Overexpression of the ThTPS gene enhanced T. hispida salt and drought stress tolerance

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Abstract

Background: Trehalose is a nonreducing disaccharide with high stability and strong water absorption properties that can improve the resistance of organisms to various abiotic stresses. Trehalose-6-phosphate synthase (TPS) plays important roles in trehalose metabolism and signaling.

Results: A full-length cDNA of *ThTPS* was cloned from *Tamarix hispida*. The phylogenetic tree among ThTPS and 11 AtTPS in Arabidopsis indicates that the ThTPS protein had a close evolutionary relationship with AtTPS7. However, the function of *AtTPS7* has not been determined. To analyze the abiotic stress tolerance function of *ThTPS*, the expression patterns of *ThTPS* were monitored under salt and drought stress and JA, ABA and GA3 hormone stimulation in *T. hispida* by qRT-PCR. The results showed that *ThTPS* expression was clearly induced by these 5 kinds of treatments at at least one studied point. Particularly under salt stress, *ThTPS* was highly induced in the roots of *T. hispda*. Furthermore, the *ThTPS* gene was transiently overexpressed in *T. hispida*. The results of physiological indexes and staining showed that overexpression of the *ThTPS* gene increased *T. hispida* salt and drought stress tolerance.

Conclusion: The *ThTPS* gene can respond to abiotic stress such as salt and drought, and overexpression of *ThTPS* gene can significantly improve salt and drought tolerance. These findings establish a foundation to better understand the response of *TPS* genes to abiotic stress in plants.

Background

Trehalose is a nonreducing disaccharide composed of two glucose molecules linked by α,α 1–1 glycosidic bonds [1–2]. Trehalose was first discovered in bacteria by Wiggers in 1832, and the French chemist Berthelot subsequently discovered the sugar in molasses secreted by weevils in the Asia Minor Desert and named it trehalose [3]. Currently, trehalose is widely found in various living organisms, such as bacteria, yeasts, molds, edible fungi, lower plants, insects and invertebrates, as well as some higher plants [3–6].

Trehalose in the living body can increase the resistance of organisms to adverse conditions. The resistance of many species to adverse environmental conditions is directly related to the concentration of trehalose in their bodies [7–8]. Trehalose is a typical stress metabolite. When the organism grows well, it does not accumulate trehalose in the body, while when the organism is in a stressful environment (such as starvation, dryness, high temperature and high salinity), trehalose can rapidly accumulate [9–10]. These trehaloses were degraded when the adverse environment was removed. In addition, the added trehalose also has clear protective effects on active substances, such as proteins, enzymes and cell membranes [11]. In plants, trehalose is an important substance in regulating diverse processes, such as development [12–16], response to biotic stresses [17–21] and abiotic stresses [22–26].

The synthesis of trehalose in plants was based on the synthesis of trehalose-6-phosphate (Tre6P) from UDP-glucose and glucose-6-phosphate catalyzed by trehalose-6-phosphate synthase (TPS) and then

catalyzed by trehalose-6-phosphate phosphatase (TPP) to trehalose. However, when trehalose is synthesized in plants, there are many nonphosphatase enzymes that can catalyze Tre6P into trehalose, and it can directly catalyze the dephosphorylation of Tre6P to produce trehalose without TPP [4]. However, the TPS protein has an irreplaceable role in plant trehalose synthesis, and the successful transcription and expression of the TPS gene was decisive for the synthesis of trehalose in plants.

Eleven TPS homologs were found in the Arabidopsis Thaliana genome [27]. The 11 AtTPS genes were classified into two classes [28–29]. Class I includes TPS1-4 genes and is closely related to the yeast TPS1 gene, whereas class II includes TPS5-11 genes and contains sequences corresponding to phosphatase and synthase domains [30–31]. In addition, 8 TPS genes were identified in potato, 53 in cotton and 20 in the soybean genome [32–34]. In many other plants, such as Plasmodiophora brassicae [18], Magnaporthe grisea [19] and Saccharum officinarum L. [23], TPS genes were also cloned.

Some TPS transgenic plants can significantly improve abiotic stress tolerance. For example, the A. thaliana TPS1 gene enhanced the osmotic, drought, desiccation and temperature stress resistance of transgenic tobacco [35]. Transformation of the yeast TPS1 gene into potato significantly improved the drought resistance of transgenic plants [36–37]. Garg et al transferred the trehalose synthesis genes (otsA and otsB) of Escherichia coli into rice and improved salt, drought and low-temperature stress resistance [38]. Jang et al. showed that transformation of the E. coli trehalase synthase gene into rice can increase trehalose accumulation and drought, high salt and cold tolerance [39]. Overexpression of the TPS1 gene sorghum enhanced tolerance to salt stress [40]. Grifola frondosa Fr. TPS gene transformation in tobacco enhanced resistance to drought and salt [41].

Tamarix hispida is a woody halophyte with developed roots and strong sprouting ability. This species has strong drought, cold, salt and alkali resistance, making it a good plant to grow in sandy soil and various degrees of salinized soil. Therefore, this halophyte is an ideal material for anti-reverse gene cloning and the study of stress resistance mechanisms. In this study, the ThTPS gene was cloned from T. hispida, and the sequence characteristics of the gene and the expression pattern after abiotic stress were analyzed. Furthermore, ThTPS was transiently overexpressed in T. hispida. The related physiological index and staining analysis were carried out and compared between the overexpression and control T. hispida under salt stress. This study will establish a theoretical foundation to further analyze the stress tolerance function of the TPS gene and use genetic engineering to improve plant stress resistance.

Results

Sequence and evolution analysis of the *ThTPS* gene in *T. hispida*

A full-length *ThTPS* gene sequence was obtained from *the T. hispida* transcriptome. The GenBank number is MN615274. The ORF of the *ThTPS* gene was 2577 bp and encoded 858 amino acids. ProtParam predicted that the molecular weight of ThTPS protein was 96.81 KD with a theoretical isoelectric point of 5.86, which is an acidic protein. The ThTPS protein belongs to the HAD hydrolase family IIB subfamily (IPR006379) and contains a glycosyltransferase domain (IPR001830, 61-544) and a

HAD-like domain (IPR023214, 591-842). Multiple sequence alignment results indicated that ThTPS has high sequence homology with the selected amino acid sequences, and the similarities were 83.41%-86.42% (Fig. 1A). The results of the phylogenetic tree among the ThTPS protein sequence together with 11 TPS members in the *Arabidopsis* family indicate that the ThTPS protein belongs to the class II subfamily and has a close evolutionary relationship with AtTPS7 (Fig. 1B). Similar to other class II members, ThTPS contains three conserved motifs (motif 1, motif 2 and motif 3).

Expression analysis of the *ThTPS* gene under different treatments in *T. hispida*

To preliminarily analyze the function of the *ThTPS* gene, we analyzed the expression levels of the *ThTPS* gene in the roots and leaves of *T. hispida* under 150 μmol/L ABA, 100 μmol/L JA, 50 μmol/L GA3, 0.4 mol/L NaCl and 20% (w/v) PEG_{6000} by qRT-PCR. The results showed that *ThTPS* exhibits different expression patterns under hormone, salt and drought treatments (Fig. 2).

Under ABA treatment, the expression of the *ThTPS* gene was upregulated at 6 h and 48 h, which was 54.66- and 3.1-fold that of the control, respectively. At 12 h, 24 h and 72 h, it showed a downward trend in roots. In leaves, the expression of *ThTPS* showed an opposite expression pattern from roots (Fig. 2A). Under JA treatment, the *ThTPS* gene showed an upregulated expression trend in roots, reaching the highest expression level at 6 h, which was 10.02 times that of the control. While this gene mainly showed a downregulated expression trend in leaves, it reached its lowest point at 12 h, which was 5.93% of the control (Fig. 2B). Under GA3 treatment, the expression of the *ThTPS* gene showed an upregulated trend at 12 h and 48 h in roots, which was 9.74 and 30.14 times that of the control. At 24 h, the gene expression changed little but showed a downregulated expression trend at 6 h and 72 h. In leaves, the *ThTPS* gene showed the opposite trend (Fig. 2C).

Under NaCl stress, in addition to the downward trend at 6 h, the *ThTPS* gene showed a trend of upregulation at other time points in roots and leaves, reaching the highest values at 24 h and 48 h, respectively, which were 361.33 and 7.55 times that of the control (Fig. 2D). After PEG_{6000} stress, in roots, the *ThTPS* gene showed an upregulation trend at 6 h and 12 h. In leaves, the gene mainly showed a downregulated trend (Fig. 2E). The above results showed that the *ThTPS* gene can respond to the above five treatments, but the expression patterns were not exactly the same.

ThTPS overexpression in *T. hispida* can enhance salt and osmotic tolerance

To further explore the resistance function of the *ThTPS* gene, the pROKII-*ThTPS* strain was transiently transformed into *T. hispida* (OE). The OE and the control were treated with 150 mM NaCl and 200 mM mannitol stress. The *ThTPS* gene expression results showed that the expression level of the *ThTPS* gene in OE plants was 100.3, 3.5 and 13.8 times that of the control after NaCl treatment for 12, 24 and 36 h, respectively. After mannitol treatment for 12, 24 and 36 h, the expression levels of the *ThTPS* gene in OE plants were 23.6, 10.1 and 13.9 times those of the control, respectively. These results indicated that transient transgenic *T. hispida* were successfully obtained, and the expression level of the *ThTPS* gene was higher after treatment for 12 h (Fig. 3A).

Based on the results of qRT-PCR, DAB, NBT and Evans blue staining and physiological indexes were performed for the transiently transgenic *T. hispida* treated with NaCl and mannitol for 12 h. The results showed that there were no obvious color and physiological index content differences between OE and control plants under normal conditions. However, the DAB and NBT staining colors of OE plants were clearly lighter than those of the control under NaCl and osmotic stress (Fig. 3B). Correspondingly, H_2O_2 contents were measured and compared. In OE plants after NaCl and mannitol stress, it was 4.08- and 5.1-fold of normal conditions, while in control plants, it was increased 6.5- and 6.96-fold (Fig. 3C). These results indicated that H_2O_2 or O^{2-} content was lower in OE plants than in the control under the two stresses. Evans blue staining results showed that OE plants had a lighter color than the control (Fig. 3B). At the same time, MDA contents in OE plants were 3.14- and 3.1-fold of normal conditions, and they were 4.7- and 3.55-fold in control plants (Fig. 3C), indicating that the cells of OE plants were less damaged. In addition, the cell protection product trehalose increased 3.98- and 4.42-fold in OE plants, which is higher than that in the control. The plant growth indicator chlorophyll decreased 33% and 26% in OE plants, which is less than the control (54% and 50%) (Fig. 3C). The above results showed that overexpression of the *ThTPS* gene can enhance the ability of transgenic *T. hispida* cells to scavenge reactive oxygen species (ROS) and promote the accumulation of less O^{2-} and H_2O_2, thereby reducing cell death and enhancing the salt and osmotic tolerance of *T. hispida*.

Discussion

Trehalose accumulates in the plant and exerts a protective function to improve certain undesirable traits of the plant. Trehalose-6-phosphate synthase (TPS), another gene related to stress resistance after glutamate, proline and betaine synthase genesis [44], functions in catalyzing the conversion of substrate to trehalose. In this study, a ThTPS gene was cloned from T. hispida. Sequence analysis showed that the ThTPS protein contains a glycosyltransferase domain and HAD-like domains. Multiple sequence alignment showed that the ThTPS protein and other TPS proteins have conserved motifs at the N-terminus and C-terminus, indicating that the TPS protein is highly conserved in different plants. Phylogenetic tree analysis with the A. thaliana TPS family showed that the ThTPS protein was closely related to the Arabidopsis family member AtTPS7 and belonged to the class II subfamily.

Class II subfamily members may have a regulatory function and are regulated transcriptionally by carbon status and stress [6]. In recent years, several approaches have found that A. thaliana TPS family proteins, class II subfamily member AtTPS6, regulate plant architecture, epidermal pavement cell shape and trichome branching [14]. AtTPS5 plays a role in thermo tolerance through its interaction with the transcriptional coactivator MBF1c [45]. However, there have been no reports describing AtTPS7 function to date. Other class II TPS proteins seem to lack significant enzymatic activity, many of which are extensively regulated by hormones, light and nutrient availability at the transcriptional level [46–52].

qRT-PCR results showed that the expression of the ThTPS gene in T. hispida was significantly changed after high salt, drought, JA, ABA and GA3 treatment. After JA, ABA and GA3 treatment, the expression of

the ThTPS gene mainly showed opposite expression trends in roots and leaves, except at the individual time points. However, after NaCl and PEG_{6000} treatment, the expression of the ThTPS gene was mainly upregulated in roots and leaves, indicating that the ThTPS gene may be involved in drought, salt stress and hormone stimulation responses, while the function may differ in root and leaf responses to hormone stimulation. In previous studies, it has been shown that AtTPS1 participates in the Glc and ABA signaling pathways controlling germination and vegetative development [22]. Overexpression of the TSase gene in Grifola frondosa reduced water loss, cell damage and photosynthesis and preserved SOD and POD activities, thereby improving drought tolerance in sugarcane [23]. OsTPS1-overexpressing rice improved the tolerance of seedlings to cold, high salinity and drought treatments without other significant phenotypic changes [24]. In this study, the physiological indicators and staining results of transient overexpression ThTPS also showed that the contents of O^{2-} and H_2O_2 were lower in OE plants, and plant cells were less damaged. This finding indicates that overexpression of the ThTPS gene can increase the salt and osmotic tolerance of T. hispida. In the following experiments, the specific resistance mechanism of the ThTPS gene will be further studied.

Conclusions

TPS protein is an irreplaceable protein in plant trehalose synthesis, and the TPS gene is successfully transcribed and expressed in plants. In this study, a ThTPS gene was cloned from T. hispida. The expression results showed that the ThTPS gene could be induced by drought, salt stress and hormone stimulation, revealing that the ThTPS gene may play a vital role in the response to stresses and hormone singling. Overexpression of the ThTPS gene in T. hispida promotes the biosynthesis of trehalose, decreases the accumulation of O^{2-} and H_2O_2, and therefore enhances salt and drought tolerance in plants. These results help to establish a foundation for the study of TPS functions and research on T. hispida stress resistance.

Methods

Plant material and stress treatment

T. hispida seeds used in the study have been formally identified, and voucher specimens of this material have been deposited in the Turpan Desert Botanical Garden (Xinjiang, 293 China). *T. hispida* seeds were uniformly sown in a plastic basket with culture medium (vegetative flower soil: vermiculite1:1). The greenhouse culture conditions were humidity 70-75%, average temperature 25°C and photoperiod 14 h/10 h. The seedlings grown to approximately 5 cm were moved to pots and cultured in the greenhouse. After 2 months, the seedlings were irrigated with 0.4 mol/L NaCl, 20% (w/v) PEG_{6000}, 150 μmol/L ABA, 50 μmol/L GA3 and 100 μmol/L JA. Normally watered seedlings were used as a control. After treatment for 6, 12, 24, 48 and 72 h, the roots and leaves of each treatment were taken separately, immediately frozen in liquid nitrogen, and then stored in a -80°C freezer for subsequent experiments. All treatments were replicated three times.

Bioinformatics analysis of the *ThTPS* gene in *T. hispida*

Through searching the transcriptome data of *T. hispida* using "trehalose-6-phosphate synthase" as a key word, a full-length *ThTPS* gene sequence was obtained. Through BLASTx alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the *ThTPS* sequence and ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) analysis, the open reading frame (ORF) and amino acid sequence of the *ThTPS* gene were identified. The relative molecular mass and theoretical isoelectric point of ThTPS protein were identified through ProtParam software. The domain of the ThTPS protein was identified by using the InterProscan online tool. The ThTPS amino acid sequences were aligned using the BLASTP program in NCBI to obtain nine other plant TPS proteins with higher homology, including *Ziziphus jujuba*, *Coffea eugenioides*, *Quercus suber*, *Vitis vinifera*, *Juglans Regia*, *Gossypium raimondii*, *Actinidia chinensis var. Chinensis*, *Camellia fraterna* and *Chenopodium quinoa*, and then multiple sequence alignment analysis was carried out using ClustalX 1.83. In addition, the amino acid sequences of 11 TPS proteins in the *Arabidopsis* family were selected, and phylogenetic tree construction analysis was performed using MEGA5.0 software. Through MEME analysis, the conserved motifs of the ThTPS protein and *Arabidopsis* TPS family members were analyzed.

RNA extraction and qRT-PCR analysis

The RNA of each sample was extracted using a plant RNA extraction kit (BioTeKe corporation), and the procedure was carried out according to the kit instructions. The RNA extraction concentrations and masses were measured using a Nanovue microphotometer and 0.8% agarose gel electrophoresis. Then, the total RNA of each sample was reverse transcribed into cDNA using TransScript One-step gDNA Remvoal and cDNA Synthesis SuperMix according the instruction manual. Then, qRT-PCR was carried out using the *Actin* (FJ618517), *α-tubulin* (FJ618518) and *β-tubulin* (FJ618519) genes as internal controls (reference genes). The internal control and *ThTPS* gene primer sequences are shown in Table 1. The reaction system of qRT-PCR was SYBR Green Mix 10 μl, 1 μl of each of the forward and reverse primers (10 μmol/L), cDNA 2.0 μl, and ddH_2O supplemented to 20 μl. The reaction procedure was 95°C for 3 min, 95°C for 20 s, 58°C for 15 s, and 72°C for 30 s for 45 cycles. Each sample was repeated three times. qRT-PCR experiments were performed using an Opticon Monitor 2 real-time PCR machine manufactured by Böhler. Data were analyzed using the $2^{-\Delta\Delta(Ct)}$ method [42].

ThTPS gene cloning and plant overexpression vector construction

According to the multiple cloning site of the plant overexpression vector pROKII and the gene sequence of *ThTPS*, Xba I and Kpn I restriction endonuclease sites were introduced at the 5' and 3' ends of the *ThTPS* gene, respectively. The primers TPS-CF (5'CTAGTCTAGAATGATGTCCAGATCTTATACC3') and TPS-CR (5'CGGGGTAC CCTAAGAGGGGCTGCCGCTAC3') were used to obtain the *ThTPS* gene by RT-PCR amplification. Then, the digested gene and the vector fragment were ligated and transformed into *E. coli* competent Top10 cells by the heat shock method. After culturing at 37°C for 8-12 h, single colonies were picked for PCR verification using vector primers and gene primers. The strains with the correct fragment

sizes were sent for sequencing analysis. The sequenced correct overexpression vector strain was designated pROKII-*ThTPS,* and the plasmid was transformed into *Agrobacterium tumefaciens* EHA105 to obtain an overexpression strain.

ThTPS gene transient transform into *T. hispida* and stress resistance analysis

According to the method of Ji et al. [43], the pROKII-*ThTPS* overexpression strain (OE) and the pROKII empty vector (Con) strain were transiently transformed into *T. hispida*. After culture in MS medium for 24 h, the *T. hispida* seedlings were cultured in MS with 150 mM NaCl and 200 mM mannitol and treated for 12, 24 and 36 h, respectively. At the same time, seedlings cultured on normal MS medium were used as controls. The RNA of each sample was extracted, and the expression of the *ThTPS* gene was analyzed by qRT-PCR. Furthermore, the physiological indexes of each sample, including MDA, chloroplast, trehalose (plant trehalose, trehalose determination by ELISA kit) and H_2O_2 (hydrogen peroxide assay kit, NanJing Jiancheng) content, were measured according to the manufacturer's protocol. Nitrotetrazolium chloride (NBT), diaminobenzidine (DAB) and Evans blue staining analyses were carried out after the seedlings were treated for 12 h. Each experiment was repeated at least three times.

Declarations

Ethics approval and consent to participate Tamarix hispida is a woody halophyte. Therefore, no voucher specimens were prepared. No specific permits were needed for the described experiments, and this study did not involve any endangered or protected species. Consent for publication Yes. Availability of data and material Relevant data analyzed during this study are included in this published article. Competing interests The authors declare that they have no conflict of interest. Funding This work was supported by the National Natural Science Foundation of China (No. 31370676), The Province in Heilongjiang Outstanding Youth Science Fund (JC2017004) and Heilongjiang Touyan Innovation Team Program(Tree Genetics and Breeding Innovation Team). The National Natural Science Foundation of China project provided financial support to the gene sequencing and physiological indicators analysis and manuscript writing. The Province in Heilongjiang Outstanding Youth Science Fund project supported in part on the physiological indicators analysis and manuscript writing. Heilongjiang Touyan Innovation Team Program(Tree Genetics and Breeding Innovation Team) project supported in part on manuscript writing. The funding bodies played no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript. Authors` contributions PLW carried out all the experiments and data analysis. PLW and CQG conceived the project, designed the experiments and drafted the manuscript. CQG supervised the analysis and critically revised the manuscript. XJL and JXL provide help during the experiment process. All authors read and approved the final manuscript. Acknowledgements Not applicable.

Abbreviations

DAB: Nitrotetrazolium chloride; NBT: Diaminobenzidine; *T. hispida*: *Tamarix hispida;* TPS: Trehalose-6-phosphate synthase; TPP: Trehalose-6-phosphate phosphatase; ORF: Open reading frame; ABA: Abscisic acid; GA3: Gibberellic acid.

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Table

Table 1 The primer sequences of real-time RT-PCR

Genes	Primers names	Sequences （5'→3'）
ThTPS	TPS-D-F	ATGGGCCAGATTCAGTCTGT
	TPS-D-R	CCTGCAGCTTTCCTGAATCTCG
Actin	*β-Actin-F*	AAACAATGGCTGATGCTG
	β-Actin-R	ACAATACCGTGCTCAATAGG
α-tubulin	*α-tubulin-F*	CACCCACCGTTGTTCCAG
	α-tubulin-R	ACCGTCGTCATCTTCACC
β-tubulin	*β-tubulin-F*	GGAAGCCATAGAAAGACC
	β-tubulin-R	CAACAAATGTGGGATGCT

Figures

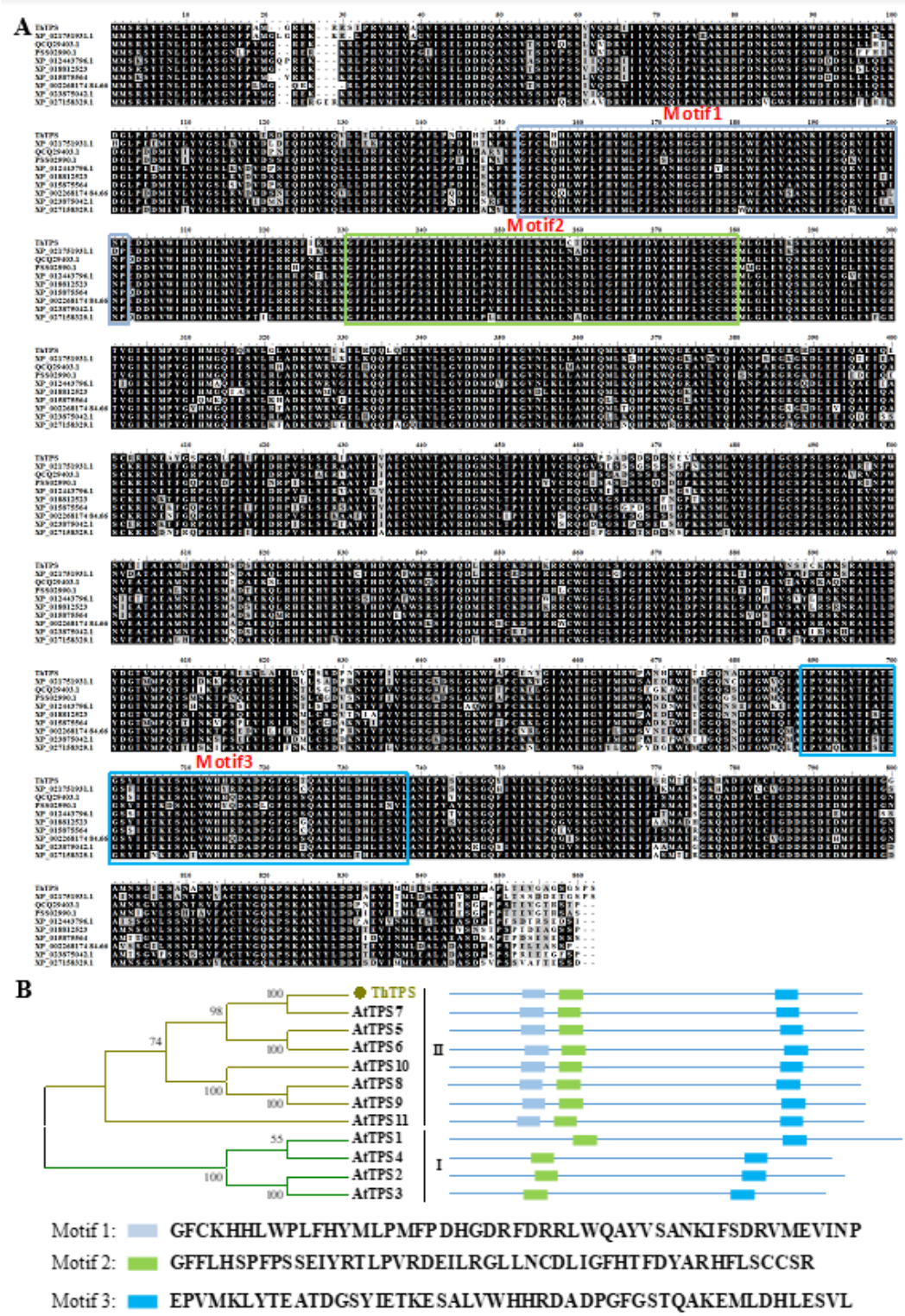

Figure 1

Multiple Sequence Alignment and Evolution Analysis of ThTPS Gene (A) Multiple sequence alignments of ThTPS protein sequence and other TPS proteins were performed with ClustalW using BioEdit software. The conserved motifs are indicated by box . (B) Phylogenetic tree constructed with the neighbor-joining method using ThTPS protein and Arabidopsis TPS family proteins. And MEME analysis of these sequences.

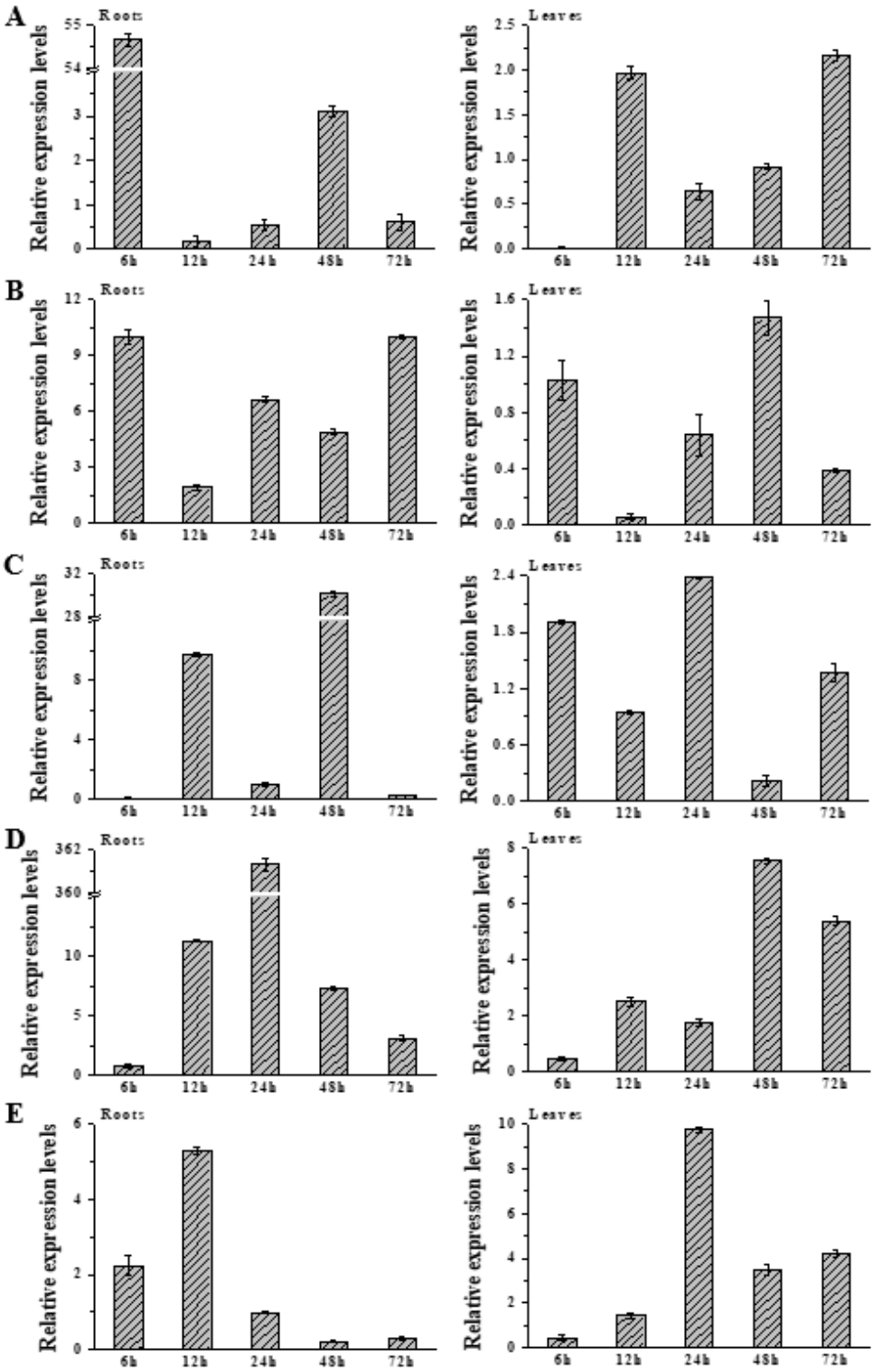

Figure 2

Expression analysis of ThTPS gene in T.hispida under several abiotic stresses and hormone treatment (A) 150 μmol/L ABA, (B) 100 μmol/L JA, (C) 50 μmol/L GA3, (D) 0.4 mol/L NaCl and (E) 20% (w/v) PEG6000 treatment. (Each sample contain three replicates and the data were treated by 2-ΔΔ(Ct))

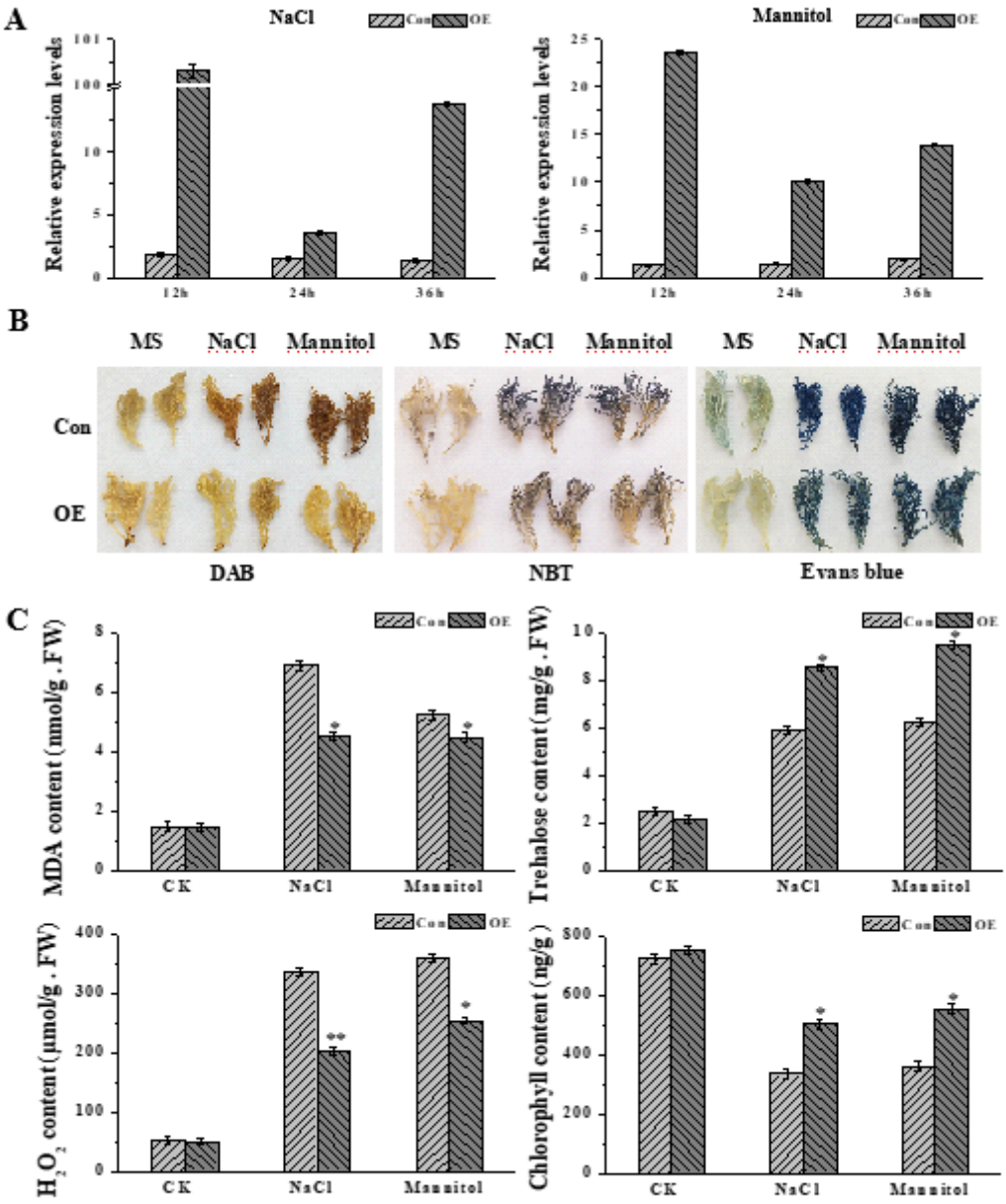

Figure 3

Resistance tolerance analysis of ThTPS gene in instantly overexpression T.hispida under NaCl and mannitol treatment. (A) qRT-PCR analysis of ThTPS gene in transient overexpressed ThTPS T.hispida and the control under NaCl and mannitol at different stress time point (Each sample contain three replicates and the data were treated by 2-ΔΔ(Ct)) B) Chemical staining analysis of T. hispida under 12h stress. (C) Related physiological indexes analysis of T. hispida under 12h stress.