be a modified form of OsTPS1. On the one hand, the 360 kDa complex containing Class II OsTPS5 and OsTPS8 proteins may aid in the post-transcriptional modification of OsTPS1. On the other hand, OsTPS5 and OsTPS8 could specially stabilize the modified form of OsTPS1 in the 360 kDa complex. Although we did not distinguish different forms of Myc-tagged truncated OsTPS1 in transgenic plants, the post-translation modification was possibly affected by the N-terminal truncation or the additional C-teriminal Myc-tag.

Obviously, OsTPS5 and OsTPS8 were mainly incorporated into the 360 kDa complex associating with OsTPS1b, but a small fraction of Myc-tagged OsTPS5 could be detected in high molecular weight fractions cofractionating with OsTPS1a, which suggests that Class II OsTPS protein can also act as components of the larger complexes. There are several other Class II OsTPS proteins, and it is ill-defined whether and how they are related to the TPS complexes, so the definitive modes of formation of the TPS complexes in rice remain shrouded in mystery.

Our gel fractionation assay showed that the OsTPS1 protein (OsTPS1a and OsTPS1b) was mainly found in the complexes. Similarly, in *Arabidopsis*, AtTPS1 was also predominantly present in a protein complex (Geelen et al. 2007). Furthermore, purified SlTPS1 protein complexes from *S. lepidophylla* showed three aggregation states of about 230, 440 and 660 kDa, and only the 440 kDa form was visible through activity-staining after native gel electrophoresis (Marquez-Escalante et al. 2006; Valenzuela-Soto et al. 2004). However, in *S. cerevisiae*, a significant part of free ScTPS1 protein does not bind to the trehalose synthase complex, which might mainly control the T6P content (Bell et al. 1998). The same study showed ScTPS2 (TPP enzyme) and two regulatory subunits with both the TPS and the TPP domains were deeply embedded within the TPS complex (Bell et al. 1998). In *S. cerevisiae*, the TPS complex (which has TPS and TPP activities) may facilitate the synthesis of trehalose, which was possibly correlated with the survival of *S. cerevisiae* cells under adverse circumstances (Hottiger et al. 1987; Hounsa et al. 1998; Lewis et al. 1997). Compared with the sole TPS domain of ScTPS1, the active TPS in plants, such as AtTPS1 and OsTPS1, possess an additional TPP-like domain, which might result in a more stable complex. Although the existence of monomeric OsTPS1 or AtTPS1 protein was not ruled out, the mechanism of modifying T6P levels by free TPS may weaken or disappear in plants. Compared to the scarce trehalose accumulation in most of higher plants (Muller et al. 1995), T6P levels carry out versatile roles in regulating plant metabolism and development (Kolbe et al. 2005; Lunn et al. 2006; Schluepmann et al. 2003, 2004; Zhang et al. 2009), which suggests that plant TPS complexes might control T6P levels rather than rapidly produce trehalose. Furthermore, plant Class III TPPs which possess only a TPP domain bear the TPP activity, such as AtTPPA, AtTPPB (Vogel et al. 1998), OsTPP1 (Pramanik and Imai 2005), OsTPP2 (Shima et al. 2007) and RAMOSA3 (Satoh-Nagasawa et al. 2006), but these proteins lacking the TPS domain may not take part in the formation of the TPS complex. In this study, the data from deletion construction of OsTPS5 and OsTPS8 showed that the TPS domain, rather than the TPP domain, is the interactive region of OsTPSs. ScTPS1, which has only a TPS domain, interacted with other subunits of the TPS complex of *S. cerevisiae*, thus confirming the essentiality of the TPS domain for the formation of the complex. In yeast two-hybrid assays, we did not find that Class III OsTPP1 interacted with any OsTPS proteins. Plant Class III TPPs released from the TPS complex, may also play an important role in the modification of T6P levels or the production of trehalose in specific stages or cells to regulate plant development.

In summary, plant trehalose metabolism, likely via T6P, plays a multifarious role in plant metabolism and development. The perplexing expression models of plant Class II *TPS* genes in response to intrinsic and adventitious signals or in the certain tissues might coincide with the precise modification of T6P levels. Without TPS and TPP activities, the functions of the Class II *TPS* genes in plants were assumed as regulatory proteins. In this study, we successfully isolated 11 TPS members in rice, and found that ten of them (OsTPS2–11) which lacked TPS and TPP activities belong

to Class II. Significantly, our collective results indicated that rice Class II TPS proteins, at least OsTPS5 and OsTPS8, directly interacted with OsTPS1 (the active TPS enzyme). Moreover, OsTPS5 and OsTPS8 could be incorporated into the OsTPS1 complexes, and might be key to forming the 360 kDa complex. These findings established a direct relationship between plant Class I and Class II TPS proteins, which might facilitate the understanding of the mechanisms for modulation of T6P levels in plants.

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