Expression of a Bifunctional Fusion of the *Escherichia coli* Genes for Trehalose-6-Phosphate Synthase and Trehalose-6-Phosphate Phosphatase in Transgenic Rice Plants Increases Trehalose Accumulation and Abiotic Stress Tolerance without Stunting Growth[1]

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Trehalose plays an important role in stress tolerance in plants. Trehalose-producing, transgenic rice (*Oryza sativa*) plants were generated by the introduction of a gene encoding a bifunctional fusion (TPSP) of the trehalose-6-phosphate (T-6-P) synthase (TPS) and T-6-P phosphatase (TPP) of *Escherichia coli*, under the control of the maize (*Zea mays*) ubiquitin promoter (*Ubi1*). The high catalytic efficiency (Seo et al., 2000) of the fusion enzyme and the single-gene engineering strategy make this an attractive candidate for high-level production of trehalose; it has the added advantage of reducing the accumulation of potentially deleterious T-6-P. The trehalose levels in leaf and seed extracts from *Ubi1::TPSP* plants were increased up to 1.076 mg g fresh weight^{-1}. This level was 200-fold higher than that of transgenic tobacco (*Nicotiana tabacum*) plants transformed independently with either *TPS* or *TPP* expression cassettes. The carbohydrate profiles were significantly altered in the seeds, but not in the leaves, of *Ubi1::TPSP* plants. It has been reported that transgenic plants with *E. coli TPS* and/or *TPP* were severely stunted and root morphology was altered. Interestingly, our *Ubi1::TPSP* plants showed no growth inhibition or visible phenotypic alterations despite the high-level production of trehalose. Moreover, trehalose accumulation in *Ubi1::TPSP* plants resulted in increased tolerance to drought, salt, and cold, as shown by chlorophyll fluorescence and growth inhibition analyses. Thus, our results suggest that trehalose acts as a global protectant against abiotic stress, and that rice is more tolerant to trehalose synthesis than dicots.

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranose) is a nonreducing diglucoside that is found in various organisms, including bacteria, algae, fungi, yeast (*Saccharomyces cerevisiae*), insects, and some plants (Elbein, 1974). Trehalose serves not only as a carbohydrate reserve, but also as a protective agent against a variety of physical and chemical stresses in various organisms (van Laere, 1989; Wiemken, 1990; Eleutherio et al., 1993; Strøm and Kassen, 1993). Trehalose is known to have high water retention activity, which maintains the fluidity of membranes under dry conditions (Leslie et al., 1995). Thus, this sugar allows desert plants to tolerate naturally occurring stresses during cycles of dehydration and rehydration (Drennan et al., 1993; Müller et al., 1995).

A role for trehalose in stress tolerance has been demonstrated for cryptobiotic plant species, such as the desiccation-tolerant *Selaginella lepidophylla*. In this case, trehalose accumulation represented 12% of the plant dry weight during dehydration, which probably protected the proteins and membrane structures. Upon rehydration, *S. lepidophylla* regained complete viability and the trehalose levels declined (Goddijn and van Dun, 1999). Plants accumulate a number of osmoprotective agents, such as Pro, in response to NaCl stress. During osmotic stress in rice (*Oryza sativa*), trehalose or similar carbohydrates appear to be more important than Pro. It has been shown that treatment of rice with exogenous trehalose caused a decrease in NaCl accumulation and growth inhibition (Garcia et al., 1997).

Transgenic plants that expressed the trehalose-6-phosphate (T-6-P) synthase (TPS) and/or T-6-P phos-

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phatase (TPP) genes from microorganisms, not only exhibited increased drought tolerance, but also showed strong developmental alterations (Holmström et al., 1996; Goddijn et al., 1997; Romero et al., 1997; Pilon-Smits et al., 1998). These pleiotropic phenotypes were present even in the absence of trehalose accumulations (Müller et al., 1999). All of the transgenic plants reported to date have been dicot plants, which generally produce very low levels of trehalose (Holmström et al., 1996; Goddijn et al., 1997; Romero et al., 1997; Pilon-Smits et al., 1998). Interestingly, rice appears to be more tolerant to trehalose than dicot plants because exogenous application of trehalose produced no growth inhibition or visible changes in the appearance of rice plants, whereas Pro inhibited growth by approximately 15% (Garcia et al., 1997). To develop stress-tolerant transgenic plants through elevated production of trehalose, we transformed rice plants with a gene that encodes a bifunctional fusion enzyme (TPSP) of TPS and TPP from *Escherichia coli* (Seo et al., 2000). The catalytic efficiency of TPSP was 3.5- to 4.0-fold higher than that of a mixture of the individual enzymes, which demonstrates the kinetic advantage of the fusion enzyme (Seo et al., 2000). The resultant transgenic plants produced trehalose levels that were up to 0.1% of the fresh weight, and the plants showed no visible growth inhibition. The production of trehalose in these plants resulted in increased tolerance to drought, salt, and cold stresses.

RESULTS

Transformation of Rice with the Recombinant Fusion Gene *TPSP*

Overexpression of a heterologous *TPS* gene from *E. coli* or yeast in dicot plants results in significant morphological growth defects and altered metabolism (Goddijn et al., 1997; Romero et al., 1997). The yeast T-6-P inhibits hexokinase in vitro (Blazquez et al., 1993), thereby partly regulating Glc influx into glycolysis (Thevelein and Hohmann, 1995). These observations have led us to speculate that T-6-P might cause phenotypic alterations in transgenic plants. To produce high levels of trehalose while maintaining relatively low levels of T-6-P in plants, we transformed rice with a gene that encodes a bifunctional fusion (TPSP) of the TPS and TPP of *E. coli* (Fig. 1A). The K_{cat} value, the turnover number, of TPSP for UDP-Glc and Glc-6-phosphate was similar to that of TPS plus TPP. However, the catalytic efficiency of TPSP was 3.5- to 4.0-fold higher than that of a equimolar mixture of the individual enzymes, which demonstrates the kinetic advantage of the fusion (Seo et al., 2000). The high catalytic efficiency that resulted from simultaneous catalysis of two-step synthesis by a single enzyme probably reduces the accumulation of potentially deleterious T-6-P.

The components of the plasmid used for rice transformation are shown in Figure 1B. The maize ubiquitin promoter was linked to the recombinant fusion gene *TPSP* (Seo et al., 2000), which was constructed by connecting the *TPS* and *TPP* genes from *E. coli* after the stop codon of the *TPS* gene had been removed by PCR. The chimeric *Ubi1::TPSP* gene was then ligated to the expression cassette that carried the coding region of the phosphinothricin acetyl transferase gene (*bar*) under the control of the 35S promoter, thereby generating the plasmid pSB-UTPSP. Fourteen independent transgenic lines were obtained by the *Agrobacterium tumefaciens*-mediated method, and grown to maturity in the greenhouse. Phosphinothricin acetyl transferase can detoxify phosphinothricin-based herbicides (Duan et al., 1996). All of the transformants were herbicide resistant, as tested by painting leaves with the commercial herbicide Basta (Jang et al., 1999). Of the 14 plants, 11 were fertile, and their T_1 and T_2 seeds were collected. The copy numbers and integration events relating to the transgene were determined by genomic Southern blots. The 11 lines contained one to three copies of the transgene. Five homozygous T_2 lines containing one or two copies of *TPSP* were chosen for further analysis (Fig. 1C).

Analysis of Transgenic Rice Plants

To investigate *TPSP* expression levels, RNA-blot hybridization was carried out using total RNA samples from leaf tissues. As shown in Figure 2A, the probe (Fig. 1B) detected a single mRNA band of approximately 2.4 kb in the five transgenic lines tested. Transcript levels of *TPSP* varied within a range of 2-fold among the lines, as judged by values of *TPSP* to *rbcS* ratio. To examine whether the *TPSP* expression could activate other stress-inducible genes in the *Ubi1::TPSP* plants, we analyzed transcript levels of some candidate genes including s*alT* (Claes et al., 1990), *Lip19* and *Lip5* (Aguan et al., 1991), and the Arabidopsis *cor47* homolog *Dip1* (GenBank accession no. AU095986). *Lip5* and *Dip1* were the ones that were largely induced upon exposure of untransformed rice to drought and salt stresses for 2 h, as depicted in Figure 2B. Therefore, RNAs from transgenic and non-transgenic plants were hybridized with *Lip5* and *Dip1* probes. In the case of *Ubi1::TPSP*-1 and -5 plants under normal growth conditions, transcript levels of *Lip5*, but not those of *Dip1*, were increased by 1.5- and 1.6-fold, respectively, as compared with non-transgenic controls (Fig. 2A). Thus, the stress-inducible genes are partly induced by trehalose synthesis, but not as much as by stress treatments in rice.

To examine the accumulation levels of trehalose and T-6-P in transgenic plants, quantitative carbohydrate analysis was carried out by high-performance ion chromatography (HPIC), as described in "Materials and Methods." The carbohydrate profiles of *Ubi1::TPSP* plants were similar, but distinct from those of untransformed controls (Fig. 3). Trehalose

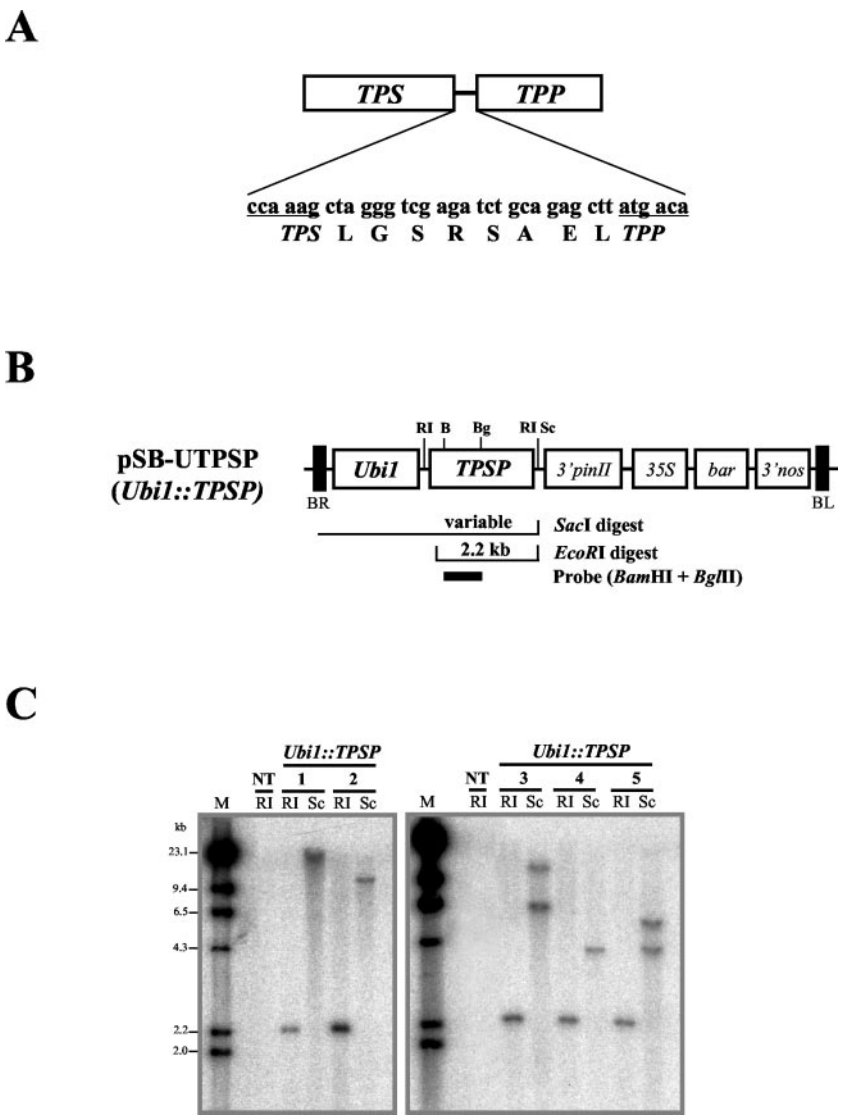

Figure 1. The bifunctional *TPSP* fusion, a transformation vector, and genomic Southern-blot hybridization of transgenic rice plants. A, The predicted amino acid sequence of the fusion boundary of *TPSP* is shown. The *TPSP* construct was made by in-frame fusion of the *E. coli otsA* and *otsB* genes, which encode TPS and TPP, respectively. B, pSB-UTPSP (*Ubi1::TPSP*) consists of the maize (*Zea mays*) ubiquitin promoter (*Ubi1*) linked to the *TPSP* coding region, the 3′ region of the potato proteinase inhibitor II gene (*3′pinII*), and a gene expression cassette that contains the 35S promoter, the *bar*-coding region, and the 3′ region of the nopaline synthase gene (*nos*). The restriction enzymes, the expected fragment sizes, and the hybridization probe (probe) used for genomic DNA-blot analyses are shown below the map. C, Genomic Southern-blot analysis of *Ubi1::TPSP* transgenic rice plants. Genomic DNAs from the leaves of five *Ubi1::TPSP* plant lines and from untransformed control plants (NT) were digested with *Eco*RI (RI) or *Sac*I (Sc), fractionated on an agarose gel, blotted onto a nylon membrane, and hybridized with the probe for *TPSP* coding region (described in B).

was present in the leaf and seed extracts of transgenic plants at levels of 0.31 to 1.076 mg g fresh weight^{-1} depending on lines, which contrasted with the negligible levels of trehalose in untransformed control plants (Table I). The transcript levels of *TPSP* did not correlate with those of trehalose accumulation. For example, the *Ubi1::TPSP*-2 plants had lower expression of *TPSP* by about 2-fold than the *Ubi1::TPSP*-1 plants (see *TPSP* to *rbcS* ratios in Fig. 2A), but contained relatively similar levels of trehalose (Fig. 3). This is probably because transgenic plants can tolerate levels of trehalose accumulation within a limited range that allows them to grow and develop normally, thereby restricting the trehalose levels of the higher expressor. As summarized in Table I, several previous studies showed that transgenic plants expressing *TPS* and/or *TPP*, either from *E. coli* or yeast, had lower levels of trehalose accumulation than *Ubi1::TPSP* plants. Our *Ubi1::TPSP* plants produced trehalose at levels that were up to 200-fold higher than those reported for transgenic tobacco plants that were transformed independently with *E. coli TPS* or *TPP* expression cassettes (Goddijn et al., 1997). As shown in Figure 3B, T-6-P was not detected in leaf tissues of both transgenic and non-transgenic rice plants. We also measured trehalase activities in young rice leaves by estimating the amounts of Glc produced by hydrolysis of trehalose and corresponding decrease in trehalose. As shown in Table I, trehalase activity of rice is lower than that of tobacco and comparable with that of potato tuber. Taken together, these results suggest that the high levels of trehalose accumulation in *Ubi1::TPSP* plants is because of the enzymatic activity of TPSP, rather than lower activity of trehalase.

Effect of Trehalose Production on the Carbohydrate Content and Growth Phenotype of Transgenic Plants

Exogenous application of trehalose to Arabidopsis strongly reduced root elongation with a concomitant

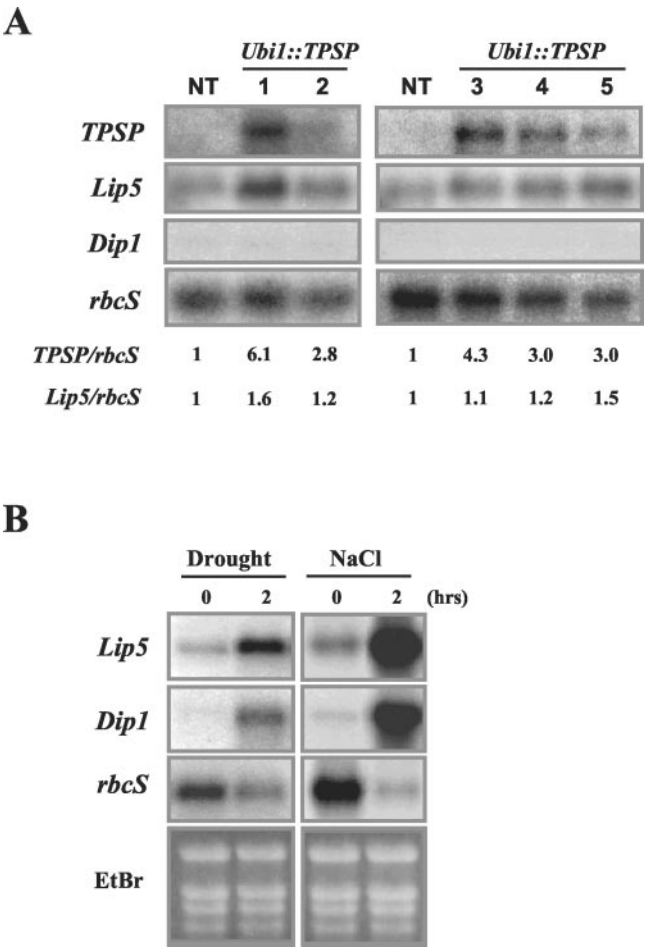

Figure 2. Transcript levels of *TPSP* and stress-inducible rice genes in the leaves of *Ubi1::TPSP* and untransformed plants. A, Northern-blot analysis was performed using total RNA from young leaves of five *Ubi1::TPSP* plant lines (shown in Fig. 1C) and from untransformed control plants (NT). The blots were hybridized with probes for *TPSP* (as described in Fig. 1B), *Lip5* (Aguan et al., 1991), and *Dip1* (GenBank accession no. AU095986). Equal loading of total RNA samples was verified by reprobing the membrane with the rice *rbcS* gene for Rubisco (Kyozuka et al., 1993). Transcript levels of *TPSP* and *Lip5* in the *Ubi1::TPSP* lines were calculated using those of corresponding *rbcS* as a reference and the resultant values were then normalized to 1 for that from NT. B, Northern blots of total RNA from untransformed plants immediately before and after stress treatments. The blots were hybridized with probes for *Lip5*, *Dip1*, and *rbcS*. Transcript levels of *rbcS* were previously reported to be decreased upon exposure to drought and salt stresses (Weatherwax et al., 1996). For drought stress, 14-d-old seedlings were air dried for 2 h at 28°C; for salt stress, 14-d-old seedlings were exposed to 400 mM NaCl for 2 h at 28°C. All of the experiments were carried out under continuous 150 μmol m^2 s^{-1} light conditions. Ethidium bromide (EtBr) staining of total RNA was used to ensure equal RNA loading.

increase in starch accumulation in shoots, but the soluble sugar content remained unchanged. These results suggest that trehalose interferes with carbon allocation to the sink tissues by inducing starch synthesis in the source tissues (Wingler et al., 2000). To examine the effect of trehalose production on carbohydrate content in transgenic rice, extracts from leaves and seeds of the *Ubi1::TPSP* plants were analyzed. Quantitative carbohydrate analysis by HPIC showed no significant changes in the carbohydrate content of the leaves, whereas several of the carbohydrate peaks were changed in the seeds. In particular, the Suc and multiglucoside concentrations in the seeds were significantly reduced. Three additional peaks (P1, P2, and P3) appeared in the HPIC profiles of the transgenic seeds (Fig. 3A).

In previous reports, constitutive expression of *TPS* and/or *TPP* from either *E. coli* or yeast in tobacco or potato plants resulted in undesirable pleiotropic effects, including stunted growth and altered root systems under normal growth conditions (Holmström et al., 1996; Goddijn et al., 1997; Romero et al., 1997; Pilon-Smits et al., 1998). These pleiotropic growth phenotypes were present even in the absence of bulk accumulations of trehalose (Müller et al., 1999). Although the *Ubi1::TPSP* plants produced trehalose levels that were up to 0.1% of the plant fresh weight, they showed neither growth inhibition nor visible changes in appearance. As depicted in Figure 4, the *Ubi1::TPSP* plants showed normal vegetative phenotype and fertility as compared with untransformed control plants. A slight delay in germination of *Ubi1::TPSP* seeds was observed at 3 d after the start of germination, but the growth rates converged at later stages without notable difference in shoot and root growth. We also made *35S::TPSP* potato plants that hardly grew with altered phenotypes and died prematurely (data not shown). These results lead us to speculate that the overproduction of trehalose is not as toxic for rice as it is for dicot plants.

Stress Tolerance Is Significantly Improved in the Transgenic Plants

In nature, trehalose serves as a protectant against a variety of stresses in different organisms (Eleutherio et al., 1993; Strøm and Kassen, 1993; Garcia et al., 1997). To investigate whether the accumulation of trehalose in *Ubi1::TPSP* plants was correlated with increased stress tolerance, 6-d-old T_2 seedlings were grown in a greenhouse and watering was stopped for up to 12 d. After 12 d without watering, differences in drought tolerance were evident between the untransformed control and *Ubi1::TPSP* plants (Fig. 5A). After prolonged exposure to drought stress, the *Ubi1::TPSP* plants survived and displayed vigorous root and shoot growth; over the same treatment period, the untransformed plants were nearly dead because of severe damage of leaves and concomitant loss of chlorophyll. The increased tolerance of the *Ubi1::TPSP* plants was confirmed by measuring changes in chlorophyll fluorescence. Most of the chlorophyll fluorescence in leaves arises from chlorophyll and is associated with the PSII. The ratio of F_v to F_m was used to estimate the quantum yield of PSII

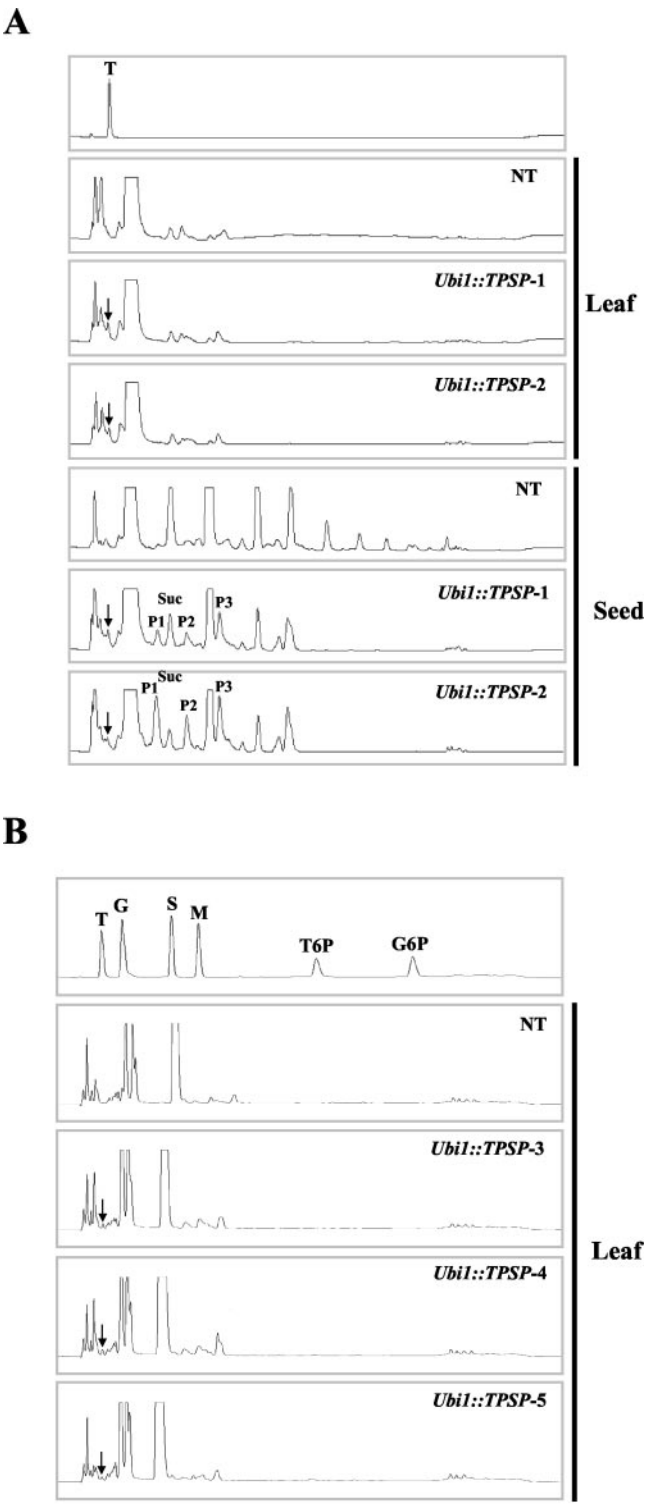

Figure 3. HPIC analysis of trehalose accumulation in *Ubi1::TPSP* plants. A, The chromatograms show carbohydrate profiles from a standard containing 1 μg of trehalose (T), leaf and seed extracts that were prepared from untransformed controls (NT), and two transgenic lines (*Ubi1::TPSP*-1 and -2). B, Carbohydrate profiles from a standard containing 1 μg each of trehalose (T), Glc (G), Suc (S), maltose (M), T-6-P, and Glc-6-phosphate (G-6-P), leaf extracts that were prepared from untransformed controls (NT), and three transgenic lines (*Ubi1::TPSP*-3, -4, and -5).

(Strasser and Butler, 1977). Environmental stresses that damage the efficiency of PSII result in decreases in the F_v/F_m ratio (Artus et al., 1996). To examine stress tolerance using the F_v/F_m ratio, 14-d-old seedlings were exposed to various stresses under continuous 150 μmol m^2 s^{-1} light (see "Materials and Methods"). A decrease in the F_v/F_m ratio was observed after the plants were subjected to dehydration, salt, or low-temperature stresses. As shown in Figure 5B, the F_v/F_m ratios were 15% to 19% higher in *Ubi1::TPSP* plants than in the untransformed control plants.

To investigate the increased tolerance of *Ubi1::TPSP* plants against salinity, we measured the growth during germination of five homozygous T_2 seedlings in hydroponic solutions that contained 100 mM NaCl. In the absence of NaCl, *Ubi1::TPSP* seedlings grew similarly to non-transgenic seedlings during 13 d after germination, as shown in Figure 4. In the presence of NaCl, in contrast, both shoot and seminal root growth of the *Ubi1::TPSP* seedlings was much faster than that which occurred in those of the non-transgenic seedlings (Fig. 6, A and B).

Thus, the constitutive expression of *TPSP* in transgenic plants leads to increased levels of trehalose accumulation, which correlated with enhanced tolerance against drought, salinity, and low temperature, suggesting that trehalose acts as a global protectant against abiotic stress in rice.

DISCUSSION

Trehalose is a nonreducing disaccharide that functions as a stress protection metabolite and carbohydrate reserve in many organisms (van Laere, 1989; Wiemken, 1990; Eleutherio et al., 1993; Strøm and Kassen, 1993; Goddijn and van Dun, 1999). To generate stress-tolerant transgenic rice plants, we transformed rice with a gene encoding the bifunctional enzyme TPSP, which was derived from an in-frame fusion of TPS and TPP from *E. coli*. The high catalytic efficiency of the fusion enzyme (Seo et al., 2000) and the single-gene engineering strategy made this an attractive candidate for the high-level production of trehalose combined with reduced accumulations of potentially deleterious T-6-P. This is probably because physical proximity of two enzymes increases the reaction rate by facilitating transfer of the reaction intermediate T-6-P when they are present in a complex. The resultant transgenic plants (*Ubi1::TPSP*) produced trehalose levels that accounted for up to 0.1% of the plant fresh weight, which was 200-fold higher than the levels in transgenic tobacco plants that were cotransformed with *E. coli TPS* and *TPP* on independent expression cassettes (Goddijn et al., 1997). The fact that trehalase activity of rice is comparable with that of potato tubers (Table I) led us to conclude that the high levels of trehalose accumulation in *Ubi1::TPSP* plants is because of the enzymatic activity of TPSP, rather

Table I. *Trehalose contents and trehalase activities in monocot and dicot transgenic plants*

Leaf tissues were ground in liquid nitrogen and extracted at 100°C with water. After the extract was clarified, trehalose was quantitated by HPIC with a Carbo-Pak PA1 column using the DX500 HPIC system (Dionex 500). Trehalase activity was measured by estimating both the glucose produced by hydrolysis of trehalose and trehalose reduced.

Transgenic Plants	Transgene	Trehalose Production	Trehalase Activity[a]	References
		mg g fresh wt^{-1}	*μmol h mg protein*$^{-1}$	
Rice	*E. coli otsA/otsB* fusion	0.31–1.076	0.174 ± 0.05	This study
Tobacco (*Nicotiana tabacum*)	Yeast *TPS1*	0.16–0.64 (0.8–3.2)[b]	NT[c]	Holmström et al. (1996)
Tobacco	Yeast *TPS1*	0.17	NT	Romero et al. (1997)
Tobacco	*E. coli otsA* and *otsB*	0.038–0.04 (0.19–0.20)[b]	NT	Pilon-Smits et al. (1998)
Tobacco	*E. coli otsA* and *otsB*	0.005–0.07	0.442–0.679	Goddijn et al. (1997)
Tobacco	*E. coli otsA*	0.02–0.11	0.442–0.679	Goddijn et al. (1997)
Potato (*Solanum tuberosum*)	*E. coli otsA* and *otsB*	0.003–0.02	0.048 ± 0.006 (0.125 ± 0.003)[d]	Goddijn et al. (1997)

[a]Tested in leaf tissues. [b]Nos. in parentheses refer to milligram per gram dry wt, as described in the literature. [c]NT, Not tested. [d]Tested in tuber.

than the lower activity of trehalase. This is because trehalose was not detected at all in transgenic potato tubers with *E. coli otsA* and *otsB* even though they contained a similar level of trehalase activity to that of rice (Goddijn et al., 1997). This becomes clearer if the trehalase activity in rice was underestimated because of the usage of unpurified, non-desalted extracts in our assay conditions. Interestingly, our *Ubi1::TPSP* plants showed no growth inhibition or visible phenotypic alterations despite the high-level production of trehalose, in contrast with the results obtained for transgenic dicots, such as potato and tobacco (Goddijn et al., 1997; Romero et al., 1997).

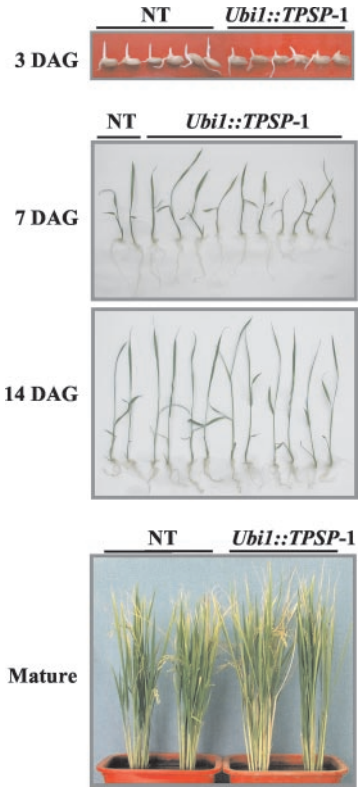

Figure 4. Growth phenotypes of T_2 plants of *Ubi1::TPSP*-1 and untransformed control plants (NT), 3 d after germination (3 DAG), 7 d after germination (7 DAG), 14 d after germination (14 DAG), and in mature plants setting seeds (mature).

Trehalose may also function as a regulator of plant metabolism and development (Goddijn and Smeekens, 1998; Vogel et al., 1998; Goddijn et al., 1999). For example, the growth of Arabidopsis seedlings on trehalose-containing medium led to the inhibition of root elongation and an accumulation of starch in the shoots (Wingler et al., 2000). An Arabidopsis mutant that was disrupted in the gene encoding TPS showed an embryo-lethal phenotype (Eastmond et al., 2002). These results are seemingly consistent with observations that overexpression of a heterologous *TPS* and/or *TPP* gene in dicot plants results in severely stunted growth (Goddijn et al., 1997; Romero et al., 1997). To date, studies of this type have been conducted in dicotyledonous plants, such as Arabidopsis, tobacco, and potato. Very little is known about the physiological roles of trehalose metabolism in monocots. Garcia et al. (1997) treated rice plants with exogenous trehalose or Pro, and found that, unlike the situation in dicots, trehalose produced no growth inhibition or visible changes in plant appearance, but instead reduced the inhibitory effects of NaCl. In contrast, Pro inhibited growth by approximately 15%. These observations led us to speculate that trehalose synthesis might not function in monocot plants as it does in dicot plants. It seems likely that monocots are more tolerant to the biosynthesis of trehalose than dicots because our *Ubi1::TPSP* plants produced trehalose at relatively high levels without any phenotypic alterations. This is further evidenced by our *35S::TPSP* potato plants that were severely stunted and died prematurely.

In yeast, T-6-P affects glycolysis and sugar signaling through its interaction with hexokinase, which is a putative sensor (Thevelein and Hohmann, 1995; Paul et al., 2001). Although it remains to be determined whether T-6-P in plants interacts with hexokinase as it does in yeast, T-6-P appears to be important in sugar signaling in plants (Paul et al., 2001). Our

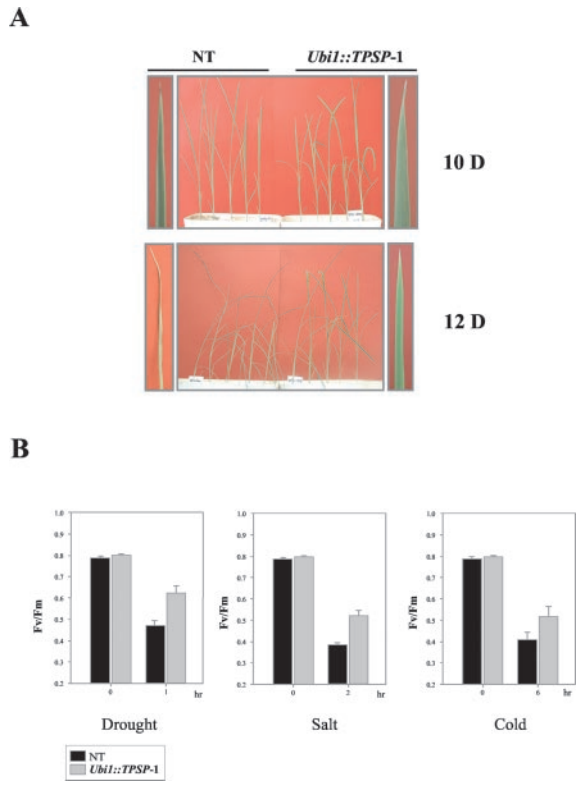

Figure 5. Stress tolerance of T_2 plants of *Ubi1::TPSP*-1 and untransformed control plants (NT). A, Six-day-old seedlings were grown in the greenhouse for 10 d (10 D) and 12 d (12 D) after watering stopped. Photos of the upper leaves of corresponding plants are shown at either side of the figures. B, For drought stress, 14-d-old seedlings were air dried for 1 h at 28°C; for salt stress, 14-d-old seedlings were exposed to 150 mM NaCl for 2 h at 28°C; and for cold stress, 14-d-old seedlings were exposed to 4°C for 6 h. All of the experiments were carried out under continuous 150 μmol m^2 s^{-1} light conditions. Chlorophyll fluorescence (variable fluorescence [F_v] and maximal fluorescence [F_m]) was measured using a pulse modulation fluorometer. Six seedlings were measured and averaged for each treatment protocol.

bifunctional enzyme TPSP was designed in such a way that it not only gave high catalytic efficiency (Seo et al., 2000) for trehalose production, but it also restricted T-6-P accumulation to minimum levels. In our *Ubi1::TPSP* plants, T-6-P was present at levels below detection. Although this might be because our assay method (about 1-ng sensitivity) for T-6-P was not sensitive enough, we believe that the fusion gene assisted the transgenic rice plants in achieving normal growth.

Exogenous application of 25 mM trehalose to Arabidopsis induced strong accumulations of starch in the shoots, whereas the Glc and Fru levels were not affected and the Suc content was reduced. Thus, trehalose appears to affect starch biosynthesis by inducing directly the components of the starch biosynthetic pathway (Wingler et al., 2000). Inhibition of trehalase in vivo by validamycin A led to the accumulation of trehalose and to strong reductions in the Suc and starch contents of the flowers, leaves, and stems. Thus, Arabidopsis trehalose and trehalase may play significant roles in regulating carbohydrate allocation in plants (Müller et al., 2001). We performed carbohydrate profile analysis to examine the effect of trehalose accumulation on carbohydrate allocation in the *Ubi1::TPSP* plants. This method enabled us to detect significant changes in the soluble carbohydrate content of the seeds, but not of the leaves. In the transgenic seeds, the concentrations of Suc and multiple-glucoside carbohydrates were reduced, whereas three new carbohydrate peaks (P1, P2, and P3 in Fig. 3) were detected. Although the constituents of the three peaks remain to be determined, our data suggest that production of trehalose does not affect carbohydrate allocation in leaf tissues. This could be one reason why our transgenic rice plants grew normally in the presence of accumulated trehalose. One possible explanation for the difference in carbohydrate contents between seeds and leaves could be that trehalose inhibits carbon allocation to the sink tissues by increasing starch synthesis in the source tissues, as observed in trehalose-treated Arabidopsis seedlings (Wingler et al., 2000). It is also possible that trehalose affects activities of enzymes involved in starch biosynthesis in a sink-specific manner, thereby altering the pool sizes of soluble carbohydrates in rice seeds. Trehalose has been shown to interfere with carbohydrate-mediated gene regulation in soybean (*Glycine max*; Müller et al., 1998), barley (*Hordeum vulgare*; Wagner et al., 1986), and Arabidopsis (Wingler et al., 2000).

Trehalose has been found to be more effective than other sugars in increasing lipid bilayer fluidity (Crowe et al., 1984a, 1984b) and in preserving enzyme stability during drying (Colaco et al., 1992). In rice, trehalose promotes resistance to salt stress (Garcia et al., 1997). Under conditions of dehydration, and salt or cold stress, the F_v/F_m ratios of our *Ubi1::TPSP* plants were 15% to 19% higher than those of control plants (Fig. 5B), which indicates that the transgenic plants are performing efficient photosynthesis under the adverse conditions. Consistent with our observations is that transgenic rice plants overexpressing *OsCDPK7*, a gene for a protein kinase, showed 10% higher levels of F_v/F_m ratio for up to a 24-h period of cold treatment, yet the extent of tolerance to the stress was significant (Saijo et al., 2000). Moreover, under drought- and salt-stressed conditions, growth of *Ubi1::TPSP* seedlings was much faster than that of the non-transgenic seedlings (Figs. 5 and 6). Transcript levels for *Lip5* in *Ubi1::TPSP* plants were slightly elevated under normal growth conditions, whereas they were greatly induced upon exposure to drought and salt stresses (Fig. 2), suggesting that the enhanced stress tolerance was not mainly because of the induction of stress-inducible genes. Taken together, these results demonstrated that trehalose functions as a global protectant against abiotic stress in rice.

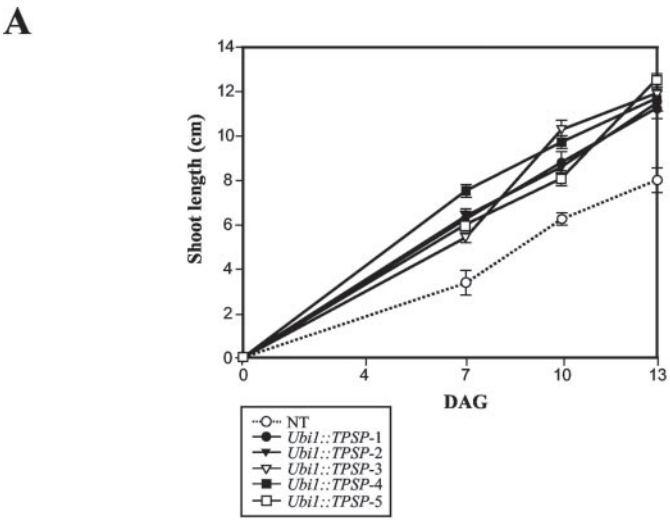

Figure 6. Salt tolerance of non-transgenic and *Ubi1::TPSP* seedlings grown in the presence of 100 mM NaCl. Ten T_2 seeds from each of the five (1–5) *Ubi1::TPSP* lines and the non-transgenic (NT) plants were germinated and grown in hydroponic solutions that contained 100 mM NaCl under continuous 150 μmol m^2 s^{-1} light conditions. A, The shoot length was scored at various intervals. Each data point represents the mean ± SE of triplicate experiments (n = 10). B, Representative seedlings at 10 d after germination are shown.

MATERIALS AND METHODS

Plant Materials

Transgenic and non-transgenic rice (*Oryza sativa*) plants were grown in a greenhouse or in one-half-strength Murashige and Skoog solid medium. Embryogenic callus formation was initiated from mature rice cv Nakdong embryos and maintained on solid Murashige and Skoog medium (pH 5.8) that contained 1% (w/v) agarose, 30 g L^{-1} Suc, and 2.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid.

Vector Construction and Transformation of Rice

The recombinant fusion of the *Escherichia coli* genes for TPS and TPP (Seo et al., 2000) was introduced into rice plants. The pSB-UTPSP (*Ubi::TPSP*) plasmid consisted of the maize (*Zea mays*) ubiquitin promoter linked to the TPSP coding region, and the 3′ region of the potato (*Solanum tuberosum*) proteinase inhibitor II gene (*pinII*), as well as a gene expression cassette that comprised the 35S promoter, the *bar*-coding region, and the 3′-region of the nopaline synthase gene (*nos*). The plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating, as previously described (Jang et al., 1999). For *A. tumefaciens*-mediated transformation, about 200 mature seeds of rice cv Nakdong were dehusked and sterilized with 70% (w/v) ethanol for 1 min with gentle shaking. The ethanol was discarded and the seeds were sterilized further with 100 mL of 20% (w/v) commercial bleach for 1 h with gentle shaking. The sterilized seeds were rinsed several times with sterile water. Callus induction, cocultivation with *A. tumefaciens*, and the selection of transformed calli were carried out as previously described (Jang et al., 1999).

Carbohydrate Analysis

The samples were ground in liquid nitrogen and extracted for 10 min at 100°C with 10 mL g fresh weight^{-1} water. The extract was centrifuged, and the supernatant filtered through a 0.45-μm filter unit. Quantitative carbohydrate analysis was carried out by HPIC with a Carbo-Pak PA1 column (4 × 250 nm) using the DX500 HPIC system (Dionex 500, Dionex, Sunnyvale, CA). Carbohydrate was eluted in a continuous sodium acetate gradient of 0 to 250 mM in a 150 mM NaOH solution over 30 min, and monitored with an ED40 electrochemical detector (Dionex DC Amperometry). Commercially available trehalose, Glc, Suc, maltose, T-6-P, and Glc-6-phosphate (Sigma, St. Louis) were used as the standard.

Trehalase Assay

Crude enzyme extracts were obtained by grinding frozen plant material in extraction buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM Suc, 1 mM EDTA (pH 8.0), and 10 mM phenylmethylsulfonyl fluoride. The suspension was incubated for at least 2 h at 0°C and centrifuged (5,000 rpm for 5 min). The supernatant was used for the enzyme activity assays. Trehalase activity was measured by estimating both the Glc produced by hydrolysis of trehalose and trehalose reduced using HPIC with a Carbo-Pak PA1 column (4 × 250 nm) using the DX500 HPIC system (Dionex 500). The reaction mixture containing 30 mM trehalose (Sigma) was incubated at 37°C for 1, 2, and 3 h and stopped by boiling for 2 min. Soluble protein was determined with the Bradford method (Bradford, 1976). Trehalose activity represents the mean of triplicate experiments.

Chlorophyll Fluorescence under Conditions of Drought, and Salt or Cold Stress

Rice seeds were sterilized with 70% (w/v) ethanol for 1 min with gentle shaking. The ethanol was discarded and the seeds were sterilized further with 100 mL of 20% (w/v) commercial bleach for 1 h with gentle shaking. The sterilized seeds were rinsed several times with sterile water and germinated on soil in a growth chamber (16-h-light/8-h-dark cycles at 28°C). For the cold stress treatment, 14-d-old seedlings were exposed to 4°C for 6 h under continuous 150 μmol m^2 s^{-1} light. For the salt stress treatment, 14-d-old seedlings were grown in a nutrient solution, 0.1% (v/v) Hyponex (Hyponex, Busan, Korea), for 2 d and then transferred to fresh nutrient solution containing 9% (w/v) NaCl for 2 h at 28°C under continuous 150 μmol m^2 s^{-1} light. For the dehydration stress treatment, whole plants were air dried for 1 h at 28°C under continuous 150 μmol m^2 s^{-1} light. The chlorophyll fluorescence levels of the untransformed control and of transgenic plants were measured using a pulse modulation fluorometer. The plants were kept in the dark for 2 h before fluorescence measurements and then subjected to a 1-h light period. Subsequently, the leaves were dark adapted for 10 min. At the beginning of each measurement, a small measuring light beam was turned on, and the minimal fluorescence level (F_o) was measured. F_m was then measured by applying a saturation light pulse. F_v/F_m represented the activity of PSII, and was used to assess functional damage to the plants (Artus et al., 1996).

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