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Molecular Cloning, Characterization and Expression of a Novel Trehalose-6-phosphate Synthase Homologue from *Ginkgo biloba*

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In many organisms, trehalose acts as protective metabolite against harsh environmental stresses, such as freezing, drought, nutrient starvation, heat and salt. Herein a cDNA (designated as *GbTPS*, GenBank Accession Number AY884150) encoding a trehalose-6-phosphate synthase homologue was isolated and characterized from the living fossil plant, *Ginkgo biloba*, which is highly tolerant to drought and cold. GbTPS encoded an 868-amino-acid polypeptide with a predicted isoelectric point of 5.83 and molecular mass of 97.9 kD. Amino acid sequence alignment revealed that GbTPS shared high identity with class II trehalose-6-phosphate synthase homologues (67% identical to AtTPS7), but had only 17% and 23% of identity with OstA from *Escherichia coli* and ScTPS1 from *S. cerevisiae,* respectively. DNA gel blot analysis indicated that *GbTPS* belonged to a small multi-gene family. The expression analysis by RT-PCR showed that *GbTPS* expressed in a tissue-specific manner in *G biloba* and might involve in leaf development. *GbTPS* was also found to be induced by a variety of stresses including cold, salt, drought and mannitol.

Keywords: cDNA cloning, GbTPS, Ginkgo biloba, RACE, Trehalose

Database Accession No: AY884150

Abbreviations: GbTPS: *Ginkgo biloba* trehalose-6-p synthase homologue; NUP: nested universal primer A; RACE: rapid amplification of cDNA ends; RT-PCR: reverse transcription polymerase chain reaction; TPP: trehalose-6-phosphate phosphatase; TPS: trehalose-6-phosphate synthase; UPM: universal primer mixture.

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Introduction

Trehalose is a non-reducing disaccharide in which the two glucose units are combined in an alpha, alpha-1, 1-glycosidic linkage. Due to the absence of its reducing ends, many studies have convincingly demonstrated that trehalose distributes to organism's acclimation with various environmental stresses (Wiemken, 1990; Ribeiro *et al.*, 1997; Crowe *et al.*, 1998), such as heat shock (De Virgilio *et al.*, 1994; Nwaka *et al.*, 1994), nutrient starvation, dehydration (Gadd, 1987), salt (Garcia *et al.*, 1997) and oxidative stress. Furthermore, its role as protector against environmental stresses has been proven by transgenic studies. The transformation of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP), derived either from *Escherichia coli* or yeast, endows tobacco or tomato with elevated tolerance to drought with the accumulation of a limited amount of trehalose. However, other unexpected pleiotropic phenotypes, including stunted growth and altered metabolism under normal growth conditions, were reported (Romero *et al.*, 1997; Pilon-Smits *et al.*, 1998; Yeo *et al.*, 2000; Zhao *et al.*, 2000). Overexpression of *E. coli* trehalose biosynthetic genes (*OtsA* and *OtsB*) as a fusion under the control of either tissue-specific or stress-dependent promoters in transgenic rice enhanced the tolerance of pants to salt, drought, and low-temperature. The transgenic rice plants accumulated trehalose at levels 3-10 times higher than those of the nontransgenic controls without pleiotropic effects (Garg *et al.*, 2002). These results indicated that accumulated trehalose increased the tolerance of plants to stresses and highlighted the potential agriculture application of trehalose. In addition, trehalose is also involved in the glucose metabolism and signal sensing (Eastmond *et al.*, 2003; Elbein *et al.*, 2003; Avonce *et al.*, 2004), and even plays a role in plant growth and development (Muller *et al.*, 1999).

The biosynthesis of trehalose consists of two enzymatic

steps. The first step is to synthesize trehalose-6-P from Glc-6-P and UDP-Glc by TPS, and the second step is to dephosphorylate trehalose-6-P to synthesize trehalose by TPP (Cabib and Leloir, 1958). In *E. coli*, these two enzymatic counterparts are encoded by *OtsA* and *OtsB*, respectively (Goddijn and van Dun, 1999). In *Saccharomyces cerevisiae*, the formation of trehalose depends on a trehalose synthase complex, which contains a regulatory subunit encoded by *TSL1*, a homologue of *TPS3* (Bell *et al.*, 1998), besides a TPS (Sc*TPS1*) and a TPP (Sc*TPS2*) subunit. These genes have been isolated from yeast and bacteria (Luyten *et al.*, 1993; Kaasen *et al.*, 1994). TPS and TPP have also been isolated from higher organisms including plants. *TPS* has been identified from many plant species such as lower plant fern (Zentella *et al.*, 1999) and higher plants *Arabidopsis thaliana* (Vogel *et al.*, 1998) and tobacco (Wang *et al.*, 2005) through functional complementation of heat-sensitive yeast *tps2* mutants (Thevelein and Hohmann, 1995). However, no plant homologues of yeast *TSL1-TPS3* have been found in plants so far (Leyman *et al.*, 2001). It is unknown yet that whether a similar complex as in *S. cerevisiae* is also present in plants.

Furthermore, after *A. thaliana* genomic sequence is published, 11 putative TPS genes have been found and grouped into two classes, depending on their homologies to *S. cerevisiae* TPS1 (TPS activity) and TPS2 (TPP activity) (Leyman *et al.*, 2001). Class I consists of four genes (*AtTPS1-4*), and class II consists of seven genes (*AtTPS5-11*). Among Class I TPSs, only AtTPS1 has a proven TPS, but not TPP activity (Blazquez *et al.*, 1998). Two of the class II homologues, although detected at transcriptional level, showed neither TPS nor TPP activity. However, the members of class II were suggested to be involved in the regulation of trehalose metabolism in specific developmental stages and specific tissues or formation of a trehalose complex, even though no straightforward evidence has been present to support the hypothesis (Leyman *et al.*, 2001). *Arabidopsis* also has 10 TPP homologues (class III), each of which only contains a TPP domain (Eastmond and Graham, 2003). Complementary functions of two class-III proteins to heat-sensitive phenotype of the *S. cerevisiae* tps2 mutant suggest that they may bear some TPP activities (Vogel *et al.*, 1998). Cloning of new plant TPS genes, particularly class-II or class III homologues, might help us to understand more the function and molecular evolution of this large gene family.

To date, the study of genes with respect to trehalose metabolism in plants has been mainly carried out in angiosperm, particularly in *Arabidopsis*. None is reported in gymnosperm. *Ginkgo biloba* is called a "living fossil" for its existence in this planet for more than 200 million years as one of the oldest gymnosperm species. Furthermore, *G. biloba* has a good resistance or tolerance to drought and low-temperature. In this paper, we reported the isolation and molecular characterization of the full-length cDNA of TPS homologue (*GbTPS*) from *G. biloba*. The expression profiles of *GbTPS* in specific issues, during different developmental stages and under various environmental stresses were also investigated.

Materials and Methods

Plant materials. Fresh four-leaf-stage seedlings of *Ginkgo biloba* grown at 25°C in a controlled-growth chamber (16 h light/8 h dark) were used for different treatments. For salt and mannitol treatments, the seedlings were supplemented with 250 mM NaCl or 200 mM mannitol solution, respectively. In cold treatment, the seedlings were grown under 4°C and samples including both roots and leaves were harvested at individual time points of 0, 4, 8, 24 and 48 h. In drought treatment, the seedlings were dehydrated and harvested at 0.5, 1, 2 and 4 h, respectively. Roots, stems and leaves from the same seedlings as well as the general fruits were sampled for tissue-specific analysis. The leaves were harvested at 2 weeks (w), 3, 4, 6 w after their shooting and used to analyze developmental expression pattern of the cloned gene. All samples were immediately frozen in liquid nitrogen and stored at −80°C prior to RNA extraction.

DNA and RNA extraction. Genomic DNA was extracted from the leaves of *G. biloba* according to the protocol as described before (Jiang, 2000). Total RNA was extracted separately from all samples by using CTAB method (Liao *et al.*, 2004), followed by incubation with RNase-free DNase I (TaKaRa) at 37°C for 30 min. The quality and concentration of DNA and RNA were measured with electrophoresis and spectrophotometer.

Internal conservative fragment cloning. To clone the internal conservative fragment, primers GbTH1 (5'-TCACGGAGGTCGCT TTGATCGG-3') and GbTH2 (5'-TCCCATCCCTCACAGCAGTT AT-3') were designed and synthesized based on the conserved amino acid and nucleotide sequences among AtTPS5, AtTPS6 and AtTPS7 (Shanghai Sangon Biotechnological Company, China). The fragment was obtained by using One-Step-RT-PCR kit (TaKaRa). RT-PCR reaction was programmed as below: 50°C for 30 min, denatured at 94°C for 2 min, followed by 25 cycles of amplification (94°C for 45 s, 52°C for 45 s, 72°C for 1 min). The PCR product was purified and cloned into pMD18-T vector (TaKaRa) followed by sequencing.

5' RACE. Primer GbTPS5' (5'-CGAAGGTATGGAAACCAATCA AATC-3') and the nested primer GbTPS5'N (5'-GCACCCAGAC ATAGTCATCATCAGG-3') were designed based on the sequence of the internal conservative fragment. The cDNA was synthesized using the SMARTTM RACE cDNA Amplification Kit (Clontech). An aliquot of 1 μg total RNA was reversely transcribed according to the kit manual to get the 5'-ready cDNA. 5'-CDS primer A and SMART II A oligonucleotide (provided in the kit) were used. The product of the first round 5'-RACE-PCR performed with the GbTPS5' and UPM primers were diluted 50-fold and used as the template for the nested PCR with primers GbTPS5'N and NUP (provided in the kit). Both primary PCR and nested PCR were performed as the following procedure: the cDNA was denatured at 94°C for 3 min followed by 30 cycles of amplification (94°C for 5 s, 68°C for 10 s, 72°C for 1 min). The product was purified and cloned to pMD18-T vectors followed by sequencing.

3' RACE. The 3'-ready cDNA was synthesized by reversely transcribing 1 μg total RNA with a cDNA synthesis primer (3'-CDS primer A

provided in the kit). Based on the sequence of the cloned internal fragment, primer GbTPS3 (5'-GTGATAACTGCTGTGAGGGATG GGA-3') was designed and synthesized. The 3' RACE was performed essentially according to the manufacturer's instructions. The PCR was carried out in a total volume of 50 μl containing 2 μl 3'-Ready cDNA, 10 pM each of GbTPS3 and NUP (provided in kit) primers, 10 μM dNTPs, 1 x cDNA reaction buffer and 5U *Taq* polymerase. The reactions were performed at 94°C for 15 s, 68°C for 30 s and 70°C for 1 min with 32 cycles. The amplified product was purified and cloned into pMD18-T vector followed by sequencing.

Full-length cDNA cloning of *GbTPS*. After comparing and aligning above three fragments with Vector NTI 8.0, the full-length cDNA of *GbTPS* was deduced and obtained through One-step RT-PCR amplification, which was performed with primers GBTPSF (5'-ATGATGTCAAGATCTTATACAAATC-3') and $poly(A)_{24}$ by using One-Step-RT-PCR kit (TaKaRa), under the following condition: 50°C for 30 min and 94°C for 2 min followed by 30 cycles of amplification (94°C for 1 min, 58°C for 1 min, 72°C for 3 min). The PCR product was purified and cloned into pMD18-T vector followed by sequencing.

Bioinformatic analysis and phylogenetic construction. The obtained sequences were analyzed by using bioinformatic tools at websites (www.ncbi.nlm.nih.gov, www.expasy.org). The software Vector NTI 8 was used for sequence multi-alignment and assembling. GbTPS and other known TPS or TPP from other species retrieved from GenBank were aligned with ClustalX1.81 (Thompson *et al.*, 1997) and subsequently a phylogenetic tree was constructed by the neighbor-joining method (Saitou, 1987) with ClustalX1.81. The reliability of the tree was measured by bootstrap analysis with 1500 trials (Felsenstein, 1985) and edited with TreeExplorer.

Southern blot analysis. The genomic DNA (30 μg per lane) was digested respectively with *Hind*III, *Dra*I, *Xba*I and *Eco*RI, separated by electrophoresis on 1% agarose gel in TBE buffer and then transferred onto a positively charged Hybond-N^+ nylon membrane (Amersham Pharmacia). The membrane was subsequently hybridized with a randomly-labeled *GbTPS* DNA fragment generated with primer GbTPS1 (5'-TCGGTCTCTCTGGCAGGCTTATGTAT-3') and primer GbTPS2 (5'-CACATCCTCCACATCTTTGCCCCTACC-3'). For probe-labeling, An aliquot of 100 ng purified product was used as the template in a total volume of 50 μl. Probe labeling, hybridization and signal detection were performed following Images Random Prime Labeling Module and CDP-Star Detection Module manufacturer's instructions (Amersham Pharmacia).

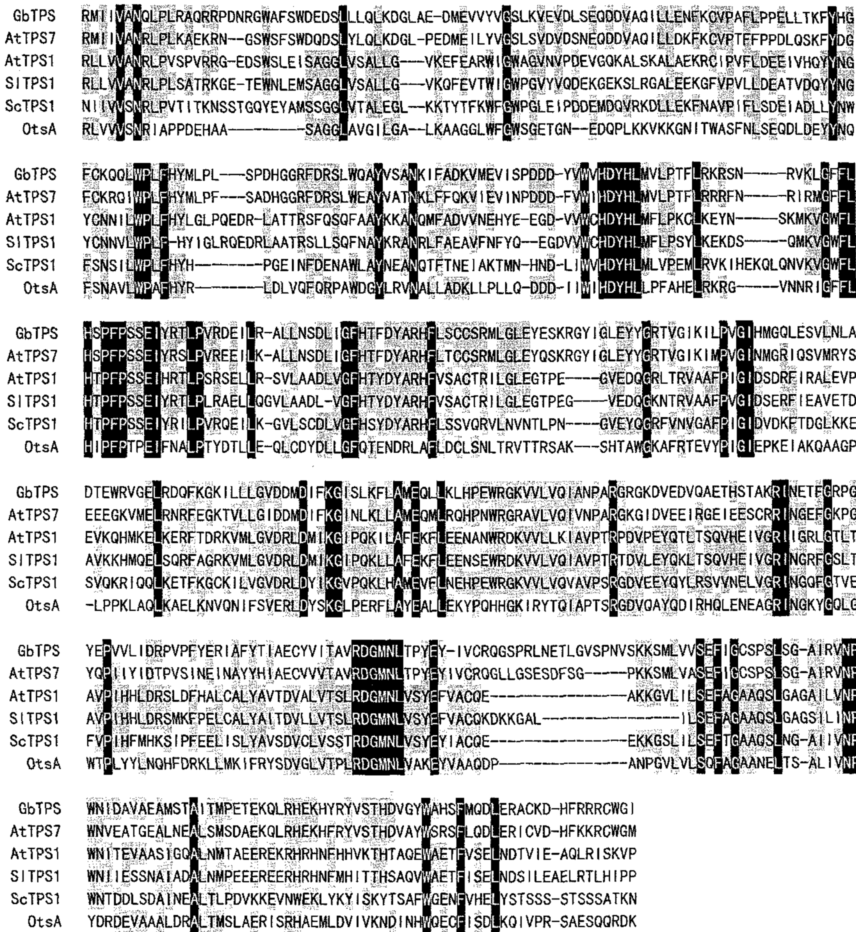
Expression profile analysis. An aliquot of 1 μg total RNA was used as the template in semi-quantitative RT-PCR analysis using One-Step-RT-PCR Kit (TaKaRa) with primers THoneF (5'-TCGGT CTCTCTGGCAGGCTTATGTATC-3') and TH-RR (5'-CATCCCA TCCCTCACAGCAGTTATCAC-3') in a 25 μl reaction mixture containing 0.5 μg RNA sample. The PCR program was performed as below: the RNA was reversely transcribed at 50°C for 30 min, denatured at 94°C for 2 min followed by 25 cycles of amplification (94°C for 45 s, 60°C for 45 s, 72°C for 1 min). Two primers, 18SF (5'-ATGATAACTCGACGGATCGC-3') and 18SR (5'-CTTGGAT GTGGTAGCCGTTT-3'), were used to amplify 18S rRNA gene in the semi-quantitative RT-PCR as controls. Ten μl out of 25 μl was loaded on an ethidium bromide-stained 1.2% (w/v) agarose gel. Quantification of band strength was accomplished by scanning the gels with the ultraviolet and visible light automobile analyzing instrument FR-200A (FuRi) and image smart view software.

Results and Discussion

Cloning and characterization of *GbTPS*. By using the RACE method, the full-length cDNA sequence of *GbTPS* gene (GenBank Accession No. AY884150) was cloned and confirmed by RT-PCR. The cDNA is 3220 bp long, including the poly(A) tail, and contains an open reading frame (ORF) of 2607 bp. There is a 136-bp 3' UTR (untranslated region) following the stop codon and a 477-bp 5'UTR upstream of the start codon. A proposed polyadenylation signal (AATAAA) resides 50 bp downstream of the stop codon.

The deduced GbTPS protein was 868 aa long with a calculated molecular weight (MW) of 97.9 kD and an isoelectric point (pI) of 5.83. BLAST in NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the multi-alignment by Vector NTI8.0 revealed that GbTPS had considerable homology to other TPSs and TPPs (Fig. 1A, Fig. 1B). GbTPS is lowly identical to OstA from *E. coli* and ScTPS1 from *S. cerevisiae* (17% and 23% identity, respectively). Unlike SlTPS1 from *S. lepidophylla* which shared an identity of 67% to the first isolated plant TPS from *A. thaliana*, AtTPS1 (Zentella *et al.*, 1999), GbTPS from *G. biloba* was only 28% identical to AtTPS1, whereas it shared 68% identity to the *Arabidopsis* TPS class-II homolog, AtTPS7, with even higher identity to AtTPS5 and AtTPS6 (Leyman *et al.*, 2001). It also had higher identity with ScTPS2 (26% identity) than with ScTPS1 (23% identity) from yeast. In contrast to OstA and ScTPS1, GbTPS possessed an additional C-terminal region homologous to ScTPS2 between residues 577 and 852, and the region was also present in SlTPS1 and AtTPS1. The N-terminal extension existing in SlTPS1 and AtTPS1 (Blazquez *et al.*, 1998; Zentella *et al.*, 1999) was not found in GbTPS. Compared with SlTPS1 and AtTPS1, the TPP homologous part of GbTPS1 contained two conserved phosphatase boxes (Thaller *et al.*, 1998; Leyman *et al.*, 2001) which were present in a large family of phosphotases, including ScTPS2, plant TPP and the class II TPS subfamily in *Arabidopsis* (Fig. 1B). This implies that GbTPS belongs to class II subfamily of TPS.

Molecular evolution analysis. A phylogenic tree of TPS and TPP from bacteria, fungi and plants were constructed to investigate the evolutionary relationships among deduced GbTPS, and other proteins related to trehalose biosynthesis. The result revealed that these proteins were grouped into three subfamilies depending on their similarities to yeast TPS1 or TPS2 (Fig. 1D). The first subfamily consisted of proteins most similar to yeast TPS1 (ScTPS1) including SlTPS, AtTPS1-4, ScTPS1 and other TPS enzymes (class I).



(A)

Fig. 1. Comparison of *G. biloba* GbTPS protein with other TPS and TPP. Amino acid sequences used in the analysis are from *A. thaliana* (AtTPS1, T30F21.9, Y08568; AtTPS2, F6I1.1, AAF99834; AtTPS3, F20D23.30, AAD50035; AtTPS4; T29A15.40, CAB38267; AtTPS5, dl4920w, NP_567538; AtTPS6, T23K23.13, NP_974105; AtTPS7, T2D23.11, AAF82169; AtTPS8, F1707.18, AAC18810; AtTPS9, T23E23.3, T02267; AtTPS10, T13D8.4, AAC24048; AtTPS11, MSF3.8, AAD08939; AtPPB, AF007779; AtTPPA, AF007778), *E. coli* (OtsB, P31678; OtsA, P31677); from *Selaginella lepidophylla* (SlTPS1, U96736), *Saccharomyces cerevisiae* (ScTPS2, X70694; ScTPS1, X68214), *M. thermoautotrophicum* (MtTPS1, AE000931), *Oryza sativa* (OsTPP, NP_922665) and from *Nicotiana tabacum* (NtTPPL, AY570725). (A) Alignment of an internal region of plant TPS or TPS-like amino acid sequences with bacteria and yeast sequences. Gaps introduced to optimize the alignment are indicated by dashes. Conservative residues are shaded and those indicated with black background represent identical residues. (B) Partial alignment of GbTPS with TPP from bacteria, fungi and plants. Two conserved domains are underlined, conservative residues are shaded and identical residues are highlighted with black background. (C) Diagram of TPS and TPP protein sequences. A comparison of protein structure is shown based on sequence-alignment data presented in A and B, together with the "rpsblast" searching results from NCBI database. (D) Phylogenetic tree analysis of GbTPS. The sequences are aligned with ClustalX and edited with Tree-explorer.

Obviously this branch of phylogenic tree was grouped in a family-specific manner. For example, two members of bacterial TPS from *E. coli* and *M. thermoautotrophicum* had closer relationship than others. GbTPS was grouped into class II which also included seven TPS homologues from *A. thaliana* (Leyman *et al.*, 2001). Given the closer relation and structural similarity between members of class II and ScTPS2, they probably derive from the same ScTPS2 ancestor. Class III was constituted of TPP enzymes, in which all plants TPPs were grouped into a cluster closer to *E. coli* OstB but separating from yeast ScTPS2, suggesting that plant TPPs originate from OstB rather than from ScTPS2.

Southern blot analysis. To determine whether *GbTPS* belongs to multi-gene family, Aliquots of genomic DNA (30 μg per sample) were digested with *Hin*dIII, *Dra*I, *Xba*I and *Eco*RI, respectively, followed by hybridization with *GbTPS* probe. There are no restriction sites of *Dra*I, *Xba*I and *Eco*RI within the probe region except for only one restriction site of *Hin*dIII present in the probe region. Southern blot analysis revealed

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GbTPS   (574) FRKLSTEHIVSAYKRTKSRAILLDYDGTMMPQTS--INKTPGSEVLFILNSLCSDPKNVVFIVSGRGRQTLSQWLSPCE-MLGIAAEHGY
AtTPS7  (568) FRKLSIPCIVSDYKRAKSRAILLDYDGTLMPQNS--INKAPSQEVLNFLDALCEDKKNSIFIVSGRGRESLSKWFTP-CKKIGIAAEHGY
ScTPS2  (554) TPALNRPVLLENYKQAKRRLFLFDYDGTLTPIVKDPAAAIPSARLYTILQKLCADPHNQIWIISGRDQKFLNKWLGGKLPQLGLSAEHGC
AtTPPA  (103) PSALTSFEKIMSFAKGKRIALFLDYDGTLSPIVEEPDCAYMSSAMRSAVQNVAKYFPTAIISGRSRDKVYEFVNLSELYYAGSHGMDIMS
AtTPPB  (100) PSALNMFDEIVNAAKGKQIVMFLDYDGTLSPIVEDPDKAFITHEMREVVKDVASNFPTAIVTGRSIEKVRSFVQVNEIYYAGSHGMDIEG
OsTPP    (99) PSALTSFEDIVNLARGKRLALFLDYDGTLSPIVDNPENAVMSDEMRSAVKHVASLFPTAIISGRSRDKVFDFVKLTELYYAGSHGMDIMG
NtTPPL  (101) PSALSSFEQITNYAKGKRIALFLDYDGTLSPIVDDPDRAFMSGAMRATVRNVAKYFPTAIISGRSRDKVYDFVGLAELYYAGSHGMDIMG
OtsB      (1) ----MTEPLTETPELSAKYAWFFDLDGTLAEIKPHPDQVVVPDNILQGLQLLATASDGALALISGRSMVELDALAKP--YRFPLAGVHGA

GbTPS   (661) FIRWTRDAEWETCVQGVDFGWKQITEPVMKLYTETTDGSVIETKESALVWHHQDADPDFGSCQAKELLDHLESVLANEPVVVKSGQHIVE
AtTPS7  (655) FLKWSGSEEWETCGQSSDFGWMQIVEPVMKQYTESTDGSSIEIKESALVWQYRDADPGFGSLQAKEMLEHLESVLANEPVAVKSGHYIVE
ScTPS2  (644) FMKDVSCQDWVNLTEKVDMSWQVRVNEVMEEFTTRTPGSFIERKKVALTWHYRRTVPELGEFHAKELKEKLLSFTDDFDLEVMDGKANIE
AtTPPA  (193) PAG--ESLNHEHSRTVSVYEQGKDVNLFQPASEFLPMIDKVLCSLIESTKDIKGVKVEDNKFCISVHYRNVEEKNWTLVAQCVDDVIRTY
AtTPPB  (190) PTN------------ENSNGQSNERVLFQPAREFLPMIEKVVNILEEKTKWIPGAMVENNKFCLSVHFRRVDEKRWPALAEVVKSVLIDY
OsTPP   (189) PVRKSDSSGQHVECIRSTDSEGKEVNLFQPASEFLPMISEVYKKLSESIKDIDGARMEDNKFCVSVHYRNVAPHDYGEVHQRVTAVLKNY
NtTPPL  (191) PV---RSVSDDYSCIRSTNKQGKEVNLFQPAGEFLPMIDEVFRSLIELTKDITGAKVENNKFCVSVHYRNVDEKSWSAIGESVDELLKHY
OtsB     (85) ERR-----DINGKTHIVHLPDAIARDISVQLHTVIAQYPGAELEAKGMAFALHYRQAPQHEDALMTLAQRITQIWPQMALQQGKCVVEIK

GbTPS   (751) VKPQGVSKGLVAERLLSIMVQND---------------KRPDFVMCIGDDRSDEDMFEGITSA
AtTPS7  (745) VKPQGVSKGSVSEKIFSSMAGKG---------------KPVDFVLCIGDDRSDEDMFEAIGNA
ScTPS2  (734) VRPRFVNKGEIVKRLVWHQHGKPQDMLKGISEKLPKDEMPDFVLCLGDDFTDEDMFRQLNTI
AtTPPA  (281) PKLRLTHGRKVLEIRPVIDWDKGKAVTFLLESLGLNNCEDVLPIYVGDDRTDEDAFKVLRDG
AtTPPB  (268) PKLKLTQGRKVLEIRPTIKWDKGQALNFLLKSLGYENSDDVVPVYIGDDRTDEDAFKVLRER
OsTPP   (279) PCLRLTHGRKVLEVRPVIDWNKGKAVEFLLESLGLCGKEDVLPIYVGDDKTDEDAFKVLKAN
NtTPPL  (278) PRLRLTHGRKVLEVRPVLNWDKGKAVEFLLESLGLKNCDDVLPIYVGDDRTDEDAFKVLREG
OtsB    (170) --PRGTSKGEAIAAFMQEAPFIG---------------R--TPVFLGDDLTDESGFAVVNRL
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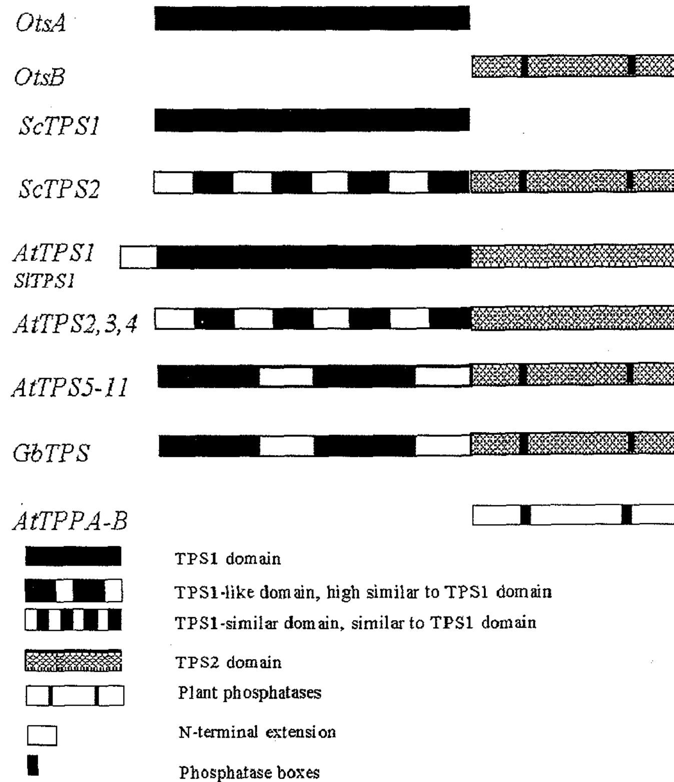
(B)

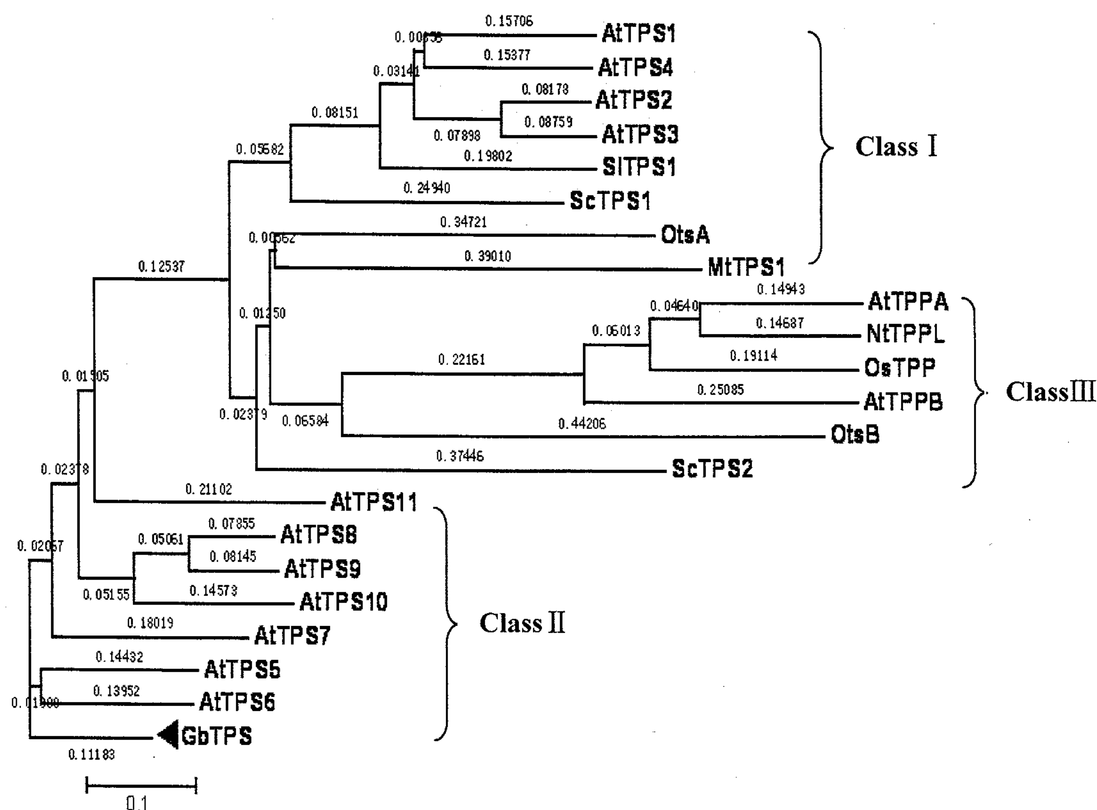


(C)

Fig. 1. Continued.

that several hybridization bands ranging from 1 kb to 9 kb were present in each lane (Fig. 2), indicating that *GbTPS* belongs to a small multi-gene family. There is a large TPS family existing in *Arabidopsis* (Leyman *et al.*, 2001), indicating TPS are widely spread in plant kingdom.

Tissue-specific and developmental expression patterns. To determine the tissue-specific and developmental expression patterns of *GbTPS* gene, semi-quantitative RT-PCR was performed in which 0.5 μg of total RNA from various tissues or at different developmental stages was used as the template.



(D)

Fig. 1. Continued.

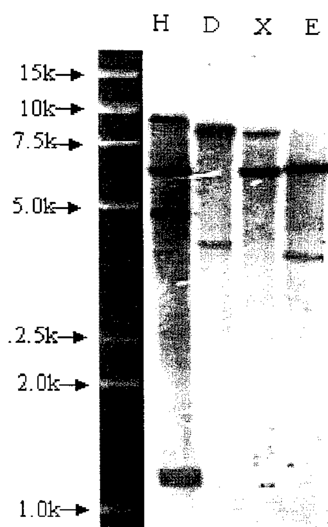


Fig. 2. DNA gel blot analysis. Genomic DNA (30 μg/lane) was respectively digested with *Hin*dIII (lane H), *Dra*I (lane D), *Xba*I (lane X) and *Eco*RI (lane E) followed by hybridization with biotin- labeled *GbTPS* probe.

As shown in Fig. 3A, *GbTPS* expressed the highest in leaf, followed by in stem and root, and no expression could be detected in fruit, indicating that *GbTPS* was a tissue-specific expressing gene, which was similar to the counterparts in *Arabidopsis* (Vogel *et al.*, 2001). Previous study shows that *AtTPS1* is required for both late embryo development and metabolism (Eastmond *et al.*, 2002). Whether other TPS homologues in plants might be involved in plants' development is unknown. In this study, the result showed that *GbTPS* expression increased along with time of leaf development (Fig. 3B), implying that the trehalose might involve in leaf development.

Expression profiles of *GbTPS* under various stress treatments. Because trehalose biosynthesis in organisms was induced by different abiotic stresses, we tested whether the expression of *GbTPS* was also affected by these stresses by using semi-quantitative RT-PCR. The seedlings of *G. biloba* were treated with cold, NaCl, mannitol and drought respectively for various durations and afterwards both roots and leaves were harvested for total RNA extraction. As shown in Fig. 3C, cold stress enhanced the expression of *GbTPS* gradually up to 8 h, after which expression decreased in roots. Expression of *GbTPS* upon NaCl and mannitol treatments in roots also gradually increased in initial 8 h and decreased between 8 h and 48 h. However, the expression level of *GbTPS* in leaves was increased dramatically after 0.5 h drought treatment and the expression of *GbTPS* was maintained at high level thereafter. The expression of *GbTPS* in roots was similar with

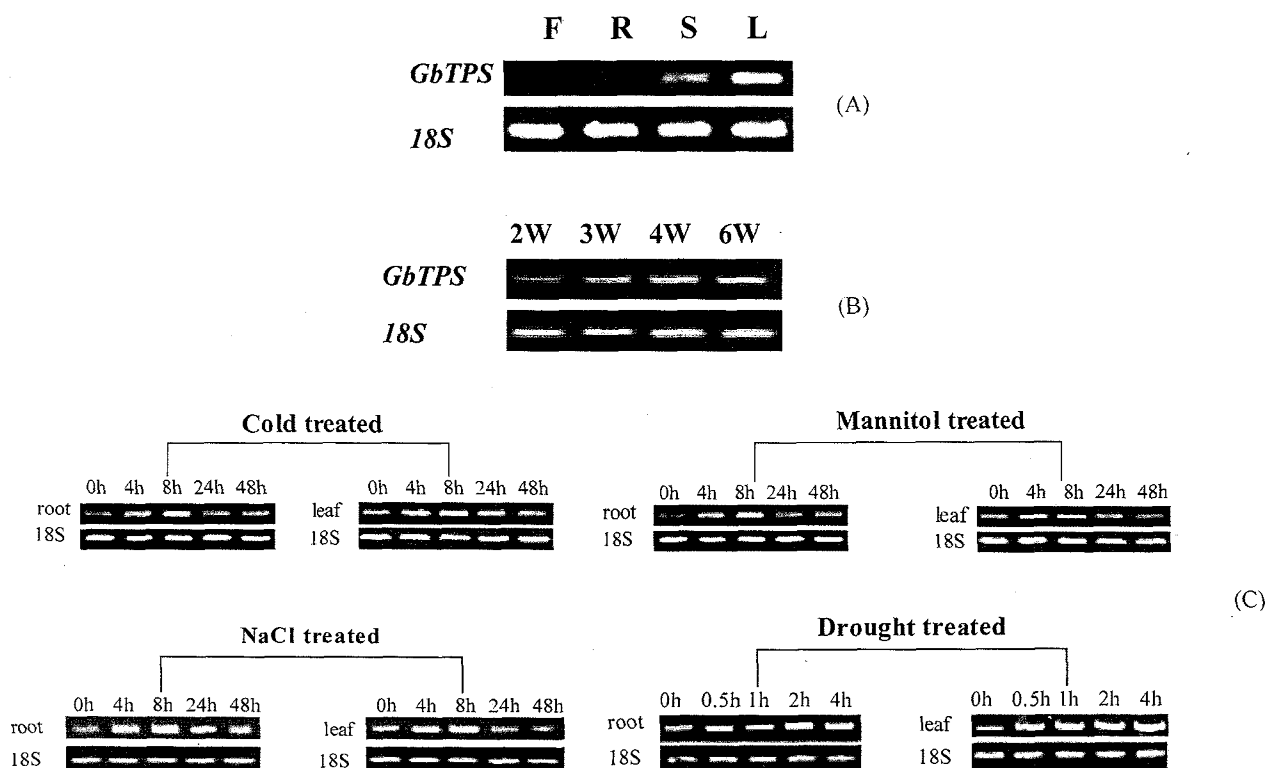


Fig. 3. Expression analysis of *GbTPS*. (A) Expression pattern of *GbTPS* in different tissues of *G. biloba* including fruit (F), root (R), stem (S) and leaf (L). (B) Developmental expression pattern of *GbTPS* in leaf (2 weeks, 3 weeks, 4 weeks and 6 weeks). (C) Expressing profiles of *GbTPS* under the treatments of cold (4°C), NaCl (250 mM), Mannitol (200 mM mannitol) and drought for various duration. The *G. biloba* 18S gene is used as control.

that in leaves under the various treatments. Low temperature, drought and high salinity are common stresses that adversely affect plant growth and crop production. All three stresses together with mannitol, an osmotic stress reagent, have been shown to induce transient Ca^{2+} influx into the cell cytoplasm (Sanders *et al.*, 1999; Knight, 2000). The Ca^{2+}, serving as one of the intracellular secondary signal molecules, then stimulate the accumulation of compatible osmolytes including trehalose (Hasegawa *et al.*, 2000). These stresses upon *G. biloba* might also activate the same signaling pathway of plant cell, which is reflected by the expression profiling of *GbTPS* under these stress conditions.

In this article, a novel full-length cDNA of a TPS gene from *G. biloba* was cloned and characterized. It is the first TPS gene isolated from gymnosperm. Multiple alignments showed that the deduced GbTPS was homologous with other TPS proteins, particularly with the members of class II TPS family (Fig. 1B), and that GbTPS contained both TPP and TPS similar domains (Fig. 1C). Evolutionary analysis further suggests that GbTPS has closer relationship with class II TPS family than with class I or class III TPS family. The expression of *GbTPS* was tissue-specific and up-regulated by cold, NaCl, mannitol and drought treatments. The cloning and characterization of *GbTPS* will be helpful to further isolate and characterize other GbTPS family members, to understand the detailed function/role of GbTPS family members in *G. biloba*, as well as to figure out the molecular evolutionary position. Furthermore, functional complementation experiment of *GbTPS* in yeast and mutation analysis upon the promoter region is underway to verify the function of *GbTPS* in various stresses and its regulatory mechanism.

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