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Characterization of a Drought-Responsive Gene, *OsTPS1*, Identified by the T-DNA Gene-Trap System in Rice

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Trehalose-6-phosphate synthase (TPS) is a key enzyme for trehalose biosynthesis. To understand the function of the *TPS* gene, we identified the T-DNA insertion in a *TPS* gene of rice. Line 1C-071-05 had T-DNA inserted at the second intron of *OsTPS1*. Its deduced OsTPS1 protein shared 52% homology with *Arabidopsis* TPS. Reverse transcriptase-PCR analysis of *OsTPS1* showed that *OsTPS1* is inducible by drought, salt, cold, and ABA. Leaves of *OsTPS1* knockout (KO) plants were more sensitive to drought or cold stress than were the wild types. Furthermore, transgenic rice of *UBI::CBF1* had high expression *OsTPS1* mRNA, suggesting that *OsTPS1* is regulated by the CBF/DREB transcription factor. Therefore, we propose that *OsTPS1* plays an important role during the abiotic stress response.

Keywords: drought, rice, T-DNA, trehalose-6-phosphate synthase

In rain-fed regions, drought stress is a serious factor that limits rice production and yield stability (Dey and Upadhyaya, 1996). As the scarcity of fresh water increases globally, many scientists are working to engineer drought-resistant plants as well as to understand the drought-responsive mechanism. Osmotic adjustment is an effective component of such manipulations. The accumulation of compatible solutes (osmoprotectants), such as betaine, proline, and sugar, is also common in plant systems (Blum, 1988). Trehalose is an osmoprotectant formed from UDP-glucose and glucose-6-phosphate. It is catalyzed by the enzymes trehalose-6-phosphate synthase (TPS) and, subsequently, trehalose-6-phosphate phosphatase (TPP). This osmoprotectant stabilizes proteins and membrane structures under stress (Iwahashi et al., 1995), and can act as a water substitute on the surfaces of macromolecules (The Genetic Engineering Newsletter, 2004). Nevertheless, its exact function remains unclear. Trehalose has been found only in certain specialized resurrection species, e.g., *Myrothamnus flabellifolius* and *Selaginella lepidophylla*, where it accumulates at rates of up to 3% and 12%, respectively, of their total dry weights (Bianchi et al., 1993; Drennan et al., 1993; Oscar et al., 1999). In most plants, however, trehalose is hardly detectable, i.e., 0.15 mg g^{-1} dry weight or lower, depending on tissue type and species (Garcia et al., 1997; Vogel et al., 1998; Roessner et al., 2000). Although this fact sheds doubt on the role of trehalose as a stress protectant, genes encoding the enzymes for its biosynthesis have been identified in a number of plant species, thereby indicating the ubiquitous distribution of the trehalose biosynthesis system in that kingdom (The Genetic Engineering Newsletter, 2004).

Interest is growing in exploiting trehalose metabolism as a means for engineering stress tolerance in plants. For example, transgenic tobacco and rice plants that express the *Escherichia coli TPS1* or TPS/phosphatase fusion gene, *TPSP*, produce trehalose and exhibit improved drought tolerance (Goddijn et al., 1997; Romero et al., 1997; Garg et al., 2002). However, although this trehalose-overproduction via metabolic engineering is of value, the role of the gene itself in such stress tolerance has not yet been reported.

The objective of this study was to identify a T-DNA tagging line of rice by searching for a *TPS* gene in the rice TES database. In addition, we examined its stress-responsive expression and characterized the *TPS* knockout plants.

MATERIALS AND METHODS

Plant Materials and Stress Treatments

Rice seeds (*Oryza sativa* ssp. *Japonica* cv. Dongjin and Hwayoung) were surface-sterilized, germinated,

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and grown hydroponically (Lee et al., 2003). For the cold treatment, 8-d-old seedlings were exposed to 4°C. For the salt or ABA treatments, the seedlings were transferred to Yoshida solution containing either 250 mM NaCl or 100 μM ABA. For drought treatment, the seedlings were air-dried, then harvested.

Identification of T-DNA Insertion Sites by Inverse PCR

Inverse PCR (IPCR) was performed as described previously (Triglia et al., 1988; Lee et al., 2004). The forward primers, located at the hygromycin phosphotransferase gene, were: first, 5′-CCATGTAGTGTATTGACCGATTC; and second, 5′-TCGTCTGGCTAAGATCGGCCGCA-3′. Reverse primers, located at the RB were: first, 5′-TTGGGGTTTCTACAGGACGTAAC-3′; and second, 5′-CAAGTTAGTCATGTAATTAGCCAC-3′. The first PCR was performed with an initial 6-min denaturation at 94°C; followed by 35 cycles of 95°C (1 min), 54°C (1 min), and 72°C (2 min); and ending with 72°C for 7 min. The second PCR was conducted with an initial 5-min denaturation at 94°C; followed by 35 cycles of 95°C (30 s), 55°C (1 min), and 72°C (2 min); and ending with 72°C for 7 min.

RT-PCR Analysis of *OsTPS1*

The gene-specific primers of *OsTPS1* for RT-PCR analysis were P5 (5′-CTGGCAACAGGCTCATCT-3′) and P2 (5′-CGCAGAGGATGAAATCTG-3′). As a control, primers specific to rice actin gene *RAc1* (McElroy et al., 1990) were used. For a positive control in the stress response, primers to *SalT* (S45168), 5′-TAAGCGACCACGAAGAGTATGA-3′ (forward) and 5′-AGTGATACCAATATGAGAAACACATAA-3′ (reverse) were included. The PCR cycles numbered 36 for *OsTPS1*, 25 for *RAc1*, and 25 for *SalT*.

Real-Time PCR

Real-time PCR was performed on a Roche Lightcycler, using the FastStart DNA Master SYBR Green I (Mannheim, Germany) according to the manufacturer's protocol. The PCR primers were: 5′-TGTGAAGATGCTCCAGGGC-3′ (forward) and 5′-ACGGGG CTCTTTCTCACG-3′ (reverse). PCR consisted of denaturation and Taq activation (95°C for 10 min) followed by 40 cycles of denaturation (95°C for 15 s), annealing (56°C for 7 s), and extension (72°C for 10 s). Afterward, the amplified products were denatured (95°C), re-natured (65°C), and progressively denatured from 65 to 95°C over 30 min, i.e., 0.1°C s^{-1}, for the fusion curve analysis.

Genotyping of OsTPS1 Knockout

To distinguish the resultant genotypes, PCR-based genotyping was conducted using genomic DNA from the progeny of our tagging line. This PCR reaction, carried out with 250 ng of genomic DNA as template and 0.5 unit of Ex-Taq polymerase, involved 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The forward (P1) and reverse (P2) primers were: 5′-CTGGCAACAGGCTCATCT-3′ (80 bp downstream of the ATG start codon) and 5′-CATCTTCACAACATCAGCCG-3′ (10 bp upstream of the TGA stop codon); The forward primer (P3) of T-DNA was 5′-GGTGAATGGCATCGTTTGAA-3′ on the LB; the reverse primer (P4) was 5′-AATCATCTAGCGAGTTTTCGAACGAG-3′ (23 bp upstream of the TGA stop codon).

Determination of Chlorophyll Fluorescence after Stress Treatment

Stress tolerance was estimated by measuring chlorophyll fluorescence after treatment. About 5-cm-long segments of the youngest expanded leaves from 2-month-old WT ('Hwayoung') and *OsTPS1* knockout (KO) plants were floated on MS liquid media. The samples were either incubated at 4°C (cold stress) or air-dried in a clip at 28°C (drought stress), with both treatments being conducted under light (260 μmol m^{-2} sec^{-1}) from a white fluorescent lamp. After incubation, the samples were dark-adapted for 30 min, and then chlorophyll fluorescence was measured with a Plant Efficiency Analyzer (Hansatech, Germany). Standard errors of the means were calculated using at least four leaves per plant from three different experiments (cold treatment) or from two different experiments (drought test).

RESULTS

Identification of the Trehalose-6-Phosphate Synthase Gene, *OsTPS1*, from T-DNA Tagged Rice

To study the role of the *TPS* gene in rice, we used the rice TES database (An et al., 2003) to conduct a computer search of its T-DNA insertion. A *TPS* tagging line, 1C-071-05, was retrieved and the tagging position was confirmed by inverse PCR coupled with

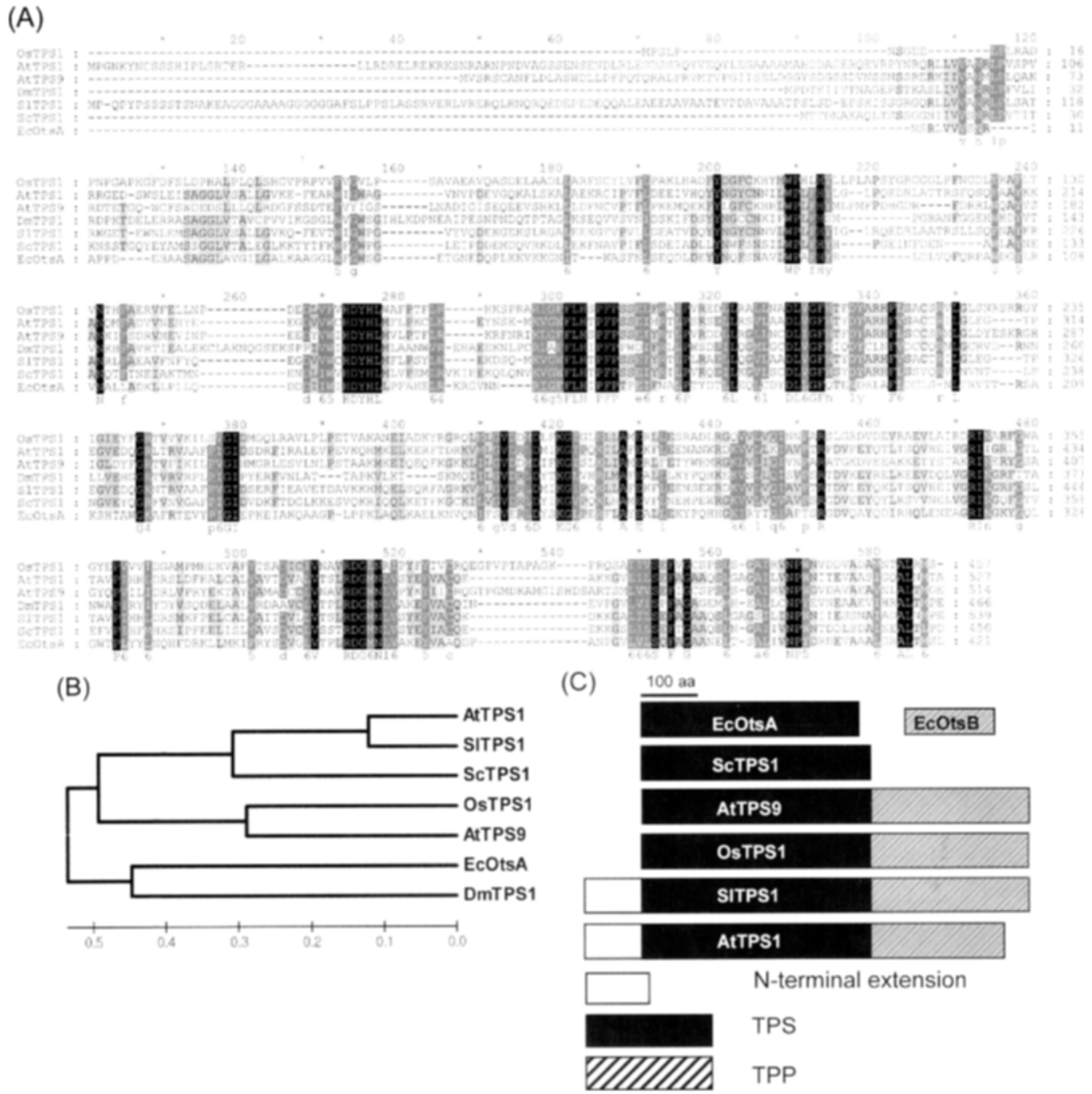

Figure 1. Comparison of OsTPS1 with other TPS protein sequences. **A,** Alignment of internal region of plant TPS amino acid sequences with bacteria, yeast, and animal sequences. Gaps introduced to optimize alignment are indicated by dashes; identical residues are shaded. **B,** Dendrogram of TPS sequences from *Arabidopsis thaliana* (AtTPS1; accession no. Y08568), *Selaginella lepidophylla* (SlTPS1; accession no. U96736), *Saccharomyces cerevisiae* (ScTPS1; accession no. X68214), *Oryza sativa* (OsTPS1; accession no. XP_482658), *A. thaliana* (AtTPS9; accession no. AAF87136Y08568), *E. coli* (EcOtsA; accession no. X69160), and *Drosophila melanogaster* (DmTPS1; accession no. AC004373). **C,** Diagram of TPS and TPP protein sequences. Comparisons of protein size and structure are based on sequence-alignment data presented in **A,** and contrasted with *E. coli* (EcOtsB) and *S. cerevisiae* (ScTPS1) sequences. Protein size is shown as length of amino acids (aa).

DNA sequence analysis (data not shown). The predicted *OsTPS1* gene, located on Chromosome 8 (P0479C12), has an ORF encoding 824 amino acids, and consists of three exons. Its deduced amino acid sequence shows homology to trehalose-6-phosphate synthase. As shown in Figure 1, the homologies of OsTPS1 to DmTPS1 (*D. melanogaster*), OtsA (*E. coli*), SlTPS1 (*S. lepidophylla*), ScTPS1 (*S. cerevisiae*), AtTPS1

and AtTPS9 (*A. thaliana*) are 20%, 22%, 29%, 32%, 30%, and 52%, respectively (see Kaasen et al., 1992; McDougall et al., 1993; Blazquez et al., 1998; Zentella et al., 1999). Two distinct regions – the TPS and TPP portions – that are found in most eukaryotes, are also conserved in OsTPS1 (Fig. 1C). The TPS portion, which spans 478 amino acids in the N-terminal, has homology to other TPSs that ranges from 24% (DmTPS1) to ~50% (AtTPS9). In contrast, the TPP portion, spanning the C-terminal for 346 amino acids, shares homology with TPPs in the range of 13% (DmTPS1) to ~54% (AtTPS9). Therefore, OsTPS1 is so named because of these characteristics.

We estimated the number of T-DNA insertions in the genome of Line 1C-071-05 by Southern blot, using the β-*glucuronidase* (*gus*) gene as a probe. Two copies of T-DNA were integrated into the genome (data not shown); thus, two tagged sequences were isolated by IPCR. A T-DNA was inserted into the co- ding region of a putative ser/thr receptor protein kinase (XP_462753), where the *gus* gene in the T-DNA was inserted in the reverse orientation of the kinase gene. Another T-DNA was inserted at the second intron of *OsTPS1* (Fig. 2), where the transcriptional direction of the *gus* gene was the same as for *OsTPS1* transcription. Therefore, we studied this *OsTPS1* gene further. Our TBLASTN search, using the OsTPS1 protein sequence (accession no. XP_482658) as a query, resulted in the retrieval of seven homologues from scaffolds 001867, 000874, 001264, 000636, 000014, 006370, and 001058.

Expression Analysis of *OsTPS1*

We performed northern blot analysis to study the expression of *OsTPS1* under various stress conditions. *OsTPS1* cDNA, provided by the Rice Genome Program (Clone No. C12688), was used as a probe. However, no hybridization signal was detected at a loading of 30 μg total RNA (data not shown). Therefore, we conducted reverse transcriptase PCR analysis, and observed that expression was induced by drought stress after as little as 10 min (Fig. 3A). *OsTPS1* was also induced by cold or salt treatment. Transcripts were induced gradually, reaching a maximum at 12 h for the drought, salt, and ABA experiments, and at 24 h for the cold treatment. Identity of the PCR bands was confirmed by DNA sequence analysis. The RT-PCR results were further verified by real-time PCR (Fig. 3B) Expression of a rice actin gene, *RAc1*, served as an internal control. In the drought-stressed seedlings, *OsTPS1* induction was detected at similar levels in both shoots and roots, although transcript amounts in the former organ type were slightly reduced after 24 h of drought (Fig. 3C).

We also examined the expression of *OsTPS1* in various tissues at different developmental stages. High levels of transcript were present in the calli, seedling shoots and roots, and the internodes between Nodes I and II at the pre-heading stage, as well as in the developing (3- to 8-cm) panicles, mature panicles prior to anthesis, and developing seeds at 3 d after pollination (DAP) and at 6 DAP (Fig. 3D). However, expression was rarely

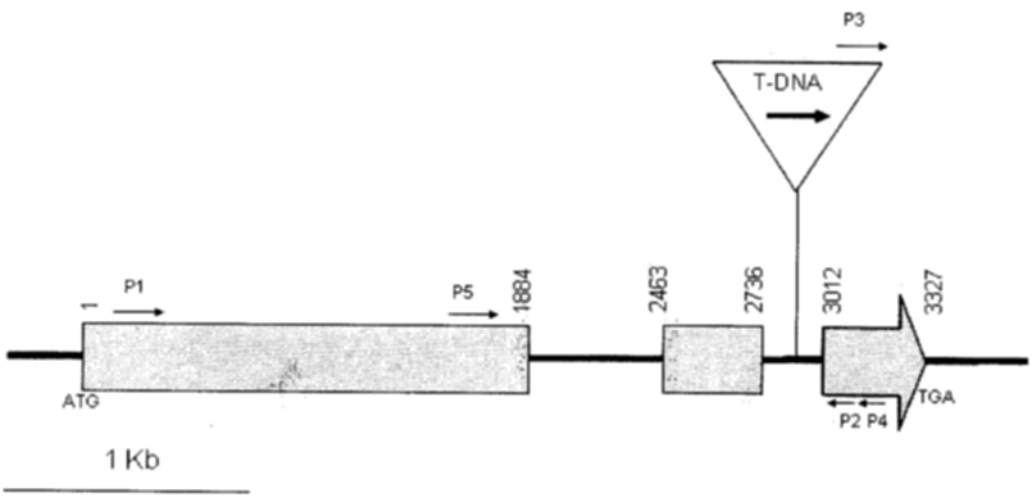

Figure 2. Schematic diagram of *OsTPS1* and position of T-DNA. Filled boxes represent exons; lines between boxes are introns. ATG start codon and TGA stop codons are indicated. Small arrows represent gene-specific primers, P1, P2, P4, and P5. P3 is located on left border of T-DNA. Direction of T-DNA is indicated.

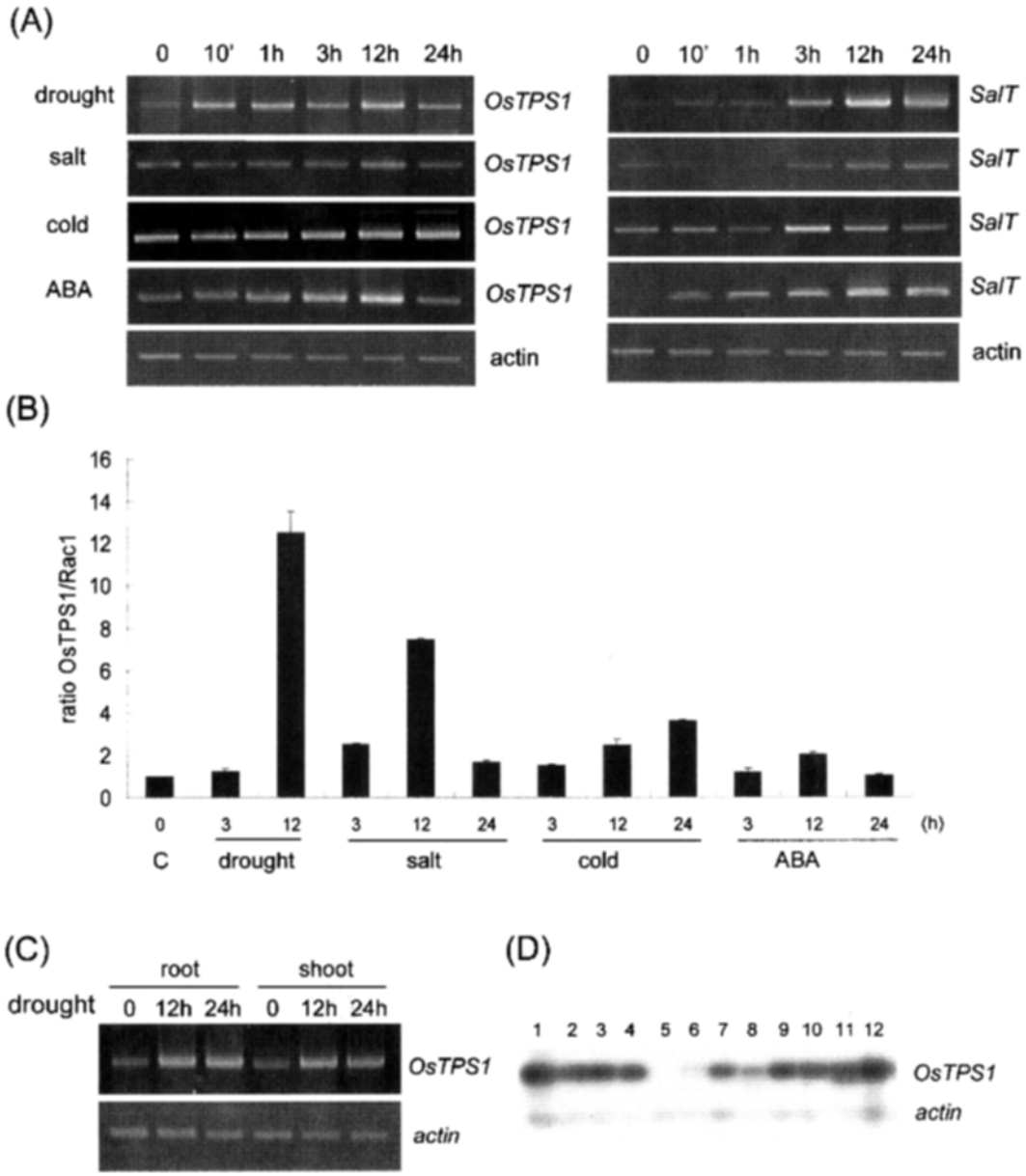

Figure 3. Analysis of *OsTPS1* expression induced by abiotic stresses and ABA. **A,** Semi-quantitative RT-PCR analysis of *OsTPS1*. *SalT* was used for drought-, cold-, salt-, and ABA-response controls. Transcript of rice *actin* gene shows internal control for PCR analysis. **B,** Analysis of *OsTPS1* expression levels using real-time PCR. Error bars represent standard deviation. **C,** Semi-quantitative RT-PCR analysis of *OsTPS1* in shoots and roots of drought-treated seedlings. Transcript of rice *actin* gene shows an internal control for PCR analysis. **D,** RT-PCR analysis of *OsTPS1* expression throughout developmental stages. Ten µl of PCR product was separated, blotted, and hybridized with radiolabeled *OsTPS1* probe. Lanes: 1, callus; 2, 7-d-old seedlings; 3, seedling shoot; 4, seedling root; 5, mature leaves; 6, sheath from flag leaves; 7, highest internode (between nodes I and II) at pre-heading stage; 8, 1- to 2-cm panicles; 9, 3- to 8-cm panicles; 10, mature panicles prior to anthesis; 11, developing seeds at 3 days after pollination (DAP); 12, developing seeds at 6 DAP.

detected in the mature leaves, and was only found at basal levels in the flag leaf sheath. This result demonstrates that the expression of *OsTPS1* is organ-specific.

Genotyping of Line 1C-071-05

Seven T1 plants of Line 1C-071-05 were grown, and the genotypes of their progeny were determined using

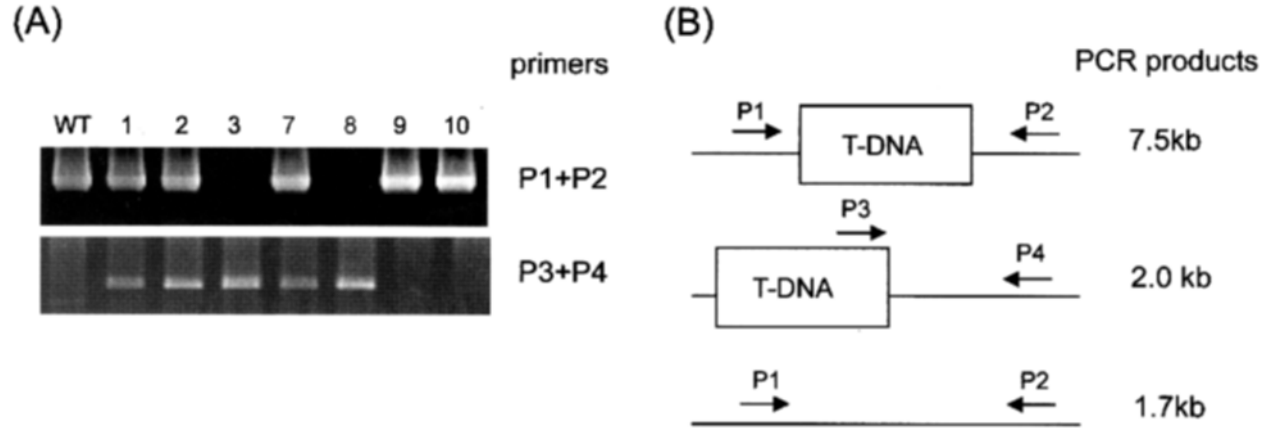

Figure 4. Genotyping of progenies from Line C-071-05. **A,** Lanes 1, 2, and 7 are heterozygous (T/W); Lanes 3 and 8 are homozygous (T/T); Lanes 9 and 10 are WT (W/W). Seven plants of T2 generation were PCR-analyzed with primers P1, P2, P3, and P4. **B,** Schematic diagrams of genotyping; P1, forward primer in *OsTPS1*; P2 and P4, reverse primers in *OsTPS1*; P3, forward primer in T-DNA.

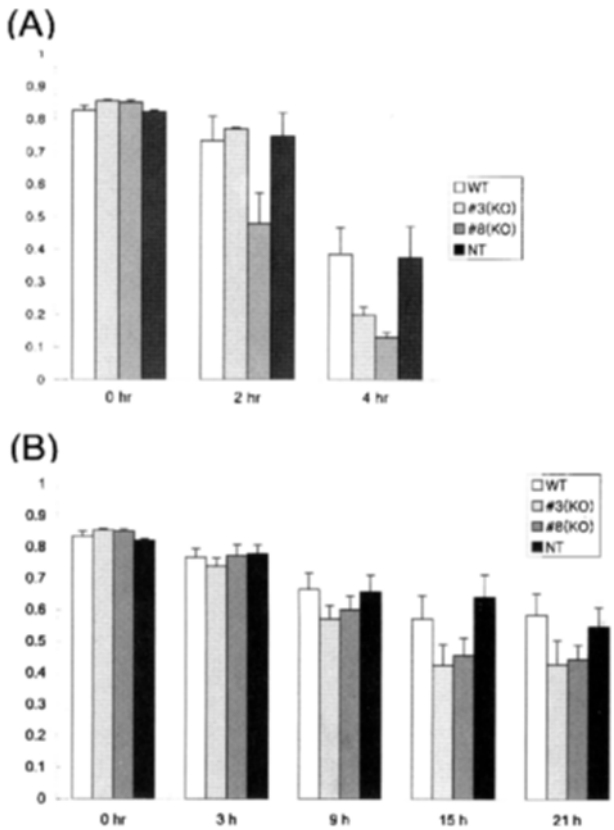

Figure 5. Stress tolerance of *OsTPS1* KO plants as defined by level of chlorophyll fluorescence. Changes in fluorescence were measured from extended leaves at indicated times after drought **(A)** and cold **(B)** stresses were applied. Functional damage to photosynthetic apparatus was estimated by measuring mean and standard error of Fv/Fm values. Bars represent standard errors of means from triplicate experiments for cold treatment and duplicate experiments for drought test.

OsTPS1-specific primers and the T-DNA primer gene (Fig. 1). We found that Plants #3 and #8 were T-DNA homozygotes, while #1, #2, and #7 were heterozygotes (Fig. 4). Numbers 9 and 10 were wild-type segregants. Based on their morphology, we could not detect any phenotypic change in those KO plants of the *OsTPS1* gene.

Stress Tolerance of *OsTPS1* KO Plants

We used chlorophyll fluorescence as an indicator of stress tolerance after cold or drought treatment. The ratio of Fv to Fm, which represents the activity of Photosystem II, defines the degree of functional damage in plants (Genty et al., 1989). Our KO plants, #3 and #8, as well as the wild types, showed progressively lower values for Fv/Fm following stress treatment. For example, prior to these experiments, the Fv/Fm ratios for both KO and WT were about 0.85 (Fig. 5). After 4 h of drought-stress treatment, values for the two KO plants were reduced significantly, to 0.16 ± 0.024 and 0.12 ± 0.021, whereas those for the WT and the non-transgenic segregants (NT) were about 0.47. Nevertheless, after 6 h, all plants were similarly withered. For the cold-stress trials, Fv/Fm values for the KOs were reduced to 0.43 ± 0.07 and 0.46 ± 0.05 after 15 h. In contrast, those calculated for NT and WT were 0.64 ± 0.07 and 0.57 ± 0.07, respectively. These results indicate that, at certain time points, our knockout plants are more sensitive to cold or drought stress than are the WT.

Expression of *OsTPS1* in Transgenic *UBI::CBF1* Rice

Because *OsTPS1* is responsive to abiotic stress, and

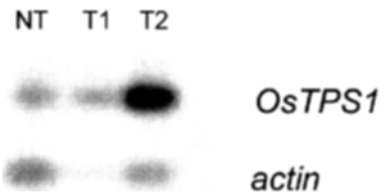

Figure 6. Analysis of *OsTPS1* expression in transgenic rice (T1, T2) expressing *CBF1*. RNA was isolated from transgenic plants grown under normal conditions. Transcript of rice *actin* gene shows internal control for PCR analysis.

the KOs are more sensitive to cold or drought stress than are the WT, we hypothesized that *OsTPS1* is regulated by stress-signaling components. CBF genes control the expression of stress-inducible genes that contain the C-repeat/drought response element (CRT/DRE) in their promoters (Taji et al., 2002). Here, a putative CRT/DRE core sequence, CCGAC, was located at 935 b upstream of the ATG start codon of *OsTPS1*. We previously have generated and analyzed transgenic rice plants that express *Arabidopsis CBF1* cDNA (Lee et al., 2004). In the current study, semi-quantitative RT-PCR analysis also showed that *OsTPS1* expression was elevated in those transgenics that strongly expressed *CBF1* (Fig. 6).

DISCUSSION

Arabidopsis has 11 *TPS* homologues (Barbara et al., 2001), which can be grouped in two subfamilies depending on their similarity to yeast *TPS1* or *TPS2*. The first subfamily (Class I) comprises four genes, *AtTPS1* through *4*, of which *AtTPS1* encodes TPS activity (Blazquez et al., 1998). The second subfamily (Class II, *AtTPS 5* through *11*) contains phosphatase activities in the C-terminal portion, as well as TPS activity in the N-terminal (Barbara et al., 2001). Here, OsTPS1 had 48% to 50% homology with Class II sequences, compared with only 24% to 25% homology to the Class I sequences.

Using semi-quantitative RT-PCR and real-time PCR analysis, we found that *OsTPS1* transcript was up-regulated by drought, cold, salt, and ABA stresses. Both analyses were repeated three times. Although studies concerning trehalose overproduction in plants have been reported, the stress response by the gene itself has not been described except for a cotton EST clone homologous to *Arabidopsis TPS*, which is up-regulated by water stress (1999). Here, *OsTPS1* was induced in both the roots and shoots of our drought-treated seedlings. Garcia et al. (1997) have shown that trehalose begins to accumulate in small amounts not in the leaves, but in the roots, of rice after 3 d of salt stress. Their research, however, did not present any molecular genetic data. Therefore, it would be interesting to determining whether *OsTPS1* is responsible for trehalose production in salt-stressed rice roots.

Our assessment revealed that drought or cold tolerance in *OsTPS1* KO plants was about 28% to 66% lower than that in NT. In contrast, the Fv/Fm ratios for transgenic rice expressing *E. coli trehalose biosynthesis* genes are 15% to 39% higher than those of control plants under stress conditions (Garg et al., 2002; Jang et al., 2003). Although trehalose promotes tolerance to salt stress (Garcia et al., 1997), the reason for lower stress tolerance by the KOs is unclear. Therefore, quantification of trehalose in those plants should be conducted in the future. Trehalose is more effective than other sugars in increasing lipid bilayer fluidity (Crowe et al., 1984a, b) and in preserving enzyme stability during drying (Colaco et al., 1992). Although that functioning has been studied with *E. coli* or yeast trehalose biosynthesis genes, it is not well known how the rice *TPS* gene is involved in plant stress responses. To our knowledge, we are the first to demonstrate that the *TPS* gene is indeed related to stress tolerance in rice.

One suggested function for trehalose is as a regulator of plant metabolism and development (Goddijn and Smeekens, 1998; Vogel et al., 1998; Goddijn and Dun, 1999). Our study demonstrated that the *OsTPS1* KO mutants exhibited no phenotypic alterations. However, an *Arabidopsis* mutant that is disrupted in the gene encoding TPS1 manifests an embryo-lethal phenotype (Eastmond et al., 2002). Rice plants with exogenous trehalose show no growth inhibition or visible changes in their appearance (Garcia et al., 1997). Furthermore, transgenic rice that expresses *E. coli TPSP* has normal to sustained growth (Garg et al., 2002; Jang et al., 2003).

Here, we also demonstrated that expression of *OsTPS1* is up-regulated in transgenic rice expressing *CBF1*. Under non-stressed conditions, overexpression of *CBF* in transgenic *Arabidopsis* has been shown to cause the overexpression of many stress-inducible genes, e.g., *RD, ERD,* and *COR* (Kasuga et al., 1999; Gilmour et al., 2000; Seki et al., 2001). Therefore, based on the putative CRT/DRE sequence present in the *OsTPS1* promoter region, as well as the high expression of *OsTPS1* in *Ubi::CBF1* rice, we can postulate that *OsTPS1* might be a target of the rice orthologue of *CBF1*.

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