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Trehalose 6–Phosphate Synthase and Trehalose 6–Phosphate Phosphatase from *Nicotiana tabacum* Function in Trehalose Biosynthesis and Environmental Stress Tolerance of Yeast

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To investigate functions of trehalose 6–phosphate synthase (TPS) and trehalose 6–phosphate phosphatase (TPP) from a tobacco plant, *Nicotiana tabacum*, the corresponding cDNA clones were isolated. Those genes were designated *NtTPS* and *NtTPP*, respectively. NtTPS included a N–terminal extension according to comparison of deduced amino acid sequence of *NtTPS* with those of *TPSs* from *Escherichia coli* and yeast. The *NtTPS* was genetically modified to lack a region for the N–terminal extension and the modified gene was designated *ΔNNtTPS*. The genes were expressed in yeast *tps1* mutant as two separate proteins and as a NtTPS (or ΔNNtTPS)–NtTPP fusion protein. Western blot analysis showed that the NtTPS, NtTPP, and NtTPS–NtTPP were expressed abundantly in yeast, while the ΔNNtTPS and ΔNNtTPS–NtTPP were not detected. Interestingly, high levels of trehalose were accumulated in yeast expressing *ΔNNtTPS* and *ΔNNtTPS–NtTPP* in spite of their low–level expressions. Furthermore, stress tolerances of yeast against osmotic, freezing–thawing, and heat stresses were significantly improved by the expression of the tobacco gene, and the increased levels in tolerance were proportional to their trehalose levels. Our results showed that NtTPS and NtTPP functioned in trehalose synthesis by the removal of N–terminal extension of NtTPS and several environmental stress tolerances.

INTRODUCTION

Trehalose is a soluble, non–reducing disaccharide of glucose. Trehalose biosynthesis consists of two enzymatic steps catalyzed by trehalose 6–phosphate synthase (TPS), which generates trehalose 6–phosphate (T6P) from UDP–glucose and glucose 6–phosphate, and trehalose 6–phosphate phosphatase (TPP), which generates trehalose from T6P (Londesborough and Vuorio, 1993). Trehalose is found in a large variety of microorganisms and invertebrate animals and can serve as a stress protectant (Elbein, 1974). Therefore, it can be applied to construct stress tolerant plants by expressing those genes in plants and also lead to stable food production. Proposed functions of trehalose are protection of cells against denaturation of proteins and fusion of membranes to stabilize proteins and membranes under stress conditions, such as desiccation and heat stress (Wingler, 2002). To accumulate trehalose in plants, bacterial and fungal genes have been introduced since most plants have not been considered to accumulate trehalose (Jang *et al*., 2003; Miranda *et al*., 2007). Trehalose accumulation has been found in bacteria, animals, and fungi, however, origin of the introduced genes may cause anxieties about the influence of the exogenous genes on human's health and the environment. On the other hand, few reports have shown that accumulation of trehalose has been detected in plants, such as resurrection plants (Adams *et al*., 1990). Also in other higher plants, such as *Arabidopsis* and potato, trehalose can be detected at very low levels in the presence of the inhibitor of trehalase (TRE), which converts trehalose to glucose (Goddijn *et al*., 1997; Vogel *et al*., 2001). Those reports may help us to solve the biggest problem, which is the anxiety of consumers against origin of introduced genes to produce transgenic agricultural products.

In the present study, we have investigated functions of TPS and TPP from a tobacco plant, NtTPS and NtTPP, in trehalose biosynthesis. Although accumulation of trehalose has not been observed in most plants, TPS and TPP in the organisms are reported to function in trehalose biosynthesis (Zentella *et al*., 1999). Full–length cDNA clones encoding NtTPS and NtTPP were isolated from *N. tabacum* and their trehalose synthetic activities were examined using yeast expressing the corresponding genes. Furthermore, their effects on several environmental stresses were investigated using yeast as an eukaryotic model.

MATERIALS AND METHODS

Organisms and conditions

A tobacco plant (*Nicotiana tabacum* var. Samsun) was cultured on a MS agar medium (Murashige and Skoog, 1962) supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose in a growth chamber (LX–3000; TAITEC, Tokyo, Japan) at 25 °C under a photosynthetic photon flux density of about 70 μmol/m^{2}s with a 16 h photoperiod.

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Saccharomyces cerevisiae YPH500 (*Matα, ura3–52, lys2–801^{amber}, ade2–101^{ochre}, trp1–Δ63, his3–Δ200, leu2–Δ1*; Stratagene, La Jolla, CA, USA) was used in this study. Yeast cells were cultured with shaking at 30 °C in synthetic raffinose media (SR), which contained 0.67% (w/v) yeast nitrogen base (Difco Laboratories, Detroit, MI, USA), 2% (w/v) raffinose, and 0.2% (w/v) complete supplement mixture (CSM) without His, Leu, Trp, and uracil (CSM–HIS–LEU–TRP–URA) supplemented with 0.02% (w/v) histidine, 0.02% (w/v) leucine, 0.02% (w/v) tryptophan, and 0.01% (w/v) uracil. Modified SR media lacking Leu (SR–Leu), or Leu and Trp (SR–Leu–Trp) were used to screen yeast transformants.

Isolation of full–length cDNA encoding NtTPS

Total RNA was isolated from ground powder of tobacco leaves using sepasol–RNA I (Nacalai Tesque, Kyoto, Japan) based on the method of Machida *et al.* (2009). Poly(A)$^+$ RNA was purified using an *Oligotex*–MAG mRNA Purification Kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. First–strand cDNA was synthesized from poly(A)$^+$ RNA with oligo(dT) primer and ReverTra Ace (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. A cDNA fragment of *NtTPS* was amplified by PCR with two degenerated primers, 5'– AGA TGA AAG T(C/T)G G(A/G) T GGT T(C/T)C TCC A –3' and 5'– T(C/G)A C(A/G)A G(A/G)T TCA T(C/G)C CAT C(A/T)C (G/T)CA–3', which were designed based on conserved regions of deduced TPSs. DNA regions that extended 5'– and 3'–ends of the full–length *NtTPS* cDNA were amplified using rapid amplification of cDNA ends (RACE) method. Full–length *NtTPS* cDNA was amplified with two primers, 5'– ATT TCA GCC ACA TTT CCA ATT TGA G –3' and 5'– AGC TAG CAG AAA GGT TAT TTC TTT ACT TTG –3'. Amplified fragments were subcloned into a pGEM–T easy vector (Promega, Madison, WI, USA), and nucleotide sequences of the fragments were determined based on the method of Sanger *et al.* (1977).

Isolation of full–length cDNA encoding NtTPP

A *NtTPP* cDNA fragment was amplified using two degenerated primers, 5'– GG(A/T) AG(C/T) CAT GG(C/T) ATG GAC AT –3' and 5'– ACC TT(A/G) AA(C/G/T) GCA TCT TC(A/G) TC –3', based on conserved regions of deduced TPSs. The amplified fragment was labeled with DIG–11–dUTP (Roche, Basel, Switzerland) and used for library screening. Full–length *NtTPP* cDNA was isolated from a tobacco cDNA library, which was constructed with poly(A)$^+$ RNA using a SMART cDNA Library Construction Kit (Clontech, Mountain View, CA, USA) and a MaxPlax Lambda Packaging Extracts (Ar brown, Tokyo, Japan), according to the manufacturer's instruction.

Sequence analysis

Homologous genes to the cloned cDNAs were searched using the tBlastX program (Altschul *et al.*, 1990) on the National Center of Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/). Phylogenetic analysis was performed with the Neighbor–Joining method (Saitou and Nei, 1987) using the Clustal W program (http://align.genome.jp/), and phylogenetic tree was visualized with the TreeView program (Page, 1996).

Gene disruption of *TPS1* gene in yeast

Yeast *TPS1* gene was disrupted by the short flanking homology (SFH)–PCR method (Lorenz *et al.*, 1995). An open reading frame of a *LEU2* gene, involved in leucine biosynthesis, was amplified by PCR using a pESC–LEU vector (Stratagene) as a template and two primers, 5'–CAG GCT GAT AGG GGT CAC CCC GCT GGG CAG GTC AGG GCA GGG GCT AGT TAA CTG TGG GAA TAC TCA GGT ACG –3' and 5'– TCA TGA TGT TTC GAA GAA GAG ATC AGC GCG GGA GAG AAA GAA AGA TCA TAG TTT CAT GAT TTT CTG TTA CAC –3', to introduce regions responsible for homologous recombination to disrupt the *TPS1* gene. One microgram of the purified DNA was introduced into *S. cerevisiae* YPH500 strain by the lithium acetate method (Ito *et al.*, 1983). The deletion mutant, designated YPH/*tps1*, was screened on SR–Leu agar plates.

Construction of transformed YPH/*tps1* expressing *NtTPS* and *NtTPP*

The *NtTPS* and a modified *NtTPS* (*ΔNNtTPS*), which lacked a DNA region corresponding to 87 amino acids from its N–terminus, were introduced into a downstream of GAL10 promoter (galactose–inducible promoter) in a pESC–TRP vector (Stratagene), and the *NtTPP* was introduced into a downstream of GAL1 promoter (galactose–inducible promoter) in the vector. The genes were also introduced into the vector to be expressed as a fusion protein including c–myc tagging peptide as a linker of NtTPS (or ΔNNtTPS) and NtTPP. The plasmid constructs were transformed into YPH/*tps1* by the lithium acetate method. Transformants were screened onto SR–Leu–Trp agar plates at 30 °C for 3 d.

Expression of introduced genes in yeast and protein extraction

The transformed yeast cells were cultured in SR–Leu–Trp at 30 °C overnight with shaking. Five microliters of the overnight culture was inoculated into 5 ml of SR–Leu–Trp containing 2% galactose (SG–Leu–Trp) and incubated at 30 °C with shaking until the OD_{600} reached 0.4–0.6 (mid–log phase). The induced cells were collected and suspended in 50 mM potassium phosphate buffer (pH 7.5). The suspension was mixed with an equal volume of 0.5 mm diameter glass beads and disrupted for 5 min using a vortex mixer. Then, it was centrifuged at 20,000 × *g* at 4 °C for 10 min. The supernatants were used as protein extracts.

Western blotting

Protein extracts of yeast were subjected to SDS–PAGE on a 10% polyacrylamide gel (Laemmli, 1970), and the separated polypeptides were electroblotted onto a nitrocellulose membrane (Towbin *et al.*, 1970). The membrane was blocked with TTBS (100 mM Tris–HCl buffer (pH 7.5), 0.9% (w/v) NaCl, and 0.1% (w/v) Tween

20) containing 3% (w/v) skimmed milk. To detect the NtTPS, ΔNNtTPS, NtTPS–NtTPP, and ΔNNtTPS–NtTPP proteins, the blocked membrane was incubated with anti–(c–myc) monoclonal antibody peroxidase (9E10; Sigma–aldrich, St. Louis, MO, USA) in TTBS containing 3% skimmed milk. Following washing, the membrane was used to detect peroxidase activity. To detect the NtTPP, the membrane was incubated with an anti–FLAG M2 monoclonal antibody (Sigma–aldrich) in TTBS containing 3% skimmed milk. After washing the membrane four times with TTBS, it was incubated with a goat anti–mouse IgG (whole molecule)–peroxidase conjugate (Sigma–aldrich), followed by detection of the peroxidase activity in 100 mM Tris–HCl buffer (pH 7.5) containing 0.8 mg/ml diaminobenzidine, 0.4 mg/ml $NiCl_2$, and 0.008% H_2O_2.

Determination of trehalose content

Transformed yeast cells were harvested from 5 ml of 24–h culture in SG–Leu–Trp medium containing 1 mM glucose supplemented as a raw material of trehalose. The cells were washed three times with water and lyophilized. The lyophilized samples were then weighed and suspended in 0.25 mM Na_2CO_3 at a concentration of 20 mg dry cell weight/ml. The suspended cells were boiled for 10 min. After centrifugation at 20,000 × *g* for 10 min, the supernatant was used in further assays. Extraction of trehalose, and hydrolysis of trehalose to glucose with acid trehalase from porcine kidney (Sigma–aldrich), was performed as described by Zentella *et al.* (1999), and glucose was measured enzymatically using the F–kit glucose (JK International, Tokyo, Japan). Intracellular trehalose content was expressed as μmol of trehalose per one gram of cell dry weight (*μ*mol/g dry wt).

Stress treatment

The mid–log phase of yeast culture in SG–Leu–Trp containing 1 mM Glc was harvested, washed twice with 0.9% NaCl, and resuspended in 0.9% NaCl at the OD_{600} = 0.1 (2–3×10^6 cells/ml). The suspension was subjected to stress treatment. Salt stress treatment was performed by cultivation of yeast on YPR agar plates (1% yeast extract, 2% peptone, 2% raffinose) containing 0.9 M NaCl. Four microliters of the cell suspension were spotted onto the plate and incubated at 30 °C for 4 d. Freezing treatment was performed at −20 °C for 24 h. Frozen suspension was thawed at 25 °C for 5 min, serially–diluted with 0.9% NaCl, and plated on YPR agar plates. Regarding heat treatment, aliquots (1 ml) of the suspension in a test tube was incubated in a 50 °C water bath for 30 min with shaking. The treated suspension was serially diluted with 0.9% NaCl and plated on YPR agar plates. Viability (%) was calculated by dividing the counts of treated cells by that of untreated cells. YPH500 strain (wild–type) was also tested as a control.

Statistical analysis

Statistical analysis was performed using Excel 2004 (Microsoft, USA) with the add–in software Statcel 2 (Yanai, 2002). The Tukey–Kramer test was used to evaluate significance among the groups by one–way ANOVA.

RESULTS

Isolation of a full–length cDNA encoding NtTPS

At first, a 663 bp DNA fragment was amplified by PCR using two degenerated primers (data not shown). DNA fragments of 5'– and 3'–unknown regions of *NtTPS* were amplified by RACE procedures based on the nucleotide sequence of the 663–bp DNA fragment. A full–length cDNA of *NtTPS* was obtained by PCR, subcloned into a pGEM–T easy vector, and sequenced. The full–length *NtTPS* was 3,039 bp in length and encoded 928 amino acids. Deduced NtTPS showed high sequence similarity to deduced TPSs from other organisms as shown in Fig. 1A. The nucleotide and deduced amino acid sequences of *NtTPS* were deposited at DDBJ/EMBL/GenBank under accession number AB500143.

Furthermore, the structure of the deduced NtTPS was compared to deduced TPSs from *E. coli* (OtsA), yeast (ScTps1), and *Arabidopsis* (AtTPS1) as shown in Fig. 1B. The NtTPS contained both the N–terminal and C–terminal extensions found in AtTPS1 but absent in OtsA and ScTps1. Since the N–terminal extension has been reported to be responsible for the low TPS activity in *Arabidopsis* (Van Dijck *et al.*, 2002), the region excluding the N–terminal extension was determined based on the comparison, and it was designated ΔNNtTPS. *ΔNNtTPS* was 2,526 bp in length and encoded 841 amino acids.

Isolation of a full–length cDNA encoding NtTPP

A 436–bp DNA fragment of *NtTPP* gene was amplified by PCR using two degenerated primers. The 436–bp fragment was used as a DIG–labeled probe for screening of full–length *NtTPP* cDNA clone from a cDNA library, which was constructed with poly$(A)^+$ RNA from tobacco. A cDNA in the positive clone was excised from λ bacteriophage as a plasmid DNA, and the nucleotide sequence of the insert cDNA was determined. The full–length *NtTPP* was 1,854 bp in length and encoded 384 amino acids. Deduced NtTPP showed high sequence similarity to deduced TPPs from other organisms (Fig. 2A), and it showed very high sequence identity to the deduced NtTPPL (Genbank accession no. AY570725) at 98.2% (Fig. 2B). The nucleotide and deduced amino acid sequences of *NtTPP* were deposited at DDBJ/EMBL/GenBank under accession number AB500144.

Construction of yeast mutant lacking *TPS1* gene

TPS1 gene in yeast was disrupted by the SFH–PCR method. *LEU2* gene was amplified with primers that contained the upstream and downstream regions of *TPS1* gene in yeast genomic DNA. The amplified DNA fragment (1,894 bp) was purified from agarose gel, and then directly introduced into yeast by the lithium acetate method. Transformants, designated YPH/*tps1*, were selected by PCR using primers shown in Fig. 3A. A DNA band, 516 bp in size, was amplified only in the deletion mutant (Fig. 3B). The YPH/*tps1* mutant showed sensi-

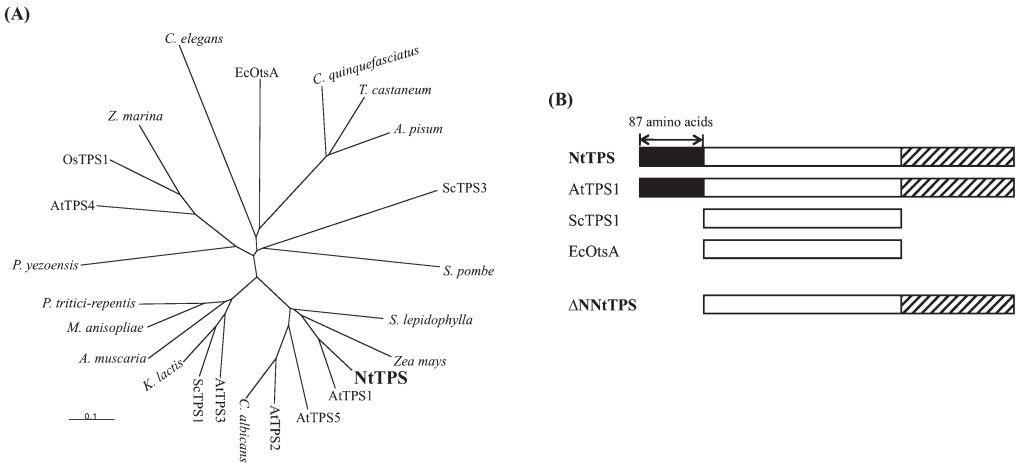

Fig. 1. Comparison of deduced amino acid sequence of *NtTPS* with those of *TPSs* from various organisms. (A) The phylogenetic tree constructed with the full–length deduced amino acid sequences of *TPS* genes. Bar, 0.1 amino acid substitutions per site. Accession numbers or locus for the sequences, shown in parentheses, are as follows: NtTPS: *N. tabacum* TPS (isolated in this study), AtTPS1: *A. thaliana* TPS1 (Y08568), AtTPS2: *A. thaliana* TPS2 (At1g16980), AtTPS3: *A. thaliana* TPS3 (At1g17000), AtTPS4: *A. thaliana* TPS4 (At1g70290), AtTPS5: *A. thaliana* TPS5 (At4g27550), OsTPS1: *Oryza sativa* TPS1 (NM_191881), ScTPS1: *S. cerevisiae* TPS1 (X68214), ScTPS3: *S. cerevisiae* TPS3 (AY692968), EcOtsA: *E. coli* OtsA (X69160), *C. elegans*: *Caenorhabditis elegans* TPS (NM_001047838), *Z. marina*: *Zostera marina* TPS (EU399760), *P. yezoensis*: *Porphyra tritici–repentis* TPS (AAW27916), *M. anisopliae*: *Metarhizium anisopliae* TPS (AAX55633), *A. muscaria*: *Amanita muscaria* TPS (CAC42133), *K. lactis*: *Kluyveromyces lactis* TPS (CAH02314), *C. albicans*: *Candida albicans* TPS (EAK92484), *Zea mays*: *Zea mays* TPS (EU659122), *S. lepidophylla*: *Selaginella lepidophylla* TPS (U96736), *S. pombe*: *Schizosaccharomyces pombe* TPS (CAB16285), *A. pisum*: *Acyrthosiphon pisum* TPS (XM_001945488), *T. castaneum*: *Tribolium castaneum* TPS (XM_970683), *C. quinquefasciatus*: *Culex quinquefasciatus* TPS (XM_001850946). (B) N–terminal extension of the deduced NtTPS protein.

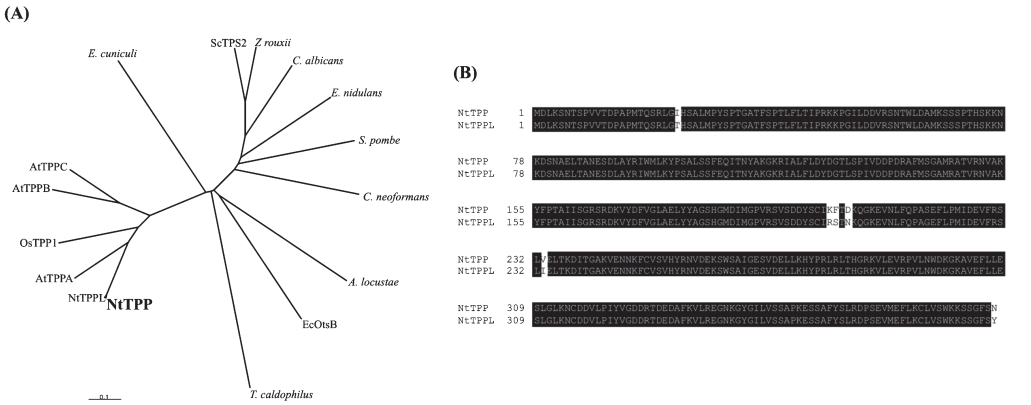

Fig. 2. Comparison of deduced amino acid sequence of *NtTPP* with those of *TPPs* from various organisms. (A) A phylogenetic tree constructed with the full–length deduced amino acid sequences of *TPP* genes. Bar, 0.1 amino acid substitutions per site. Accession numbers or locus for the sequences, shown in parentheses, are as follows: NtTPP: *N. tabacum* TPP (isolated in this study), AtTPPA: *Arabidopsis thaliana* TPPA (At5g51460), AtTPPB: *A. thaliana* TPPB (At1g78090), AtTPPC: *A. thaliana* TPPC (At1g22210), OsTPP1: *Oryza sativa* TPP1 (Os02g0661100), ScTPS2: *Saccharomyces cerevisiae* TPS2 (X70694), EcOtsB: *Escherichia coli* OtsB (ACB03091), *S. pombe*: *Schizosaccharomyces pombe* TPP (CAB10126), *E. nidulans*: *Emericella nidulans* TPP (AY190523), *Z. rouxii*: *Zygosaccharomyces rouxii* TPP (AF208030), *C. albicans*: *Candida albicans* TPP (XM_716172), *C. neoformans*: *Cryptococcus neoformans* TPP (BK005414), *E. cuniculi*: *Encephalitozoon cuniculi* TPP (AJ006825), *A. locustae*: *Antonospora locustae* TPP (AY548904), *T. caldophilus*: *Thermus caldophilus* TPP (AF135796), NtTPPL: *N. tabacum* TPP (AY570725). (B) Alignment of deduced amino acid sequences of *NtTPP* (isolated in this study) and *NtTPPL* (AY570725). Identical amino acids are shaded.

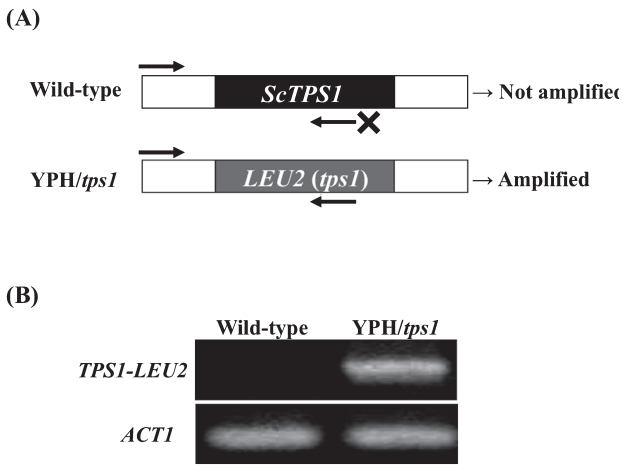

Fig. 3. Construction of yeast mutant lacking *TPS1* gene. (A) The scheme shows the procedure of PCR to confirm gene replacement of *TPS1* gene to *LEU2* gene. The arrows represent the binding sites of primers used for confirmation of *TPS1*–deletion. (B) PCR amplification to confirm deletion of *TPS1*.

tivity to high concentration of glucose, similarly to the phenotype of previously–reported *tps1* yeast (Zentella *et al*., 1999). Based on the results, disruption of *TPS1* gene in yeast was confirmed.

Expression of *NtTPS* and *NtTPP* genes in the yeast mutant

To express the *NtTPS* and *NtTPP* genes in yeast, four types of plasmids were constructed using a pESC–TRP vector and the plasmids constructs were introduced into YPH/*tps1*. The yeast transformants were designated TPS/P (expressing NtTPS and NtTPP as separate proteins), TPS–P (expressing NtTPS and NtTPP as a fusion protein, NtTPS–c–myc–NtTPP), ΔNTPS/P (expressing ΔNNtTPS and NtTPP as separate proteins), and ΔNTPS–P (expressing ΔNNtTPS and NtTPP as a fusion protein, ΔNNtTPS–c–myc–NtTPP), respectively. The pESC–TRP vector was also introduced into YPH/*tps1* to construct a control yeast strain. The transformants were cultured in SG–Leu–Trp to express the introduced genes. The protein extracts of the yeast cells were separated by SDS–PAGE, electroblotted onto a nitrocellulose membrane, and the blot was subjected to immunodetection with antibodies against c–myc tag or FLAG tag. As shown in Fig. 4A, the target proteins were detected with anti–c–myc antibodies in the protein samples of yeast carrying TPS/P and TPS–P. Expression of the protein including ΔNNtTPS was not confirmed. The target proteins were detected with anti–FLAG antibodies in the protein extracts of TPS/P and TPS–P (Fig. 4B).

Intracellular trehalose content of the transformed yeast

To confirm whether the NtTPS and NtTPP proteins are functionally active, intracellular trehalose contents in yeast transformants were investigated. As shown in Fig. 4C, trehalose was detected in all of the transformed yeasts, and the accumulated levels were higher in two transformants, ΔNTPS/P (3.81±0.93 μmol/g dry wt) and ΔNTPS–P (8.12±0.44 μmol/g dry wt), than those in other strains (*tps1*, 0.11±0.09; TPS/P, 0.50±0.23; TPS–P, 0.21±0.12 μmol/g dry wt).

Environmental stress tolerances of the transformed yeast

Stress tolerances were examined using the yeast transformants against salt, freezing–thawing, oxidative, and heat stresses. Salt stress tolerance of yeast was evaluated on YPR agar plates containing 0.9 M NaCl. The YPH/*tps1* mutant did not grow on the medium, while the wild–type and transformants grew on the medium (Fig. 5A).

Freezing tolerances of the transformants were sig-

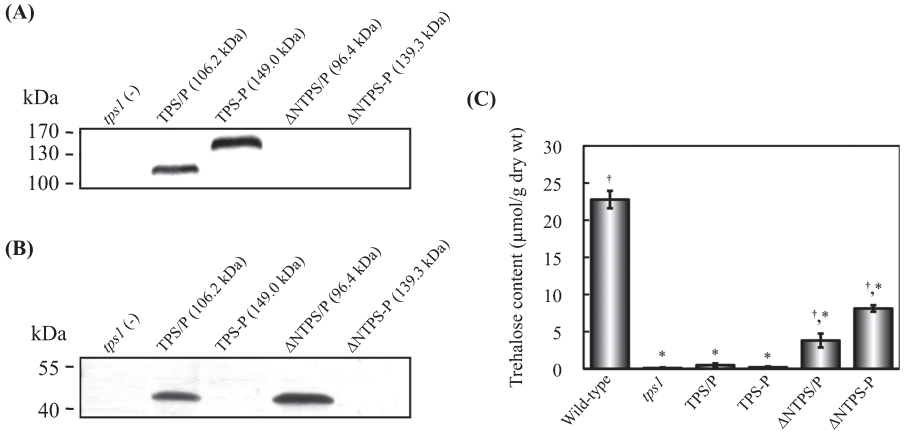

Fig. 4. Trehalose accumulation in YPH/*tps1* expressing *NtTPS* (or *ΔNNtTPS*) and *NtTPP*. (A) Western blot analysis of yeast protein extracts using anti–c–myc antibodies. (B) Western blot analysis using anti–FLAG antibodies. Predicted molecular sizes of the expressed proteins are described in parentheses. (C) Trehalose content of yeast transformants. Values are means ± SD obtained from three independent experiments. Values with daggers are significantly different from *tps1* ($^{\dagger}p<0.01$) and values with asterisks are significantly different from wild–type ($^{*}p<0.01$).

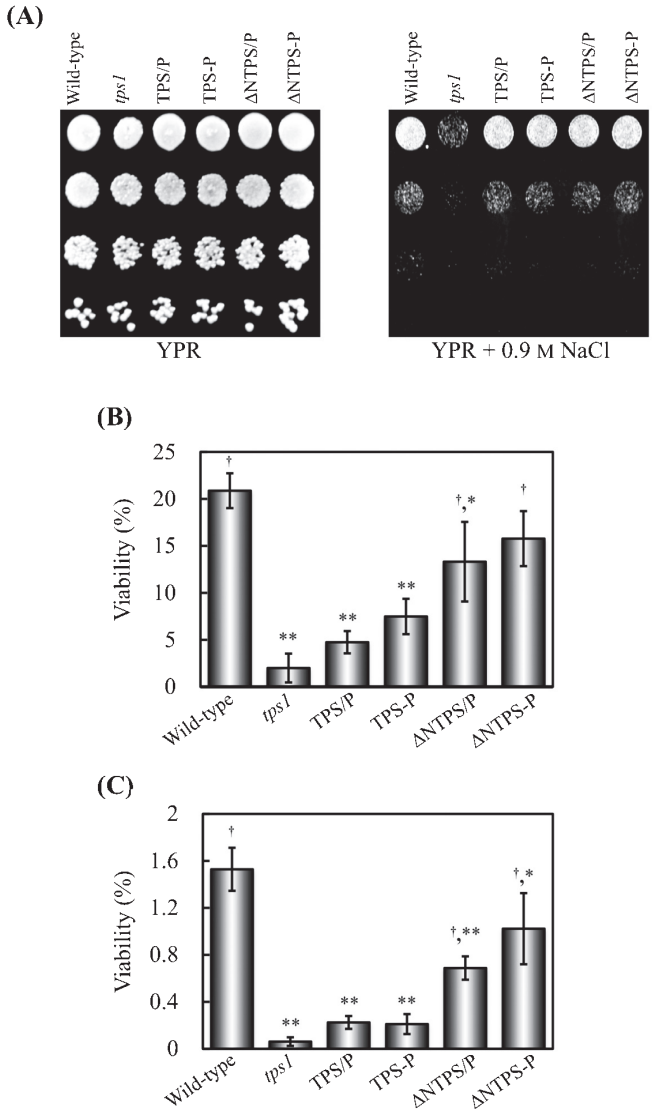

Fig. 5. Environmental stress tolerances of yeast transformants. (A) Salt stress tolerance of yeast transformants. The cell suspension (2–3×10^6 cells/ml) was serially diluted 10–fold at each step with 0.9% NaCl, and then the aliquots (4 μl) were spotted onto YPR agar plates with or without 0.9 M NaCl. (B) Freezing tolerance (−20 °C, 24 h) of yeast transformants. (C) Heat stress tolerance (50 °C, 30 min) of yeast transformants. Values are means ± SD obtained from three independent experiments. Values with daggers are significantly different from *tps1* ($^{\dagger}p<0.01$) and values with asterisks are significantly different from wild–type (*$p<0.05$, **$p<0.01$).

nificantly higher than that of the YPH/*tps1* mutant. The tolerances of transformants expressing *ΔNNtTPS* were greater than those of transformants expressing *NtTPS* (Fig. 5B). Their viabilities after freezing–thawing stress were as follows: wild–type, 20.9±1.9%; *tps1*, 2.0±1.5%; TPS/P, 4.8±1.2%; TPS–P, 7.5±1.9%; ΔNTPS/P, 13.3±4.2%; ΔNTPS–P, 15.8±2.9%.

Yeast cells were treated at 50 °C for 30 min to investigate the contribution of the tobacco enzymes to heat stress tolerance. As shown in Fig. 5C, heat tolerance of the YPH/*tps1* mutant was significantly lower than those of the wild–type and the transformants. Their viabilities after 50 °C treatment were as follows: wild–type, 1.53±0.18%; *tps1*, 0.06±0.04%; TPS/P, 0.23±0.06%; TPS–P, 0.21±0.09%; ΔNTPS/P, 0.69±0.10%; ΔNTPS–P, 1.02±0.30%.

DISCUSSION

Trehalose is known as a cellular protectant against various stresses, such as drought and heat stresses, therefore, utilization of trehalose to improve stress tolerance of agricultural products has been expected. Accumulation of trehalose has been reported in animals, bacteria, and yeast, and several researchers have achieved accumulation of trehalose in plants and improvement of environmental stress tolerance by introducing the bacterial and fungal genes involved in trehalose biosynthesis (Jang *et al.*, 2003; Miranda *et al.*, 2007; Stiller *et al.*, 2008). To apply the trehalose accumulation to practical agricultural products, origin of the introduced gene is controversial. Although plant genes encoding TPS and TPP have been introduced in plants and improved the stress tolerance

(Ge *et al*., 2008), accumulation of trehalose has not been confirmed and the acquired stress tolerance is not enough to apply to practical agricultural products. To gain further information on plant genes encoding trehalose biosynthetic enzymes, the cDNAs encoding TPS and TPP were isolated from *N. tabacum* in the present study.

The deduced NtTPS protein appeared to have N–terminal extended region that was not found in the deduced TPSs from *E. coli* and yeast (Fig. 1B), which can accumulate trehalose. The N–terminal extension was also found in TPSs from *Arabidopsis* (AtTPS1) and *Selaginella lepidophylla* (SlTPS1), and truncation of the N–terminal extension resulted in enhanced TPS activity and trehalose synthetic activity (Zentella *et al*., 1999; Van Dijck *et al*., 2002). Thus, the truncated region corresponding to 87 amino acid residues from the N–terminus of the deduced NtTPS was determined, and the remaining region containing 841 amino acid residues was designated ΔNNtTPS.

Deduced amino acid sequence of the *NtTPP* showed sequence identity to that of *NtTPPL*, a *TPP* gene isolated from *N. tabacum* in previous study (Wang *et al*., 2005). They were considered as same isotypes of TPP since the identity of the deduced amino acid sequences was very high (98.2%, Fig. 2B), while the identity of deduced *Arabidopsis* TPPs (nine isotypes) was basically 40–70%. Thus, the NtTPP was suggested to enhance heat and salt stress tolerances when expressed in yeast as well as previously–reported NtTPPL.

Expression of *TPS* and *TPP* genes has been reported to enhance stress tolerance of yeast and plants regardless of source organism of the genes, and co–expression of the genes as a TPS–TPP fusion protein has been reported to confer relatively high stress tolerance (Jang *et al*., 2003). However, even though there are such publications, trehalose accumulation in plant cells by introducing plant genes has not been achieved to our knowledge. To gain further insights on TPS and TPP from plants, we investigated functions of NtTPS (or ΔNNtTPS), NtTPP and NtTPS (or ΔNNtTPS)–NtTPP fusion proteins using YPH/*tps1* mutant. Yeast *tps1* mutant is known to be sensitive to high concentration of glucose and the mutant cannot accumulate trehalose in cells (Van Aelst *et al*., 1993). However, glucose is an essential component for trehalose biosynthesis, thus maximum glucose concentration in YPH/*tps1* culture, at which growth of the yeast was not inhibited, was determined preliminarily (data not shown). Based on the result, all yeast transformants were cultured in SR–Leu–Trp medium containing 1 mM glucose. Trehalose amounts were higher in yeast expressing ΔNNtTPS than that expressing full–length NtTPS (Fig. 4), and the accumulation levels reflected stress tolerance of yeast (Fig. 5). Although comparison of TPS activities between NtTPS and ΔNNtTPS was not examined in the present study, NtTPS appeared to function in trehalose synthesis in yeast with NtTPP when expressed in the truncated form. Our results suggest that it is possible to accumulate trehalose in plants by introducing the tobacco genes (*ΔNNtTPS* and *NtTPP*), and to enhance its environmental stress tolerances.

Suppression of TRE activity is a way that enables to enhance trehalose accumulation. We have previously identified the corresponding gene, that is *NtTRE*, from tobacco, and the encoded NtTRE protein showed trehalase activity (Machida *et al*., 2009), suggesting the NtTRE was partly responsible for the low–level accumulation of trehalose in the tobacco plant. As one of other reasons of low–level accumulation, the tobacco cannot accumulate trehalose because of its low trehalose synthetic activity. In the present study, we showed that the NtTPS and NtTPP synthesized trehalose when the NtTPS was expressed with the deletion of N–terminal extension, which was not observed in TPSs from *E. coli* and yeast. To our knowledge, there is no trial to construct a transgenic plant that expresses *TPS* and *TPP* genes, and that suppresses *TRE* gene. The trial would allow the transgenic plant to accumulate high amount of trehalose and thus to enhance its stress tolerances against various environmental stresses.

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