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Trehalose-6-phosphate synthase gene expression analysis under abiotic and biotic stresses in bottle gourd (*Lagenaria siceraria*)

Shuoshuo Wang[1], Wenli Li[1] & Han Jin[2]✉

Trehalose (Tre) is a non-disaccharide that regulates environmental stress tolerance in animals and plants, and is synthesized by Trehalose-6-phosphate synthase (TPS). This study aimed to analyze TPS genes in bottle gourd as this species has not been investigated before despite its economic importance and health benefits. Six *TPS* genes in *Lagenaria siceraria* (*LsTPS*) were identified and found to be distributed across six chromosomes. The LsTPS genes were categorized into Classes I and II based on their homology with *Arabidopsis*, rice, cucumber, watermelon, and tomato. Variable exon numbers were found in the LsTPS genes, with more exons in Class II than in Class I genes. GO term enrichment and *cis*-regulatory element analyses indicated that LsTPS genes participate in Tre synthesis and environmental stress responses. Structural analysis of TPS proteins revealed that LsTPS5 has a transmembrane helix, an α-helix and β-sheet. Gene duplication analysis indicated that purifying selection drove the evolution of the *LsTPS* family. We found that *LsTPS* genes are widely expressed in all plant tissues, and *LsTPS1/5* are constitutively expressed in all tissues. RNA-sequencing and quantitative real-time PCR data showed that *LsTPS* expression changed significantly in response to environmental stressors. This study provides to foundation for further research on the roles of the *LsTPS* gene and Tre in abiotic and biotic stress response and provides important insights for the development of genetic engineering methods to alter Tre metabolism and interactions with other molecules.

Keywords *Lagenaria siceraria*, Trehalose-6-phosphate synthase (TPS), Evolutionary, Expressed, Stress

Bottle gourd (*Lagenaria siceraria*) is an essential horticultural and ornamental plant, which was indigenously domesticated in Africa and Asia[1–4]. Bottle gourds contain a wide range of micro- and macro-elements, as well as other phytochemical compounds that are beneficial to human health; therefore, selecting for early maturing, high yielding, pest- and disease-resistant varieties/hybrids is the main goal of breeding[5]. Bottle gourds have a wide variety of fruit shapes, ranging from almost completely round to elongated, with in-between types[6,7]. The shape of the fruit often determines the type of market for the variety. Dried bottle gourds are utilized as containers, raw materials for making cucurbit flutes, or accessories for ethnic clothing[5]. Bottle gourds are also widely used as grafting rootstocks for watermelon and melon production, thereby solving the problem of poor disease resistance, as well as increasing yield and improving cold tolerance[8].

Plants are challenged by many environmental stressors during their growth and development, such as high and low temperatures, salt stress, and diseases[9,10]. Plants respond to both abiotic and biotic stressors by accumulating soluble sugars and free amino acids[11]. Trehalose (Tre) is a non-reducing disaccharide with strong hydration capacity, that replaces bound water on the surface of biomolecules thereby enhancing the stability of proteins and biofilms during environmental stresses[12,13]. Tre is widely found in living organisms such as fungi, plants, and animals[14]. Tre production is readily by under multiple stressors, such as heat stress, drought stress, salt stress, and stimulated resistance mechanisms in plants[15–20]. Metabolism of Tre and key intermediates mediates in a number of life and cellular metabolic processes, such as starch accumulation and metabolism, stomatal movement, and seed germination to regulate plant growth and development[10,21,22]. Tre is beneficial to improving plant yield and quality. Spraying foliage with 10 mM Tre significantly increased Tre content, which improved the hardness and quality of apple fruits[23]. Tre plays an essential role in boosting plant resistance to environmental

[1]College of Agriculture and Biology, Liaocheng University, Liaocheng 252000, Shandong, China. [2]School of Pharmaceutical Sciences and Food Engineering, Liaocheng University, Liaocheng 252000, Shandong, China. ✉email: jinhan@lcu.edu.cn

stresses and protecting cellular contents from damage caused by drought, heat, and cold[24]. Tre improves plant resistance by reducing oxidative damage and increasing photosynthetic capacity. Exogenous Tre sprays increase the antioxidant content in maize leaves, and the activity of antioxidant enzymes associated with drought stress resistance[17,25]. Tre mitigates high-temperature damage mainly by preserving normal photosynthesis in plants, maintaining the dynamic balance of cellular redox, and reducing the damage caused by heat stress[26–31]. Tre also improves plant tolerance to low-temperature stress, maintains intracellular oxidative balance, and protects the cell membrane structure[32,33]. Therefore, Tre application may enhance the antioxidant capacity of plants, uphold the dynamic balance of reactive oxygen species (ROS), protect the photosynthetic apparatus, and regulate osmotic regulators, which work with phytohormones to mitigate the damage caused by environmental stresses.

The monosaccharide uridine-glucose diphosphate (UDP-GLc) and glucose-6-phosphate (GLc-6-P) were used as precursors to generate alginate-6-phosphate (T6P), which is catalyzed by trehalose-6-phosphate synthase (TPS), and then by trehalose-6-phosphate phosphatase (TPP) to generate trehalose-6-phosphate (Tre)[10,21]. Two major enzymes (TPS and TPP) involved in the Tre biosynthetic pathway were identified for the first time in *Arabidopsis thaliana*[34,35]. During the TPS-catalyzed generation of T6P from UDPG and G6P, low concentrations of Ca^{2+}, $K^{+,}$ Mg^{2+}, Na^{+}, fructose, fructose 6-phosphate, and glucose enhanced the activity of the TPS enzyme, whereas proline inhibited the activity of the TPS protease[34,35]. The catabolism of Tre is simpler than its synthetic pathway, where Tre is directly hydrolyzed into two molecules of glucose, which form Glc-6-P via the action of hexokinase (HXK)[36].

The products catalyzed by TPS mainly include T6P and Tre, and T6P is involved in starch synthesis, cell differentiation and photosynthesis regulation[10,37]. Tre not only protects proteins and membrane structures in plants but also mediates stress resistance[38–40]. Therefore, the role of *TPS* genes in plants, especially their function in mitigating environmental stresses, has received increasing attention. *OsTPS1* increases abiotic stress resistance in rice by enhancing the Tre content and regulating the expression of stress-related genes[16]. *AtTPS1* is involved in the regulation of stress signals, and *AtTPS5*-dependent Tre metabolism mediates the basic defense response in *A. thaliana*[41–46]. *TPS* genes are essential for increasing plant resistance and enhancing the content of Tre, as well as for their promise in crop enhancement; for example, cotton *TPS* genes are significantly induced by drought stress[47], and *PvTPS9* regulates symbiotic root nodules in legumes[48]. Overexpression of *ClTPS3* significantly improved salt tolerance in *Arabidopsis thaliana*[49]. *SlTPS* genes positively regulate resistance to *Pst* DC3000 and *Bacillus cinerea* in tomato[50].

Many *TPS* genes have been identified and analyzed in plants, including 11 *AtTPS* genes in *A. thaliana*, 11 *OsTPS* genes in *Oryza sativa* L., 10 *SlTPS* genes in *Solanum lycopersicum* L., and 11 MtTPS genes in *Medicago truncatula*[6,51–53]. *TPS* genes have also been identified and analyzed in several cucurbits; specifically, seven *TPS* genes have been identified in cucumber, watermelon, and melon[54,55]. However, TPS genes have not yet been identified in *Lagenaria siceraria*. Owing to the increasing sophistication of gene sequencing technology, the bottle gourd genome size has been estimated as ~334 Mb with 11 chromosomes, which facilitated the identification of *TPS* genes from *L. siceraria*[5]. We identified all *LsTPS* genes in the bottle gourd genome using bioinformatics. Subsequently, RNA-sequencing data revealed that some of these genes were associated with environmental stresses, such as heat, cold, and powdery mildew. Our research offers a basis for further investigation of *LsTPS* genes and their regulatory mechanisms under abiotic and biotic stress in *L. siceraria*.

Materials and methods

Identification of *LsTPS* genes in *L. siceraria*

Bottle gourd genome and gene annotation files were obtained from the CuGenDBv2 website (http://cucurbitgenomics.org/). To detected LsTPS genes in bottle gourd genome, which were identified using an Hmmer search (*e-value* < e^{-10}), the LsTPS domain (glycosyltransferase family 20 (Glyco_transf_20); PF00982) was downloaded from the Pfam database (http://pfam.xfam.org/). All candidate LsTPS genes were detected in the TPS domain using the CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and SMART databases (http://smart.embl-heidelberg.de/) databases. Finally, proteins with at least one TPS structural domain was identified as LsTPS proteins.

Physicochemical properties, genomic localization, motif and gene structure analysis

The basic physical properties of LsTPS proteins were estimated using the online tool ExPASy (http://www.expasy.org/Tools/protparam.html) under default parameters such as molecular weight, theoretical pI, instability index, and total average hydrophilicity (GRAVY). Based on the genomic information of bottle gourds from the CuGenDBv2 website, *LsTPS* genes were renamed according to their location on the chromosomes and visualized using TBtools software[56]. The motifs of the LsTPS proteins were predicted using the online tool MEME (https://meme-suite.org/meme/), and the maximum number of motifs was limited to 15 under default parameters. Analysis and visualization of LsTPS gene structure based on the online tool Gene Structure Display Server (GSDSv2.0, https://gsds.gao-lab.org/Gsd.org/help.php).

Phylogenetic and collinearity analysis of *TPS* genes

Phylogenetic tree construction was performed using 53 TPS protein sequences from several species, including bottle gourd (*L. siceraria*; six LsTPS genes), *A*, *thaliana* (11 AtTPS genes), rice (*O. sativa*; 11 OsTPS genes), cucumber (*Cucumis sativus*; seven CsTPS genes), watermelon (*Citrullus lanatus*; seven ClTPS genes), and tomato (*S. lycopersicum*; 11 SlTPS genes) (Table S1). Amino acid sequence comparison was performed using ClustalW, and a phylogenetic tree was established using the neighbor-joining (NJ) method with MEGA X software. The bootstrap replication value was set to 1,000. LsTPS gene collinearity and selective evolutionary pressure were analyzed using TBtools software. To analyze the evolutionary selection pressure on homologous genes of the

TPS gene family in *Arabidopsis*, bottle gourd, watermelon, and cucumber, the ratio of non-synonymous to synonymous substitutions (Ka/Ks) in synonymous TPS gene pairs was evaluated using TBtools.

Protein 3D structure, gene ontology and promoter *cis*-regulatory elements

The LsTPS protein sequences were uploaded for three-dimensional (3-D) structure prediction using the online tool PHYRE2 analysis (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) with default parameters. Gene Ontology (GO) annotation files were downloaded from CuGenDBv2, and GO term enrichment was analyzed using the online tool Omicshare (https://www.omicshare.com/) for genes with a *Q-value* of < 0.05. A promotor sequence was obtained 2000 bp upstream of the start codon of the *LsTPS* gene and sent to PlantCare (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for promoter *cis*-regulatory element analysis, and visualized using TBtools software.

Plant materials and stress treatment

Bottle gourd seeds were germinated in Petri dishes lined with filter paper and sown in 50-hole trays. After the cotyledon spread, the seedlings were replanted in small pots (10 cm height × 10 cm width). The seedlings were watered with 50 ml Hoagland's nutrient solution every seven days and incubated in a plant culture room at 25 °C, 16/8 h (day/night) photoperiod and 50% relative humidity. Seedlings at the two-leaf stage were divided into two groups and subjected to either heat- or cold-stress treatments. The seedlings in the heat-stress group were further divided into groups for length of exposure to high temperatures (45 °C/40 °C, day/night). Specifically, leaf samples were collected for qRT-PCR validation at 0, 1, and 3 h after heat stress exposure, with three biological replicates. When not undergoing heat exposure, the plants were cultured at 25 °C. The cold-stress group seedlings were subjected to a simulated cold treatment at 4 °C, and leaf samples were collected for qRT-PCR validation at 0, 12, and 24 h after cold stress exposure, with three biological replicates.

LsTPS gene expression analysis

To obtain the gene expression profiles of *LsTPS* genes, we used published RNA-seq data to generate the tissue-specific expression profiles of *LsTPS* genes, including for roots, stems, leaves, flowers, and fruits of *Lagenaria vulgaris*[5]. Gene expression data are visualized as heatmaps in R software using the "heatmap" package. RNA-seq profiles of LsTPS genes in bottle gourds under heat stress, cold stress, and PM infection were obtained using previously published RNA-seq data (accession numbers: PRJNA965915, PRJNA553072, and PRJNA793252). Gene expression in bottle gourds was quantified as transcripts per million (TPM) using TBtools software. Transcript abundance was calculated using kallisto based on K-mer analysis. Genes with *P-value* < 0.05, and Log_2|Fold-change| > 1 were determined as differentially expressed genes (DEGs). Normalization (Log_2Fold-change) was generated using the DESeq2 and edgeR functions in TBtools software. For qRT-PCR, total RNA was obtained using the MiPure Cell/Tissue miRNA Kit (Vazyme, Nanjing, China), reverse transcription was performed using HiScript II Q RT SuperMix (Vazyme), and qRT-PCR was performed using a Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, city, state, USA) with ChamQ SYBR qPCR Master Mix (Vazyme). The relative expression of *LsTPS* gene was evaluated using the $2^{-\triangle\triangle CT}$ method. The primers used for qRT-PCR are listed in Supplemental Table 2.

Results

Identification and physicochemical properties analysis of LsTPS genes in *L. siceraria*

Six LsTPS genes were identified in bottle gourds using the HMMER model (PF00982). Domain analyses further demonstrated the reliability of the candidate genes (Table S3). *LsTPS* genes were located on six chromosomes, namely, chromosomes 1, 2, 4, 6, 7, and 11; these genes were renamed *LsTPS1-LsTPS6* (Fig. 1). The smallest LsTPS gene was LsTPS6, which contained 691 amino acid residues. The molecular weight of LsTPS genes ranged from 79.88 kD (LsTPS6) to 126.07 kD (LsTPS5), and isoelectric points (pI) ranged from 5.49 (LsTPS1) to 8.46 (LsTPS6); most of the LsTPS genes were weakly acidic. The total number of atoms ranged from 11,228 (LsTPS6) to 177,765 (LsTPS5). A higher instability index indicates a more unstable protein; LsTPS genes range from 40.74 (LsTPS3) to 52.27 (LsTPS6). The aliphatic index was used to assess the solubility and hydrophobicity of the protein, whereas GRAVY with larger negative values denoting better hydrophilicity, and all the LsTPS genes were hydrophilic proteins (Table 1).

Evolutionary relationship, gene structure and motif analysis of LsTPS genes

To examine the homology between the LsTPS genes, we built an evolutionary tree of the LsTPS protein (Fig. 2). Gene structure analysis indicated that the number of exons was significantly higher in Class II than in Class I (Fig. 2). The structure differences between Class I and II genes indicate their evolutionary diversity. Figure 2 shows the 15 conserved motifs of LsTPS genes. Thirteen motifs were found in all LsTPS genes, except motifs 8 and 12, both of which were present in all class I genes.

Phylogenetic analyses of TPS proteins

A phylogenetic tree was generated for TPS proteins from six species: *A. thaliana*, *C. sativus*, *C. lannatus*, *L. siceraria*, *S. lycopersicum* and *O. sativa*, revealing homology and evolutionary relationships between TPS proteins in different species. We compared all full-length protein sequences from six LsTPS genes in *L. siceraria*, 11 AtTPS genes in *A. thaliana*, 11 OsTPS genes in *O. sativa*, seven CsTPS genes in *C. sativus*, seven ClTPS genes in *C. lannatus*, and 11 SlTPS genes in *S. lycopersicum*, and constructed an NJ phylogenetic tree to further explore the homology relationships of TPS proteins in the six species (Fig. 3). Previous studies have categorized TPS genes in *Arabidopsis*, rice, cucumber, and watermelon into two groups, classes I and II[54,55]. In this study, 53 TPS proteins were similarly categorized into two classes based on phylogenetic relationships, supported by high

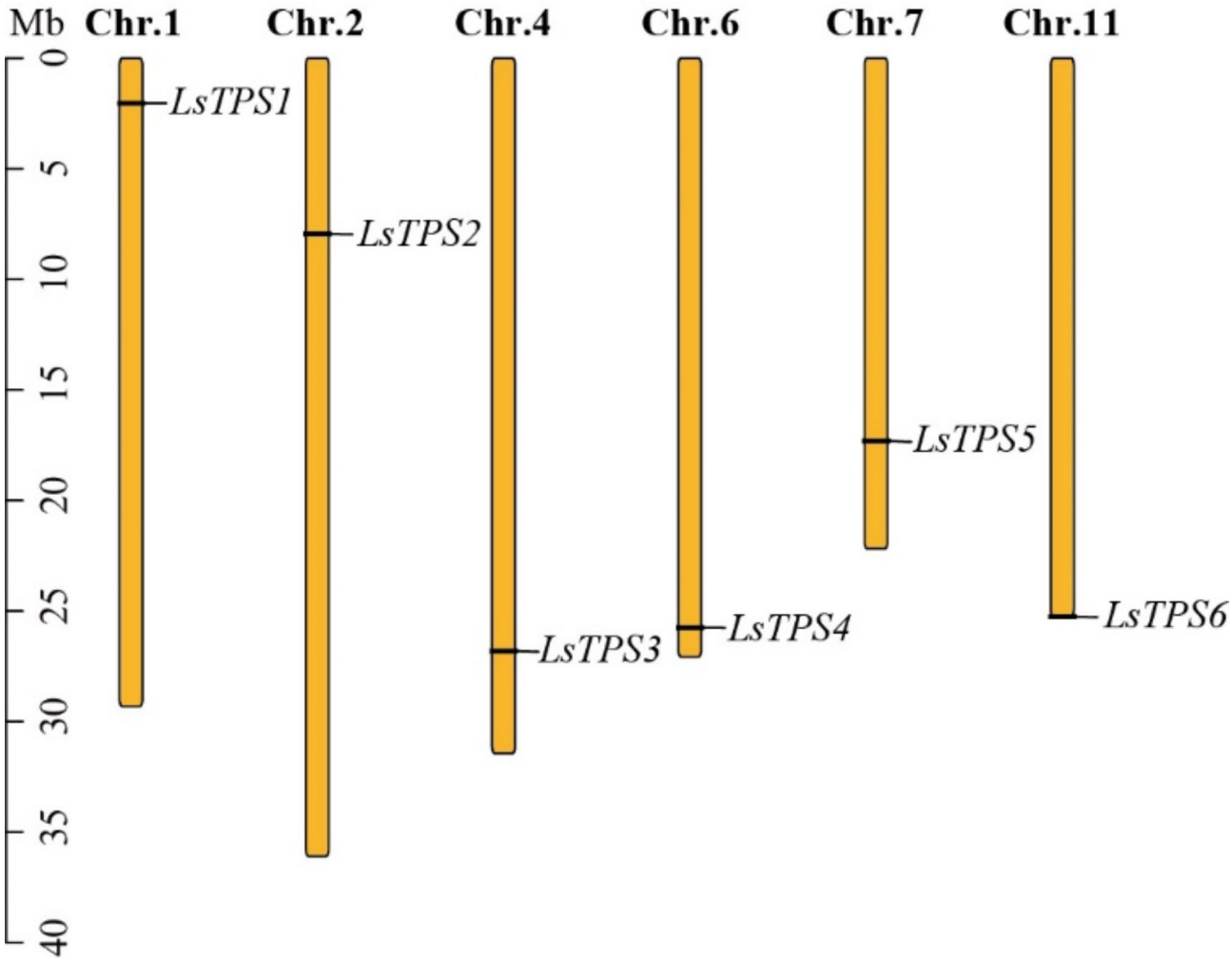

Fig. 1. Locations of LsTPS genes in bottle gourd chromosomes.

Gene ID	Gene name	Chr.	Number of amino acids	Molecular weight	Theoretical pI	Formula	Total number of atoms	Instability index	Aliphatic index	GRAVY
Lsi01G002240.1	LsTPS1	1	851	96,132.52	5.49	$C_{4279}H_{6653}N_{1165}O_{1271}S_{43}$	13,411	48.56	84.52	− 0.238
Lsi02G008480.1	LsTPS2	2	953	107,438.4	6.55	$C_{4793}H_{7520}N_{1348}O_{1405}S_{29}$	15,095	45.77	89.25	− 0.283
Lsi04G019780.1	LsTPS3	4	928	105,108.6	6.45	$C_{4674}H_{7343}N_{1317}O_{1381}S_{32}$	14,747	40.74	85.29	− 0.389
Lsi06G015400.1	LsTPS4	6	1083	122,672.6	6.68	$C_{5482}H_{8503}N_{1509}O_{1607}S_{44}$	17,145	50.29	83.8	− 0.317
Lsi07G011790.1	LsTPS5	7	1119	126,068.2	7.3	$C_{5646}H_{8897}N_{1547}O_{1624}S_{51}$	17,765	46.64	95.12	− 0.094
Lsi11G017160.1	LsTPS6	11	691	79,887.13	8.46	$C_{3607}H_{5601}N_{977}O_{1009}S_{34}$	11,228	52.27	90.26	− 0.207

Table 1. Characteristics of the identified LsTPS genes.

bootstrap values (Fig. 3). Class I contained 39 TPS members, including 7 AtTPS, 10 OsTPS, 5 CsTPS, 5 ClTPS, 10 SlTPS and 4 LsTPS genes (Table S1). Class II contained the remaining 14 TPS members. Our results showed that in each branch, there was a closer homology between bottle gourds and dicotyledonous plants compared with *Arabidopsis* and rice plants (Fig. 3). The LsTPS, CsTPS, and ClTPS genes were sorted into the same branch. These results indicate the diversity of functions in the TPS family, and suggest that greater homology exists among cucurbit species.

cis-regulatory element analyses of *LsTPS* genes in *L. siceraria*

We analyzed *cis*-regulatory elements in the LsTPS promoter to understand their transcriptional regulation. A total of 32 *cis*-regulatory elements were classified into nine responsive groups: abscisic acid (ABA)-responsive elements, auxin-responsive elements, defense and stress-responsive elements, salicylic acid (SA)-responsive

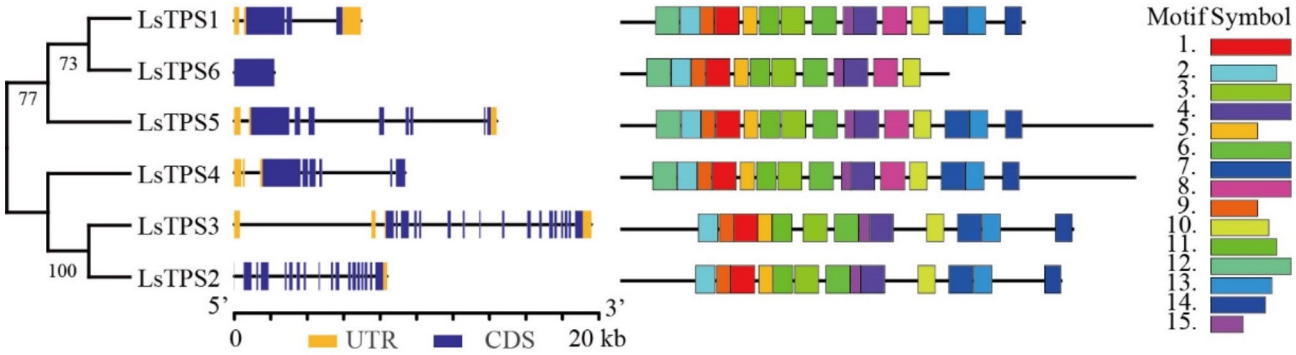

Fig. 2. Phylogenetic relationships, gene structure and motif of TPS gene family in *Lagenaria siceraria*.

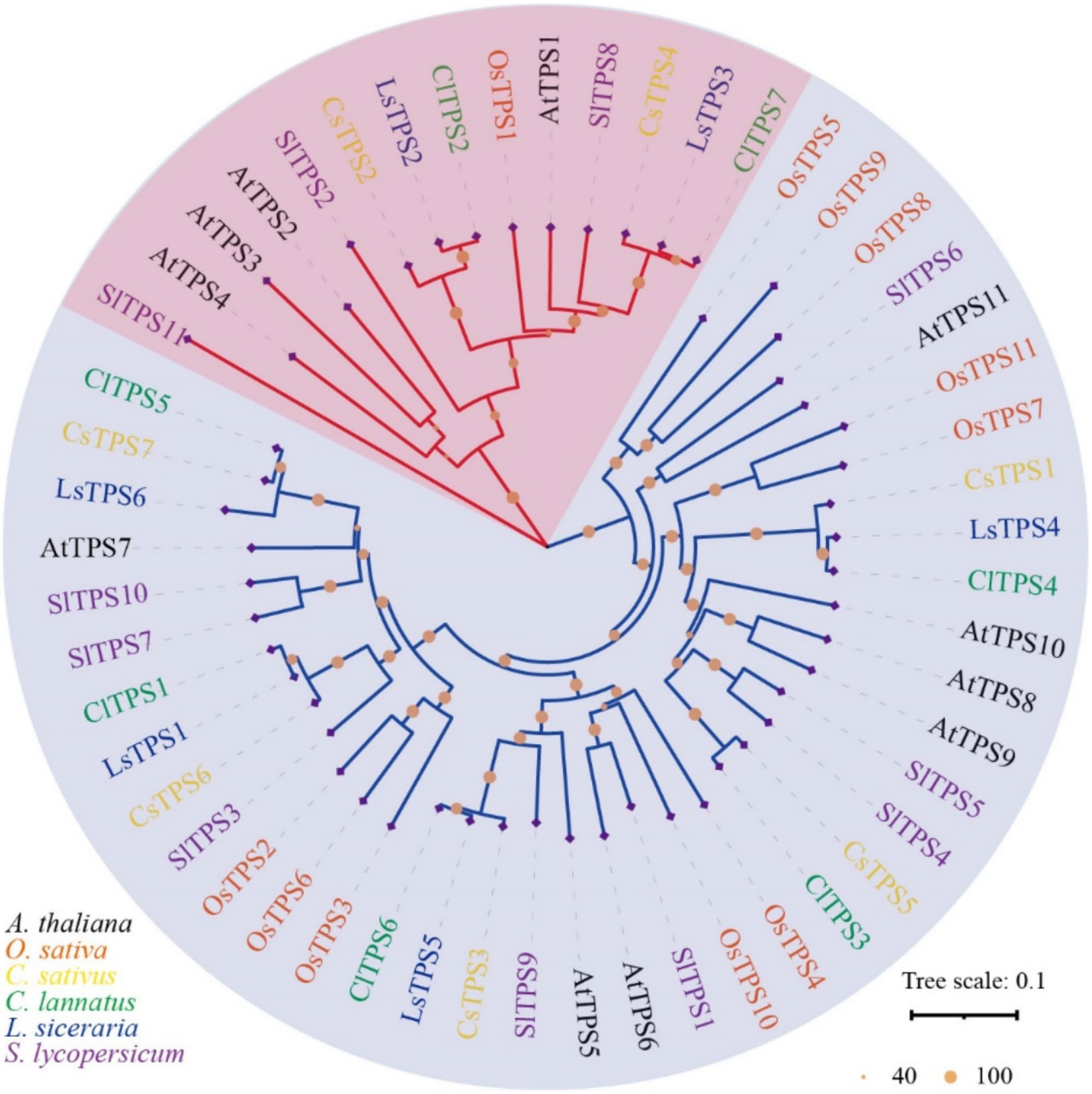

Fig. 3. Phylogenetic analysis of TPS gene family in bottle and other plants.

elements, methylated jasmonic acid (MeJA)-responsive elements, low-temperature-responsive elements, drought-responsive elements, gibberellin-responsive elements and light responsive elements (Fig. 4 and Table S4). Among them, four *cis*-regulatory elements were associated with environmental stresses, and five *cis*-regulatory elements were involved in phytohormone responses (Fig. 4). Several phytohormone response elements, including ABA-responsive elements (ABREs), SA-responsive elements (TCA-elements), and Gibberellin-responsive elements (GARE-motifs), were found in most *LsTPS* genes. Defense and stress-responsive elements (TC-rich repeats) and low-temperature-responsive elements (LTRs) were also present in most *LsTPS* genes. Drought responsive elements (MBSs) was observed in *LsTPS1* and *LsTPS4*. Nineteen light-responsive elements were identified in the *LsTPS* genes and each *LsTPS* gene contained at least eight light responsive elements (Fig. 4 and Table S4).

Three-dimensional (3-D) structure and GO annotation of LsTPS genes

To further characterize of LsTPS proteins, we analyzed the 3-D structure of LsTPS proteins. The secondary structure of TPS comprised an α-helix, a β-sheet and a transmembrane (TM-)-helix (Fig. 5). LsTPS2 and LsTPS1 had the highest percentage of α-helices (32%) and β-sheets (19%), respectively. LsTPS4 had the lowest percentage of α-helices (27%) and β-sheets (14%) (Table S5). Notably, only LsTPS5 had a TM-helix, which may be related to its biological function in the transmembrane domain (Table S5).

To gain a comprehensive understanding of gene function, *LsTPS* genes were subjected to GO term enrichment analysis. Thirty GO terms were identified, including the biological process (BP) and molecular function (MF) classes. In the BP class, the top-6 GO terms were associated with sugar synthesis and metabolism, for example 'Trehalose biosynthetic process' ($Q\text{-}value = 25 \times 10^{-16}$), 'Trehalose metabolic process' ($Q\text{-}value = 30 \times 10^{-16}$), 'Disaccharide biosynthetic process' ($Q\text{-}value = 64 \times 10^{-16}$) (Fig. 6A). Regarding the MF class, *LsTPS* genes were closely associated with enzyme activity-related terms, including 'Alpha, alpha-trehalose-phosphate synthase (UDP-forming) activity' ($Q\text{-}value = 79 \times 10^{-10}$), 'UDP-glucosyltransferase activity' ($Q\text{-}value = 72 \times 10^{-5}$) and 'Trehalose-phosphatase activity' ($Q\text{-}value = 0.0133$; Fig. 6B and Table S6).

Collinearity analyses of LsTPS genes

Gene duplication and whole-genome duplication (WGD) enhance the environmental adaptability of species[57,58]. According to the criteria for identifying gene duplication events, only one LsTPS gene pair was found in the *L. siceraria* genome: *LsTPS4* and *Lsi04G005310* (Fig. 7A and Table S7). Similar gene structure and function within each gene family may result from the amplification of ancient homologous genes or multiple independent origins of gene ancestors[59]. Therefore, a collinearity analysis of *TPS* genes was performed between *L. siceraria* and *A. thaliana*, *C. lannatus*, and *C. sativus* (Fig. 7). Five gene pairs were identified between *L. siceraria* and *A. thaliana*: *LsTPS4* and *AtTPS8/9*; *LsTPS3* and *AtTPS1/2*; and *LsTPS5* and *AtTPS5* (Fig. 7A and Table S6). Eight gene pairs were found between *L. siceraria* and *C. sativus*: *LsTPS4* and *CsTPS1/5*; *LsTPS1* and *CsTPS6/7*; *LsTPS2* and *CsTPS2/7*; *LsTPS3* and *CsTPS4*; and *LsTPS5* and *CsTPS3* (Fig. 7B and Table S7). Six gene pairs were identified between *L. siceraria* and *C. lannatus*: *LsTPS4* and *ClTPS3/4*; *LsTPS2* and *ClTPS2/5*; *LsTPS3* and *ClTPS7*; and *LsTPS5* and *ClTPS6* (Fig. 7C and Table S7). In addition, the Ka/Ks ratio in homologous *LsTPS* gene pairs was used evaluate selection pressure on *LsTPS* genes. All homologous *LsTPS* gene pairs had Ka/Ks ratios of < 1, suggesting that *LsTPS* genes underwent purifying selection (Table S8).

Expression pattern of LsTPS genes in different tissues

Transcriptional profiling in the CuGenDBv2 Genome Database was performed to analyze *LsTPS* gene expression in the roots, stems, leaves, flowers and fruits, which was used to elucidate its function and shed light on tissue-specific expression patterns (Fig. 8). Cluster analysis divided the six *LsTPS* genes into two branches, with *LsTPS1/5* having similar expression patterns in the same branch. *LsTPS* genes showed significant tissue-specific expression, and animated characterization maps showed that *LsTPS1/5* was widely expressed in all tissues, whereas *LsTPS4* was strongly expressed in the stems (Fig. 8).

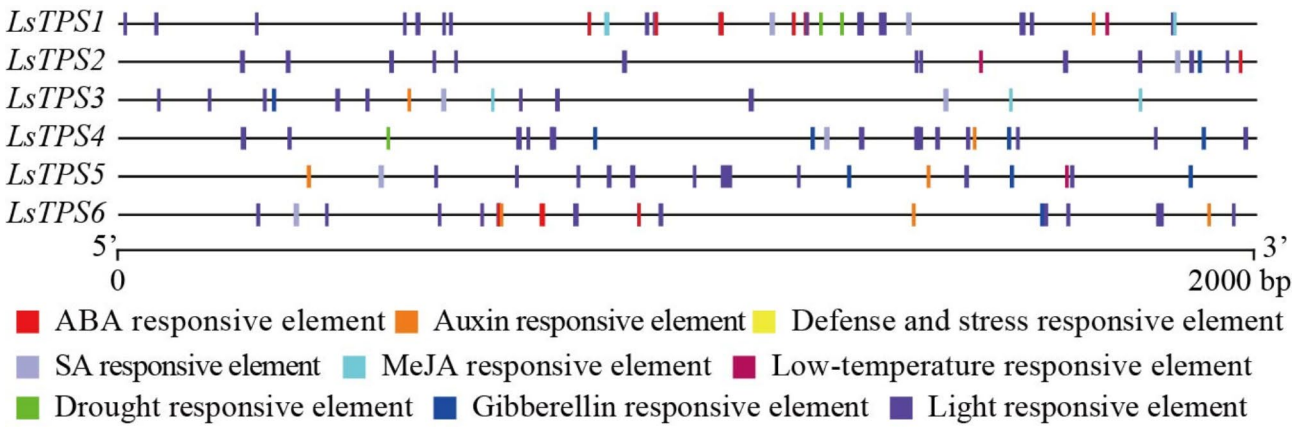

Fig. 4. Cis-regulator elements identified in LsTPS genes promoter regions.

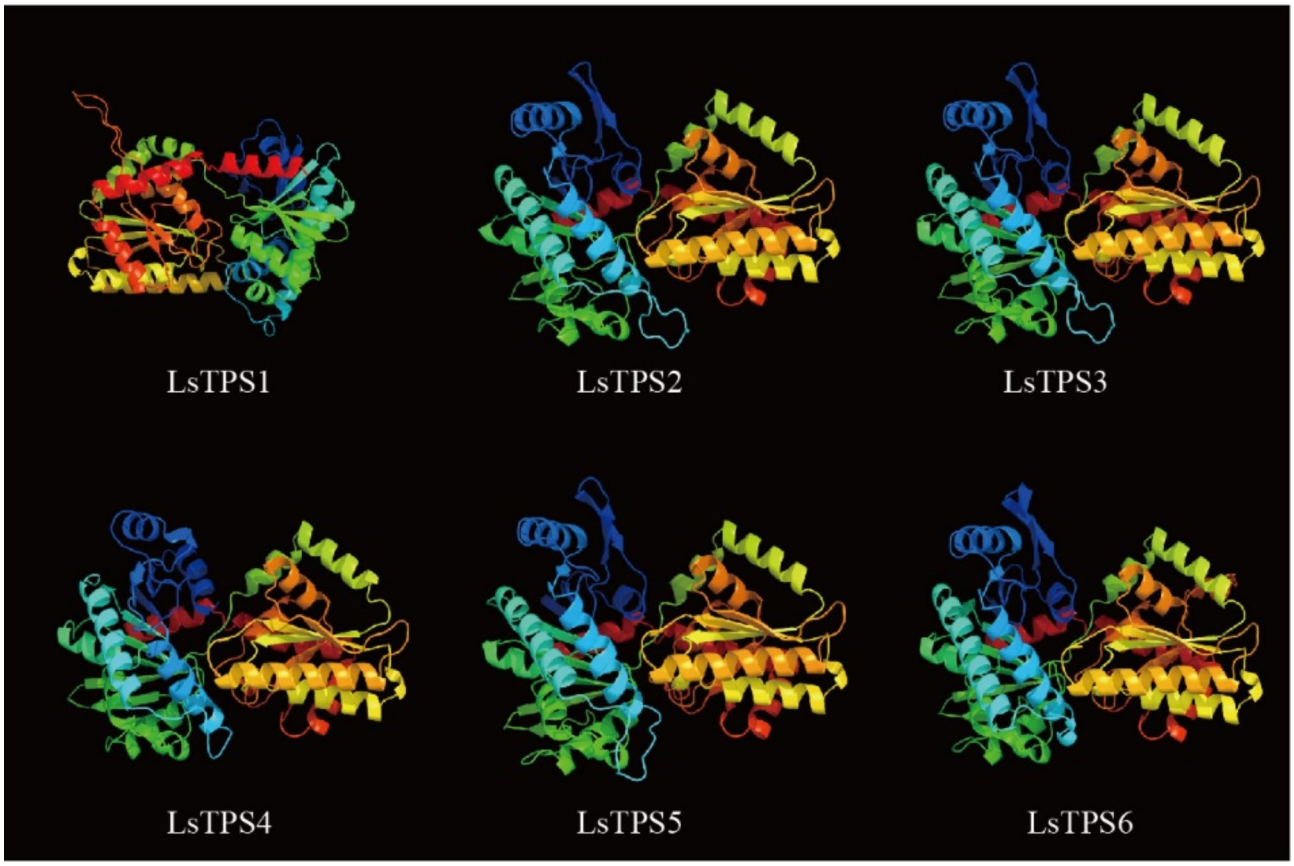

Fig. 5. Predicted 3-D structures of LsTPS proteins were constructed using Phyre2 online program.

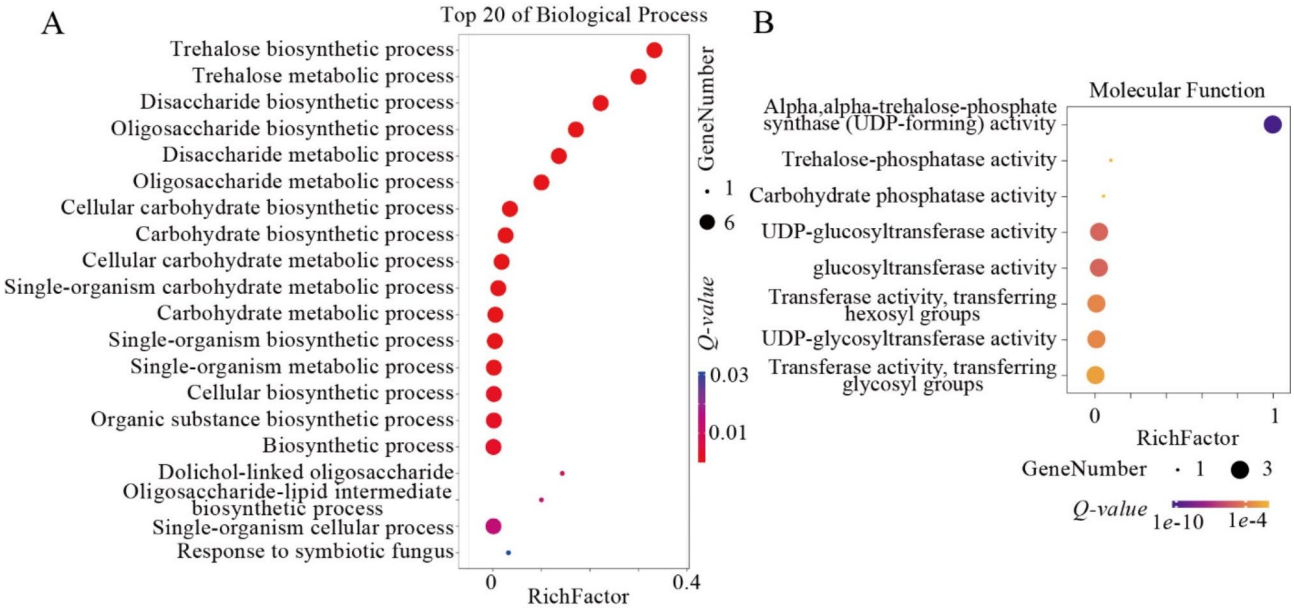

Fig. 6. Enriched GO terms for LsTPS genes in Biological Process (BP) class_R1.

Expression of stress-responsive *LsTPS* genes

To analyze the responsiveness of *LsTPS* genes to environmental stress, transcriptome data under published RNA-seq with high and low temperatures and powdery mildew infection were employed in combination with qPCR data (Fig. 9). These findings indicated that most of the *LsTPS* genes were differentially expressed in a stress-

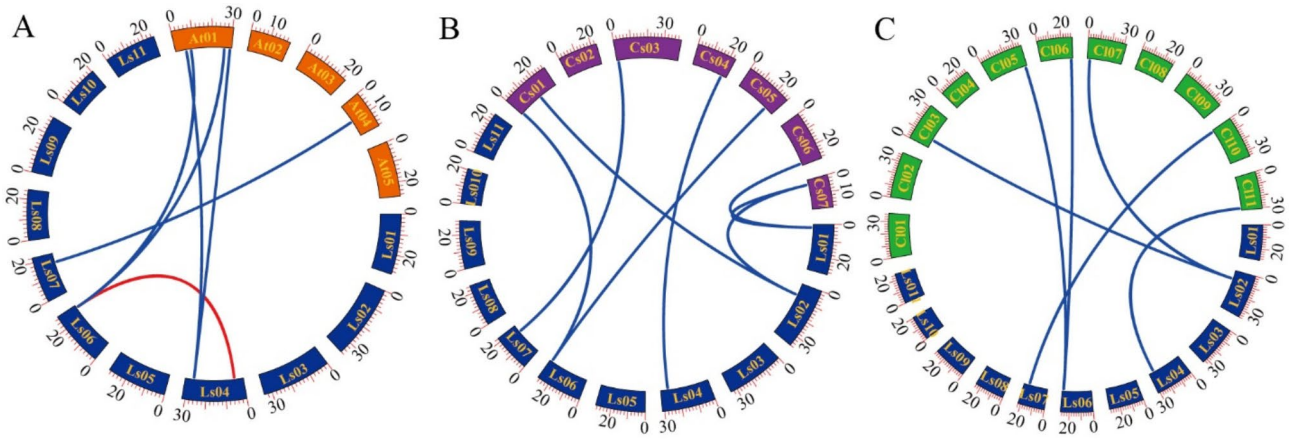

Fig. 7. Collinearity analysis of TPS genes among bottle gourd, Arabidopsis, cucumber and watermelon_R1.

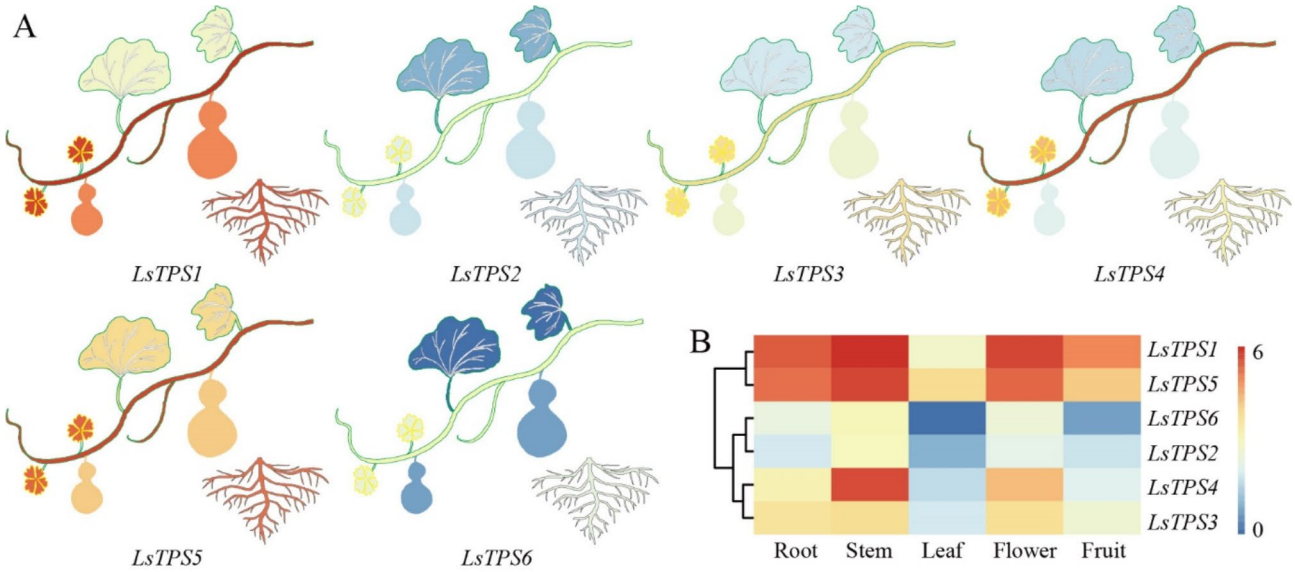

Fig. 8. Tissue-specific expression of LsTPS genes_R1.

dependent manner. Only *LsTPS1* was significantly downregulated in sensitive *L. siceraria* under heat stress, and correlation analyses revealed that *LsTPS1* was highly negatively correlated with most *heat stress genes*, *HSP genes*, *DREB* and *Ca^{2+}-related genes* (Fig. 9A and Fig. S9A). The qPCR results showed that *LsTPS1* were significantly downregulated after 1 h of heat stress (Fig. 9D). *LsTPS1* may participate in heat stress-related gene expression regulation networks. Additionally, *LsTPS1* showed a downregulation trend that corresponded with the extent of cold stress and similarly downregulated the expression of *LsTPS4/6* (Fig. 9B). Gene expression profiling showed that *LsTPS* genes responsive to chilling stress were the most abundant, and *LsTPS3/5* were significantly upregulated (Fig. 9B). The expression of *LsTPS1/4* was downregulated, whereas significant upregulation was observed for *LsTPS3/5* after cold stress (Fig. 9D). High-throughput sequencing results showed that the transcript abundance of *LTPS1/4/6* increased significantly, whereas that of *LsTPS2* decreased significantly after powdery mildew infection (Fig. 9C). Notably, *LsTPS4* expression was significantly increased in three *L. siceraria* groups (Fig. 9C). *LsTPS4* may be a key factor in the response to powdery mildew, which was supported by correlation analyses between TPS and the *R* genes utilized (Fig. S9C). During powdery mildew infection, an overall increasing trend was detected for *LsTPS1/4/6*, and the maximum expression of *LsTPS4/6* was observed 24 h after powdery mildew infection (Fig. 9C).

Discussion

Tre is a multifunctional biomolecule that participates in many metabolic processes in plants and has shown great promise in mediating plant growth and enhancing plant resistance under stress[10]. *TPS* genes are important components of Tre metabolism, and their activity is closely related to Tre metabolism under stressful conditions[9]. With genome sequencing of more species, more *TPS* gene family members has been characterized, including

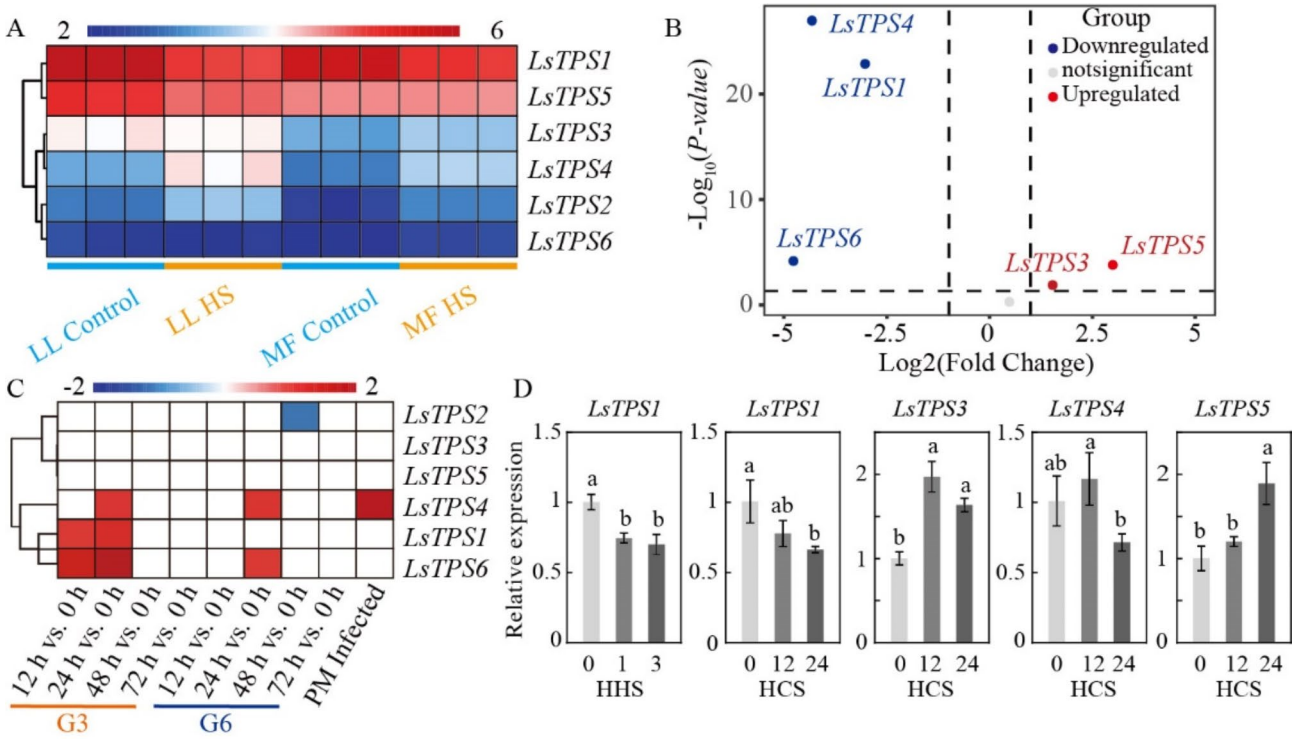

Fig. 9. Expression patterns of LsTPS genes in bottle gourd under abiotic stress and biotic stress_R1.

A. thaliana, rice, tomato, *M. truncatula*, cucumber, watermelon and melon[6,51–55]. *TPS* genes were involved in environmental stress tolerance and may enhance plant stress resistance[60,61]. Therefore, the study of *TPS* genes has attracted increasing attention, and it is important to understand the function and evolution of the *LsTPS* gene family. As genomic research continues, comparative genomic methods have being used to investigate gene families, facilitating and informing research on gene families in *L. siceraria*[5]. In the current study, six LsTPS genes were characterized and analyzed from a genome-wide perspective based on the bottle gourd genome, and the transcript levels of the *LsTPS* genes were observed in different tissues and under environmental stress. The number of *TPS* genes varied largely among species, with 53 *TPS* genes in *Gossypium raimondii*, 31 in *Brassica napus*, 11 in *Arabidopsis*, and only seven in watermelon and cucumber, respectively[51,54,55,62,63]. These results demonstrated that the *TPS* gene family is not conserved across species. In addition, All *LsTPS* proteins were hydrophilic, which is consistent with previous studies[6,63].

The comparable gene structure and function within each gene family may stem from gene amplification originating from multiple ancient paralogous homologs or gene progenitors[59]. Based on homologous evolutionary relationship analysis, the six *LsTPS* genes were categorized into two group, Class I and Class II. Plants typically have higher rates of gene duplication than other eukaryotes[64]. Previous studies identifying seven *TPS* genes in cucumber and seven in watermelon indicated that the population of *TPS* genes remained constant in Cucurbitaceae; however, duplication events still occurred. At least three, three, and five *TPS* genes in *Arabidopsis*, rice, and Trichopsis were generated by duplication events, respectively[38]. One segmental duplication event was identified in *L. siceraria*, eight segmental duplications occurred in *L. sinceraria* and *C. sativa*, and six in *L. sinceraria* and *C. lannatus*, revealing that segmental duplications in the genome are a driving factor in the expansion of gene families. In addition, gene structure and motif analyses revealed that the TPS genes on a single branch in the phylogenetic tree displayed highly similar motifs. In genetics, Ka/Ks is used as an indicator of selective pressure acting on protein-coding genes. Ka/Ks > 1 indicates strong positive selection, whereas Ka/Ks > 1 indicates purifying selection[6,65,66]. Evolutionary selection pressure analysis showed that *LsTPS* genes were subjected to purifying selection, which is consistent with findings from other species[54,55].

To analyze the evolution of *TPS* genes, the homology of TPS genes from *L. siceraria* to *A. thaliana*, *O. sativa*, *S. lyopersicum*, *C. lannatus*, and *C. sativus* was analyzed. A total of 53 TPS proteins were categorized into two different branches, with class I containing the most TPS members (39 TPS proteins), whereas class II contained a small number of TPS members (14 TPS proteins). Similar results have been reported in *M. truncatula*, *C. lannatus*, and *C. sativus*[6,54,55]. Phylogenetic differences in the TPS genes of different plants likely reflect the diversity of their functions[53]. *AtTPS5*, which can enhance *A. thaliana* resistance to *Botrytis cinerea* and *Pseudomonas syringaeby* by regulating Tre synthesis, shows close homology to *LsTPS5* and is on a large branch with *LsTPS1/6*[67].

In class II plants, *AtTPS1*, a regulator of ABA and stress signaling, shows close homology to *LsTPS3* and has been shown to promote dehydration tolerance in transgenic plants[67]. Previous studies by Li et al. revealed that *OsTPS1* enhances plant tolerance to multiple stresses by enhancing the content of Tre and proline, and is involved

in stress-related gene expression networks[16]. In addition, we observed that *L. siceraria* showed close homology to *C. lannatus* and *C. sativus* than to *A. thaliana* and *O. sativa* in each branch, suggesting functional diversification of *TPS* family among different species during plant evolution. Gene duplication drives the evolution of genes[68]. Segmental and tandem duplications are the two primary forms of gene family expansion in plants[69,70]. One LsTPS segmental duplication event was identified in the *L. siceraria* genome. This result partially supports the role of segmental duplications in the amplification of *TPS* gene family. Additionally, five and three *TPS* genes have been generated by segmental duplication events in poplar and rice, respectively[38].

The GO term enrichment analysis and *cis*-regulatory elements have been extensively applied to project the potential biological functions of genes, and to speculate on gene functions and regulatory modes. We performed GO terms analysis of *LsTPS* genes, 30 GO terms ($Q\text{-}value \leq 0.05$) were found, including 22 BPs and 8 MFs. Consistent with the function of *LsTPS* genes, which we found to be predominantly associated with Tre synthesis and metabolism of in BPs, such as 'Trehalose biosynthetic process,' 'Trehalose metabolic process,' 'Disaccharide biosynthetic process'. Consistent with previous studies, similar results were found in *Brachypodium distachyon* and *C. sativum*, where the TPS gene family played a key role in algal sugar metabolism, demonstrating functional conservation across plant species[54,71]. *Cis*-regulatory elements are closely linked to plant development and response to exogenous stress signals[72]. A total of 32 *cis*-regulatory elements belonging to nine different responsive groups were identified. None of the hormone-responsive elements or stress-related responsive elements were present in any of the *LsTPS* genes, consistent with our phylogenetic analysis that *LsTPS* genes may have functionally diversified during *L. siceraria* genome amplification.

Tre may regulate plant resistance to multiple stresses such as salt, cold, drought, and *Pseudomonas syringaeby* infection[10,41]. *TPS* genes encode regulatory enzymes involved in Tre metabolism and involved in plant stress tolerance processes[9]. Overexpression of *TPS* has been shown to improve plant resistance to stress conditions[10]. The expression profiles of *TPS* genes also revealed, their functional conservation and diversity, and the tissue expression specificity of *TPS* genes in *A. thaliana* and *O. sativa* has been revealed, suggesting that *TPS1* might play different functions in the two species[38]. In addition to plant development, *TPS* genes are responsive to multiple stressors, and have been found in both *C. sativum* and *C. lannatus*[54,55]. The transcription level of *ClTPS3* significantly increased under salt treatment, and overexpression of *ClTPS3* in *A. thaliana* improved salt stress resistance[55]. We further explored the changes in the expression of *LsTPS* genes in response to three environmental stresses, including heat stress, chilling stresss and powdery mildew infection, based on RNA-seq and qRT-PCR. Stress-dependent variation in the transcript levels of *LsTPS* genes was demonstrated; one DEG *LsTPS* was detected under heat stress, whereas five *LsTPS* genes were detected under cold stress, and three under powdery mildew infection. Notably, the overlapping responses of *LsTPS* genes to temperature stresses were found, *LsTPS1* was significantly downregulated under both high- and low temperature stresses, suggesting a possible negative regulation of temperature stress, and was also involved in powdery mildew infection. The results of this study not only offer new insights for further characterization of *TPS* gene family in plants but also provide a foundation for subsequent functional genomic investigation of *LsTPS* genes.

In this study, six *LsTPS* genes from the bottle gourd genome were characterized and analyzed for their chromosomal localization, physicochemical properties, gene structure, conserved motifs, homology relationships, *cis*-regulatory elements, GO terms and expression patterns. The expression patterns of the *LsTPS* genes in different tissues under various stress conditions were diverse and specific. Our observations provide a reference for further investigation of the mechanism underlying the role of TPS proteins in bottle gourd growth, stress tolerances and Tre metabolism pathways. However, further genetic studies are required to understand the biological functions of *LsTPS* genes.

Data availability

All data generated or analyzed during this study are included in this article. Data is provided within the manuscript or supplementary information files.

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Author contributions

H.J., and S.S.W. designed the experiments, supervised the study, and managed the projects. S.S.W. performed most of the research and drafted manuscript. S.S.W. and W.L.L. performed bioinformatics analysis and charting. H.J. analyzed and discussed the results. All authors contributed to the article and approved the submitted the version.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to H.J.

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