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Genome-Wide Analysis of the Trehalose-6-Phosphate Synthase Gene Family in Rose (*Rosa chinensis*) and Differential Expression under Heat Stress

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Abstract: Trehalose and some members of the *trehalose 6-phosphate synthase* (*TPS*) gene family play important roles in response to abiotic stress in plants. However, no studies investigating the *TPS* gene in rose have been reported. In this study, the trehalose content in the stems and roots of *Rosa chinensis* was significantly increased under heat stress, and nine *TPS* family members were identified from the genome of *R. chinensis*. The *R. chinensis TPS* (*RcTPS*) family members could be divided into two subfamilies based on the structure and phylogenetic analysis. In this study, we found that segmental duplications contributed to the expansion of the *RcTPS* gene family, and the type II subfamily gene pairs *RcTPS9–RcTPS10* and *RcTPS7a–RcTPS7b* were created by segmental duplication events. The type I subfamily *RcTPS* members contained 17 exons in the protein-coding region, whereas type II subfamily members only had 3 or 4 exons. Most *cis*-acting elements in the promoters of *RcTPS* members were related to plant hormones, especially ABA hormones. A phylogenetic tree of 78 TPS homologous amino acids from *R. chinensis* and another 7 species was constructed, which could be divided into 5 clades, and purity selection was observed to be the dominant evolutionary selection pressure. Under heat stress, except for *RcTPS1b*, the other eight *RcTPS* members were upregulated in the roots, stems, orleaves. The type II subfamily members *RcTPS7a* and *RcTPS7b* showed significantly high expression patterns in response to heat stress in all three tissues. Our findings indicate that *RcTPS7a* and *RcTPS7b* may play important roles in the heat tolerance of *R. chinensis* and are helpful for future functional studies of the two *RcTPS* members during heat stress.

Keywords: rose; *TPS* family; phylogenetic analysis; expression pattern; heat stress

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1. Introduction

Heat stress negatively affects most crops, resulting in stunted growth, abortive flowers, decreased productivity, and even death [1]. Currently known molecular mechanisms of plants' response to abiotic stress include multistage response pathways involving the stress response, signal transduction, transcription, transcription processing, translation, and post-translational protein modification [2]. As the first defense response to abiotic stress, plants accumulate a variety of compounds that act as osmoprotective agents [3]. One such compound is trehalose, a nonreducing disaccharide formed by the α-1,1-glycosidic bond between two α-glucose molecules and found in bacteria, yeast, plants, and many other organisms [4,5]. Due to its unique physical and chemical properties, such as the lack of reductive terminals related to the formation of glycosidic bonds, trehalose can stabilize enzymes, proteins, and lipids in the cell membrane under heat stress or water-limited conditions [5,6]. Many studies have shown that the trehalose metabolism pathway is an

important part of the plant sugar signaling pathway and plays an important role in plant resistance to abiotic stress [7].

At present, there are five known trehalose biosynthesis pathways, among which the trehalose-6-phosphate synthase–trehalose-6-phosphate phosphatase (TPS-TPP) pathway exists in plants. In the two-step TPS-TPP pathway, the first TPS (EC: 2.4.1.15)-catalyzed step utilizes uridine diphosphate glucose and glucose-6-phosphate to synthesize trehalose-6-phosphate (T6P), followed by TPP (EC: 3.1.3.12), which catalyzes the dephosphorylation of T6P into trehalose [3,8]. Analysis of the various mutants and transgenic plants has shown that the trehalose metabolic pathway is important for the plant sugar signaling pathway, being mainly involved in plant growth and development (embryogenesis, reproductive growth, flowering) and stress tolerance, which is mediated by transcriptional control of the *TPS* and *TPP* genes and change in the T6P level [8–11].

Trehalose biosynthetic pathways exist in all plants. The *TPS* gene family was first identified in *Arabidopsis*, which contains 11 *AtTPS* genes [7,12]. Likewise, 11 genes (*OsTPS*) were found in *Oryza sativa* [13], 12 genes (*TaTPS*) in *Triticum aestivum* [14], 8 genes (*StTPS*) in *Solanum tuberosum* [15], and 9 (*PmTPS*) in *Prunus mume* [16]. According to these research reports, it was verified that the *TPS* orthologues could respond to different abiotic stresses and play an important role in the response to stress. The *AtTPS* genes are divided into two clades: group I (*AtTPS1–AtTPS4*) and group II (*AtTPS5–AtTPS11*) [12]. The group I AtTPS members have a conserved N-terminal TPS-like domain and a less-conserved C-terminal TPP domain. Group II members, also dual-domain proteins, harbor an N-terminal TPS-like domain and a high-conserved C-terminal TPP domain [8,17]. In general, the group I AtTPS members (except AtTPS3) have been found to functionally complement yeast *tps1* mutants [18].

Transgenic plants overexpressing group I or II *TPS* genes show higher stress tolerance [7]. For example, the *AtTPS1* gene enhances osmotic, drought, dryness, and temperature stress resistance in transgenic tobacco [19]. Overexpression of *TaTPS11* in *Arabidopsis* leads to increased cold tolerance [20]. The *GhTPS11* gene in cotton responds to heat stress, drought, and salt stress, with significantly higher trehalose and T6P contents in transgenic *Arabidopsis* plants than control plants [21]. Exogenous application of trehalose was reported to improve the heat stress tolerance of wheat, and had a direct effect on the clearance of hydrogen peroxide and superoxide anion in the wheat plants [22]. In *Pleurotus tuoliensis*, exogenous application of trehalose significantly reduced the heat-stress-mediated oxidative damage of the cell membrane and showed that high-temperature stress promotes an increase in the endogenous trehalose content [23].

Roses (*Rosa* spp.) are the most popular and top-selling cut flowers in the global market. *Rosa chinensis* 'Old Blush' is a traditional famous rose species in China that participates in *R. hybrida* breeding. Moreover, *R. chinensis* is one of the high thermotolerance species [24]. For most rose varieties, the optimum temperature is 15–26 °C, and plants usually grow poorly at temperatures above 35 °C, as reproductive growth is blocked, and some species even enter a semidormancy state [24,25]. At present, the molecular mechanism of heat stress tolerance in rose plants is mostly unknown. Trehalose and its key catalytic enzyme TPS play an essential role in plants' tolerance of heat stress, such as *Arabidopsis* [26], wheat [22], and *Pleurotus tuoliensis* [23]. In this study, we first found that the trehalose content in the root and stem tissues of *R. chinensis* 'Old Blush' significantly increases during heat stress. To investigate the mechanism of the increase in trehalose in these tissues under heat stress, nine *RcTPS* gene family members were identified from the *R. chinensis* 'Old Blush' genome. Phylogenetic and selective force analysis of the *RcTPS* gene family were conducted. Moreover, the expression patterns of *RcTPS* genes were investigated in different tissues under heat stress. In this study, the basic genome bioinformatics analysis of the *RcTPS* gene family was implemented, and two *RcTPS* genes (*RcTPS7a* and *RcTPS7b*) were predicted with heat tolerance functions. This study will be helpful for analysis of the thermotolerance function and molecular mechanism of the two *RcTPS* genes in the future.

2. Materials and Methods

2.1. Plant Materials and Heat Stress Experiment

The stock plants of *R. chinensis* 'Old Blush' were cultivated in the ornamental plant germplasm center of the Hunan Agricultural University, Changsha, China. Briefly, 1-year-old healthy cuttings of the same size (the height was about 13–17cm) were planted in plastic pots (10 cm diameter) containing coconut chaff, peat, and perlite in a 4:3:3 ratio in April 2021. Water-soluble fertilizer with large amounts of elements was irrigated once a week during cuttage cultivation. About 600 cuttings eventually grew into complete plants and were used for the subsequent heat stress experiments. Each pot contained about 630 cm^3 of substrate. Firstly, the cuttings were cultured in incubators under a 12 h/12 h (day/night) photoperiod at a 25 °C/25 °C (day/night) temperature, with a 17 μmol m^{-2} s^{-1} light intensity and 60% humidity for 7 days. During this time, the plants were irrigated with neutral sterile water that contained no nutrients. Previous studies have shown that the heat stress semi-lethal temperature of most rose varieties is between 40 and 45 °C [24]. Subsequently, 30 cuttings were selected and then subjected to constant 40 °C heat stress for 72 h in the same incubators under a 12 h/12 h (day/night) photoperiod with a 17 μmoL m^{-2} s^{-1} light intensity and 60% humidity. The plants were irrigated with enough water two days before the heat treatment. Moreover, a water storage tray was placed under the plant pots, and water was added to a 1–2 cm depth to prevent water deficiency during heat stress. Mature leaves, tender stems, and lateral roots were collected from 3 biological replicates at different heat stress treatment time points (0, 1, 2, 4, 8, 12, 24, 48, and 72 h) and immediately frozen in liquid nitrogen and stored at −80 °C for subsequent trehalose measurements and reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments.

2.2. Trehalose Content Detection in R. chinensis

The rapid detection kit (Shanghai ZCIBIO Technology Co., Ltd., Shanghai, China) was used to measure the trehalose content in the samples. First, the trehalose-extracting solution was obtained from the mature leaves, tender stems, and lateral roots of *R. chinensis* according to the manufacturer's instructions, with three biological replicates. After the subsequent chromogenic reaction, a BioMate 3S Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the optical density at 620 nm and to estimate the trehalose content using the standard curve. All the collected data were finally processed using the Excel tool of Microsoft Office 2010 (Microsoft Corporation, Redmond, WA, USA), and an analysis of significant differences was conducted using SPSS Statistics 22.0 software (IBM, Armonk, NY, USA) and 1-way analysis of variance (ANOVA) and the *t*-test. GraphPad (GraphPad Software Inc., San Diego, CA, USA) was used to draw the histograms.

2.3. Identification of Rose TPS Genes

The *R. chinensis* genome DNA data were downloaded from the *R. chinensis* 'Old Blush' Hm r2.0 genome portal (RchiOBHm-V2, https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2, accessed on 27 July 2021). Combined with hidden Markov models (HMMs), BLASTN and BLASTP were used to search the genome and retrieve *TPS* family sequences. Pfam (https://pfam.xfam.org/, accessed on 22 August 2021) [27] and CDD at NCBI (NCBI, https://www.ncbi.nlm.nih.gov/cdd, accessed on 22 August 2021) were used to further verify the conserved domains. The sequences without the conserved TPS (the glycosyltransferase family 20 domain) or TPP (the trehalose phosphotase domain) domains were removed to determine the members of the *R. chinensis* TPS gene family.

2.4. Multiple Sequence Alignment and Phylogenetic Analysis

Clustal X software (http://www.cluster-x.org/, accessed on 17 August 2021) was used to compare the TPS proteins of *R. chinensis* with the AtTPS (*A. thaliana*), OsTPS (*O. sativa*), and PmTPS (*P. mume*) protein sequences. Then, MEGA X software (https://www.megasoftware.net/, accessed on 17 August 2021) was used to construct a neighbor-

joining phylogenetic tree, with bootstrap analysis (1000). The TPS members identified in *R. chinensis* were named according to their similarity to the corresponding *AtTPS* members.

Additionally, 78 protein sequences of TPS from *R. chinensis*, *A. thaliana*, and *Nelumbo nucifera*, and five *Rosaceae* species (*P. yedoensis*, *P. persica*, *P. mume*, *P. armeniaca*, and *Malus domestica*) were aligned and a phylogenetic tree was constructed using the neighbor-joining method using Mega X. The genome database and gene identifier information about all the TPS orthologues are listed in Table S1.

2.5. Selective Pressure Analysis

Using the EasyCodeML site model, the M3 and M0 models were compared using a likelihood ratio test, and synonymous (dS) and non-synonymous (dN) substitution rates, and the dN/dS(ω) ratio for each node TPS were calculated to explore the selection force of each phylogenetic group of the 78 *TPS* family members, and conduct an analysis of its molecular adaptive evolution.

2.6. Protein Physicochemical Properties and Subcellular Localization Analysis

Protparam tool (http://web.Expasy.org/protparam/, accessed on 14 September 2021) was used to analyze the physicochemical properties of the RcTPS proteins. Furthermore, the PSORT (http://psort1.hgc.jp/form.html, accessed on 14 September 2021) online tool was employed to predict the location of RcTPS protein in cells.

2.7. TPS Gene Structure and Cis-Acting Element Analyses

The number and position of the exons and introns of *RcTPS* members were analyzed by GSDS (http://gsds.cbi.pku.edu.cn/, accessed on 1 December 2021) [28]. Moreover, the 2000-bp sequence upstream of the initiation codon of *TPS* genes was extracted and submitted to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 28 August 2021) for promoter *cis*-acting element analysis, and visualized using TBtools software (https://www.tbtools.com/, accessed on 28 August 2021) [29].

2.8. Protein Motif and Conserved Domain Analysis

The conservative motifs of the *RcTPS* family were analyzed by MEME (http://meme-suite.org/tools/meme, accessed on 1 December 2021), and the motif number was set to 20. The protein sequence of RcTPS was submitted to the Pfam and NCBI CD-Search tool to further analyze the conserved domains of RcTPS members and TBtools software was used for visualization.

2.9. Gene Chromosomal Location and Collinearity Analysis

We downloaded the *R. chinensis* genome annotation gff3 file to the database and used TBtools software to extract the location information and perform visual analysis of the selected genes. MCscan X software was used to analyze the gene replication events and identify the genes with segmental duplication and tandem repeats. We used Circos (http://www.circos.ca/, accessed on 24 April 2022) to plot and show the results of the collinearity analysis.

2.10. Real-Time Quantitative PCR

A plant RNA extraction kit (Accurate Biotechnology, Changsha, Hunan, China) was used to isolate RNA samples from different tissues according to the manufacturer's instructions. After RNA purity and quality testing, complementary DNA (cDNA) was generated using the Evo *M-MLV* RT Premix (Accurate Biotechnology, China). The SYBR Green method and a 20 µL assay volume were used for RT-qPCR analysis of the *RcTPS* genes. The primers used are shown in Table S2. The expression level of the *RcTPS* genes was calculated using the $2^{-\Delta\Delta CT}$ method, and *RcCTP* was used as the reference gene [30].

3. Results

3.1. Dynamics of the Trehalose Levels in the Leaf, Stem, and Root Tissues of R. chinensis under Heat Stress

To investigate the dynamic changes in the trehalose content in *R. chinensis* during heat stress, the trehalose content in the roots, stems, and leaves of *R. chinensis* was detected. The results indicate that the basal trehalose levels varied in the roots, stems, and leaves under normal conditions, ranging from 14.81 to 33.54 mg/g fresh weight (FW) (Figure 1), with the highest levels detected in the leaves, followed by the stems and leaves. After heat stress treatment, the trehalose content in the roots and stems increased significantly during the early stage. At 8 h of heat stress, the trehalose content in the roots reached its peak (up to 1.5-fold) compared to control plants and showed a rapid decline after the 24 h time point (Figure 1A). The trehalose content in the stem increased by up to 1.4-fold at 24 h of heat stress and then returned to close to normal levels (Figure 1B). The relatively higher trehalose content in the leaves did not change much during the early stage of heat stress but rapidly decreased after the 24 h time point (Figure 1C).

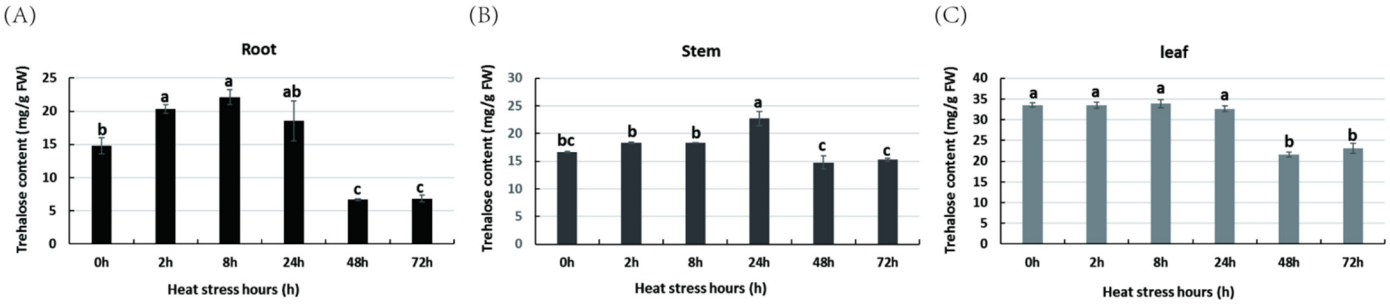

Figure 1. The trehalose content in the roots (**A**), stems (**B**), and leaves (**C**) of *Rose chinensis* in response to heat stress. Error bars represent the SD of three biological replicates. The lowercase letter represents the significance level.

3.2. Identification and Analysis of the Characteristics of TPS Family Members in R. chinensis

A total of nine *TPS* family members were identified from the *R. chinensis* genome, and named *RcTPS1a* to *RcTPS11*. The physicochemical characteristics of all nine *RcTPS* members are listed in Table 1. The number of amino acids encoded ranged from as low as 854 (RcTPS6, RcTPS7b) to 977 (RcTPS1a). The isoelectric points (pI) ranged from 5.67 to 6.51, showing weak acidity. The molecular weight (MW) ranged from 96.32 to 109.54 kDa, with an average of 100.19 kDa. The RcTPS proteins were hydrophilic (hydrophobicity < 0) and most of them were predicted in the cytosol, except RcTPS1a, RcTPS1b, and RcTPS6, which were located in the nucleus.

Table 1. Characteristics of the *TPS* gene family in *R. chinensis*.

Gene	Scaffold ID	Predicted Position	CDS (bp)	Deduced Polypeptide				Predicted Subcellular Localization
				Length (aa)	MW (kDa)	pI	GRAVY	
RcTPS1a	Scaffold_5	8460464–8470382	2934	977	109.54	6.03	−0.290	Nuclear
RcTPS1b	Scaffold_5	23007334–23020393	2793	930	105.29	6.23	−0.391	Nuclear
RcTPS5	Scaffold_7	27956967–27962352	2586	861	97.30	5.71	−0.164	Cytoplasmic
RcTPS6	Scaffold_4	64110270–64114733	2565	854	96.75	5.84	−0.184	Nuclear
RcTPS7a	Scaffold_2	47116733–47121620	2664	887	101.08	6.18	−0.264	Cytoplasmic
RcTPS7b	Scaffold_5	7851849–7856449	2565	854	96.32	5.87	−0.266	Cytoplasmic
RcTPS9	Scaffold_7	12207610–12212604	2592	863	98.38	6.11	−0.224	Cytoplasmic
RcTPS10	Scaffold_4	64989897–64994372	2601	866	97.48	5.85	−0.205	Cytoplasmic
RcTPS11	Scaffold_4	24462064–24465676	2637	878	99.56	5.89	−0.203	Cytoplasmic

pI: isoelectric points, MW: molecular weight, GRAVY: Grand average of hydropathicity.

The chromosome location results showed that *RcTPS7a* was located on Chr2; *RcTPS6*, *RcTPS10*, and *RcTPS11* were distributed on Chr4; *RcTPS1a*, *RcTPS1b*, and *RcTPS7b* were distributed on Chr5; and *RcTPS5* and *RcTPS9* were distributed on Chr7 (Figure 2A). The results of the collinearity analysis showed that there were two segmental duplication events, which occurred in the gene pairs *RcTPS9–RcTPS10* and *RcTPS7a–RcTPS7b*. These results indicate that segmental duplication may be important for the expansion of the *RcTPS* gene family and promotion of the diversity of gene functions (Figure 2B).

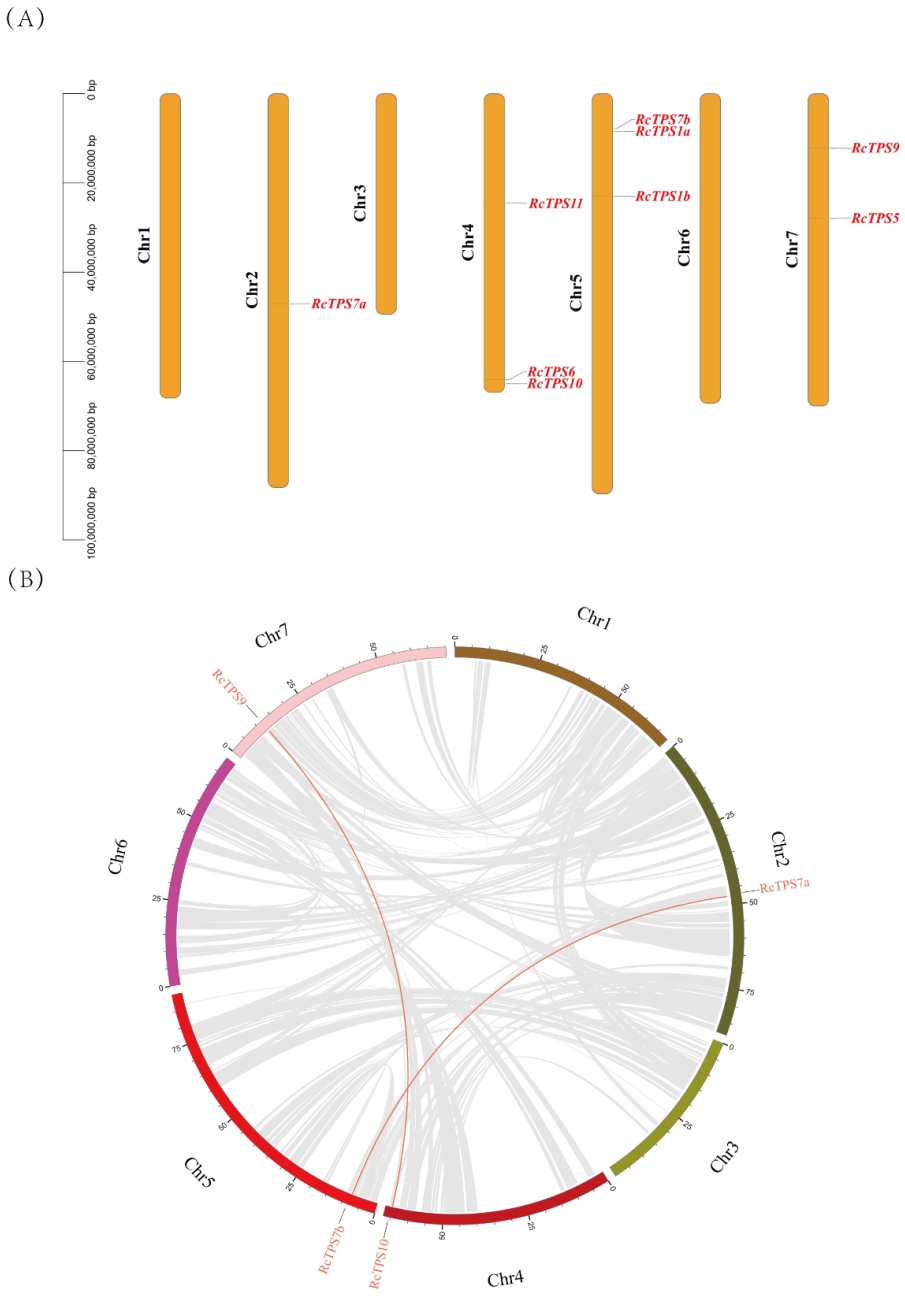

Figure 2. Chromosomal locate and collinearity analysis of the *TPS* gene family in *R. chinensis*. (**A**) The chromosomal locate diagram of the *RcTPS* gene family. The left scale represents the corresponding length of the chromosomes. (**B**) The collinearity analysis diagram of the *RcTPS* gene family. Chr1, Chr2, Chr3, Chr4, Chr5, Chr6, and Chr7 represent the seven chromosomes of *R. chinensis*, respectively.

A phylogenetic tree was constructed using a total of 40 TPS orthologs of *A. thaliana* (11 members), rice (11 members), *P. mume* (9 members), and *R. chinensis* (9 members) (Figure S1). On the basis of the phylogenetic analysis, we categorized 40 TPS members

into 2 groups: I and II TPS. *R. chinensis* TPS members were named on the basis of their similarity to the corresponding *A. thaliana* TPS members. Group II included a large number of TPS members, including 7 AtTPS (AtTPS5–AtTPS11), 10 OsTPS (OsTPS2–OsTPS11), 7 PmTPS (PmTPS5–PmTPS11), and 7 RcTPS (RcTPS5–RcTPS11) (Figure S1). The other two RcTPS members were clustered in the group I TPS subfamily and were named RcTPS1a and RcTPS1b on the basis of their highest homology level of the *A. thaliana* TPS1 member (Figure S1).

3.3. Gene Structure and cis-Acting Element Analyses of RcTPS Genes

All *RcTPS* genes contained three or more exons. Generally, the gene structures of the *RcTPS* genes clustered into the same subfamily were relatively similar. For example, group II-specific *RcTPS* contained only 3 or 4 exons while *RcTPS1a* and *RcTPS1b* of group I contained 17 exons. In addition, *RcTPS1a*, from group I, had the longest gene length while *RcTPS7a* from group II had the shortest gene length and lacked untranslated regions (UTRs) at both the 5′ and 3′ ends. The diversity of the *RcTPS* family gene structures implied a diversity regarding the evolutionary trends and gene function (Figure 3A).

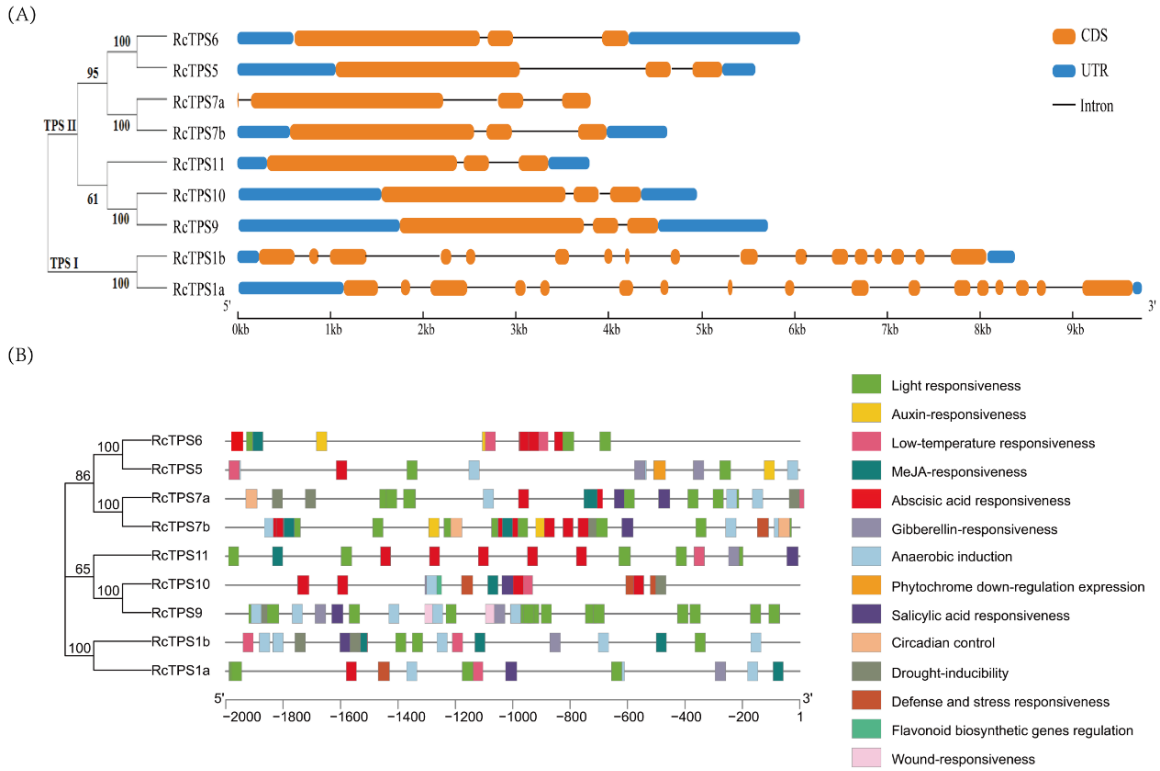

Figure 3. *RcTPS* genes' intron-exon organization and *cis*-acting elements. (**A**) Schematic diagram of the intron-exon of the *RcTPS* genes. (**B**) Schematic diagram of the *cis*-acting elements of the *RcTPS* genes.

To further understand the regulatory functions of the promoters of the *RcTPS* gene family at the transcriptional level and the functional differences between promoters of different members, analysis of the *cis*-acting elements in the PlantCARE database was carried out, which most motifs were identified responsive to environmental stress and phytohormones (Figure 3B).

Most of these *cis*-acting elements in the promoters of the *RcTPS* family were related to the light response and the response to phytohormones, especially abscisic acid (ABA). For example, G-box, Box 4, and MYB-recognition element (MRE) are related to light responsiveness while ABA-responsive element (ABRE), antioxidant response element (ARE), ethylene response element (ERE), gibberellic acid response element (GARE) motif,

and TGA-box are related to the response to phytohormones (e.g., ABA, ethylene, gibberellin, auxin, and jasmonic acid). The promoter regions of the *RcTPS* genes also contained various *cis*-acting elements related to defense and stress responsiveness, drought inducibility, and low-temperature responsiveness. Within the promoter region, three *RcTPS* genes contained defense and stress-responsiveness elements, five *RcTPS* genes contained drought-inducibility elements, and eight *RcTPS* genes contained low-temperature-responsiveness elements. Thus, various abiotic stresses could induce the expression of *RcTPS* genes, which might, in turn, induce a homeostatic response to maintain normal growth and development under abiotic stress. The promoter regions of some *RcTPS* genes also contained binding sites of MYB transcription factors under abiotic stress, suggesting that *RcTPS* genes may be interacting with MYB transcription factors to resist abiotic stress by changing the degree of cell wall lignification.

3.4. Protein Domain Analyses and Multiple Sequence Alignment of the TPS Family in R. chinensis

Conserved domain analysis revealed that the RcTPS family proteins consisted of two conserved domains: a typical N-terminal TPS (Glyco_transf_20) domain and a C-terminal TPP (Trehalose_PPase) domain (Figure 4A).

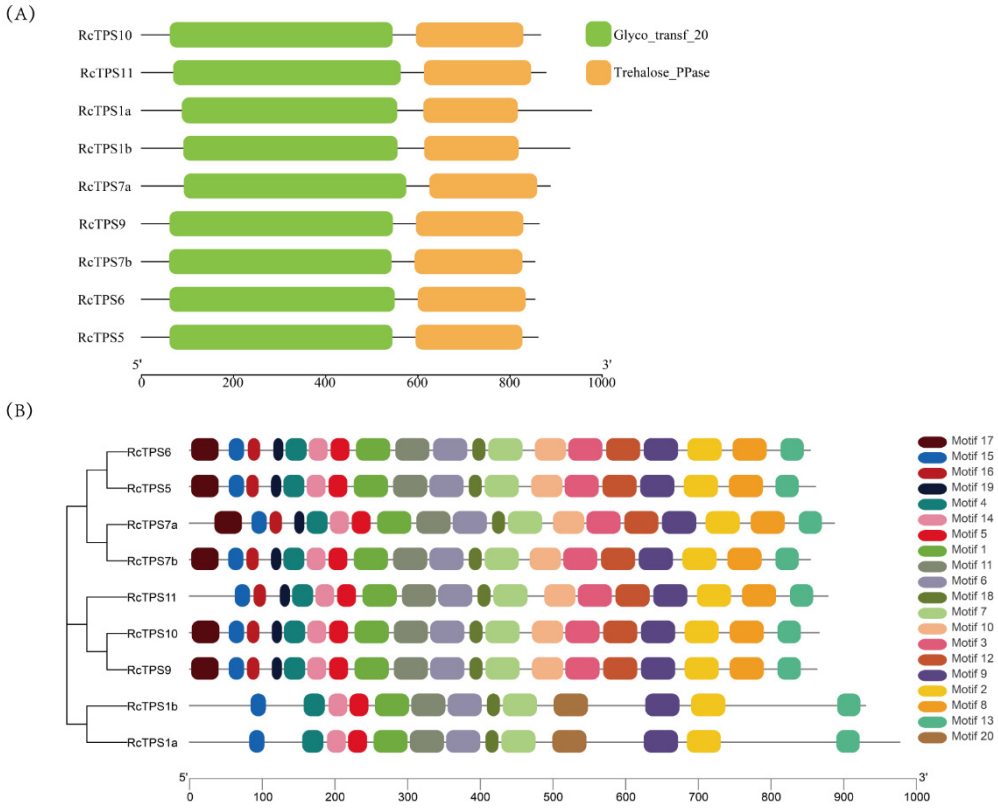

Figure 4. Schematic diagram of the conserved domains and motifs of RcTPS proteins. (**A**) The protein conserved domains, where green boxes represent Glyco_transf_20 (TPS) and orange boxes represent Trehalose_PPase (TPP) domains, respectively. (**B**) Motif analysis of RcTPS proteins in *R. chinensis*.

To further elucidate the structural features of the *RcTPS* gene family, the conserved motifs of its members were analyzed. The results indicate that a total of 12 motifs, including motifs 1–2, 4–7, 9, 11, 13–15, and 18, were highly conserved and were present in 9 members of the RcTPS protein family (Figure 4B). All motifs of the RcTPS proteins were between 17 and 50 amino acid residues in length, and the number of motifs was between 13 and 19. There were similarities in the motifs among members of the same group, especially in adjacent gene pairs, including RcTPS5/RcTPS6, RcTPS7a/RcTPS7b, RcTPS9/RcTPS10, and RcTPS1a/RcTPS1b.

In addition, the similarity of the conserved motifs among members of the same group indicated that their functions might be similar. For example, all TPS I subfamily members had 13 conserved motifs, and all TPS II subfamily members had 19 conserved motifs, except *RcTPS11*, which had 18 conserved motifs. Compared with the TPS II subfamily members, RcTPS1a and RcTPS1b in the TPS I subfamily added motif 20 but lacked motifs 3, 8, 10, 12, 16, 17, and 19, indicating that these 2 subfamilies have experienced a divergence in gene function (Figure 4B).

Multiple sequence alignment showed that the similarity of the 9 RcTPS proteins was 59.14%, with the highest identity between RcTPS1a and RcTPS1b (77.96%) and the lowest identity between RcTPS1b and RcTPS7a (31.96%). The pairwise amino acid sequence identities ranged from 31.96% to 34.87% between group I (RcTPS1a, RcTPS1b) and group II (RcTPS5–RcTPS10) and varied from 77.10% to 57.19% within group II members. The average identities of the amino acid sequences of the TPS and TPP domains were 58.02% and 54.67%, respectively. In addition, the alignment results showed that several amino acid residues in the catalytic enzyme activity domains (TPS and TPP domain) are highly conserved (Figure S2), indicating that RcTPS proteins have corresponding enzyme activity. At the same time, amino acid regions outside the TPS and TPP domains also showed high diversity. These different regions may contribute to the functional diversity of the TPS family proteins in *R. chinensis*.

3.5. Evolution Analysis of RcTPS Genes

Pairwise comparisons were analyzed among the 78 full-length TPS protein sequences from *R. chinensis*, *A. thaliana*, and *N. nucifera*, and 5 *Rosaceae* plants (*P. yedoensis*, *P. persica*, *P. mume*, *P. armeniaca*, and *Malus domestica*). The proteins shared 20.18–99.88% identities while the average pairwise sequence identities were 33.97%, 43.12%, and 37.69% in the full, group I, and group II sequences, respectively.

A phylogenetic tree of 78 *TPS* orthologues was constructed to characterize their evolutionary relationships in 8 species (Figure 5). In addition to *A. thaliana* and *M. domestica*, the *TPS* gene family of the other six species all shared nine members. The *A. thaliana TPS* family contained 11 members, and the *MdTPS* family contained 13 members. All the TPS homologous protein sequences were named and clustered into two main subfamilies: group I TPS (17 proteins) and group II TPS (61 proteins) (Figure 5). To analyze orthologous relations in each subfamily, the 61 group II TPS members were further classified into 4 clusters (II1, II2, II3, and II4) with high bootstrap support (Figures S3 and 5). Groups II1, II2, and II3 all contained at least one TPS orthologue of the eight species, while no NnTPS orthologues belonged to group I and no MdTPS orthologues belonged to group II4. The 17 group I TPS orthologues could be further assigned to 2 clades: the 3 *A. thaliana* group I TPS orthologues (AtTPS2, AtTPS3, and AtTPS4) were classified into subclade I, and the 14 other group I TPS1 orthologues were classified into subclade II (Figure 5). The quantity variance of group I TPS orthologues may suggest that subclade II TPS genes were lost from the other seven species genomes, except for *A. thaliana*.

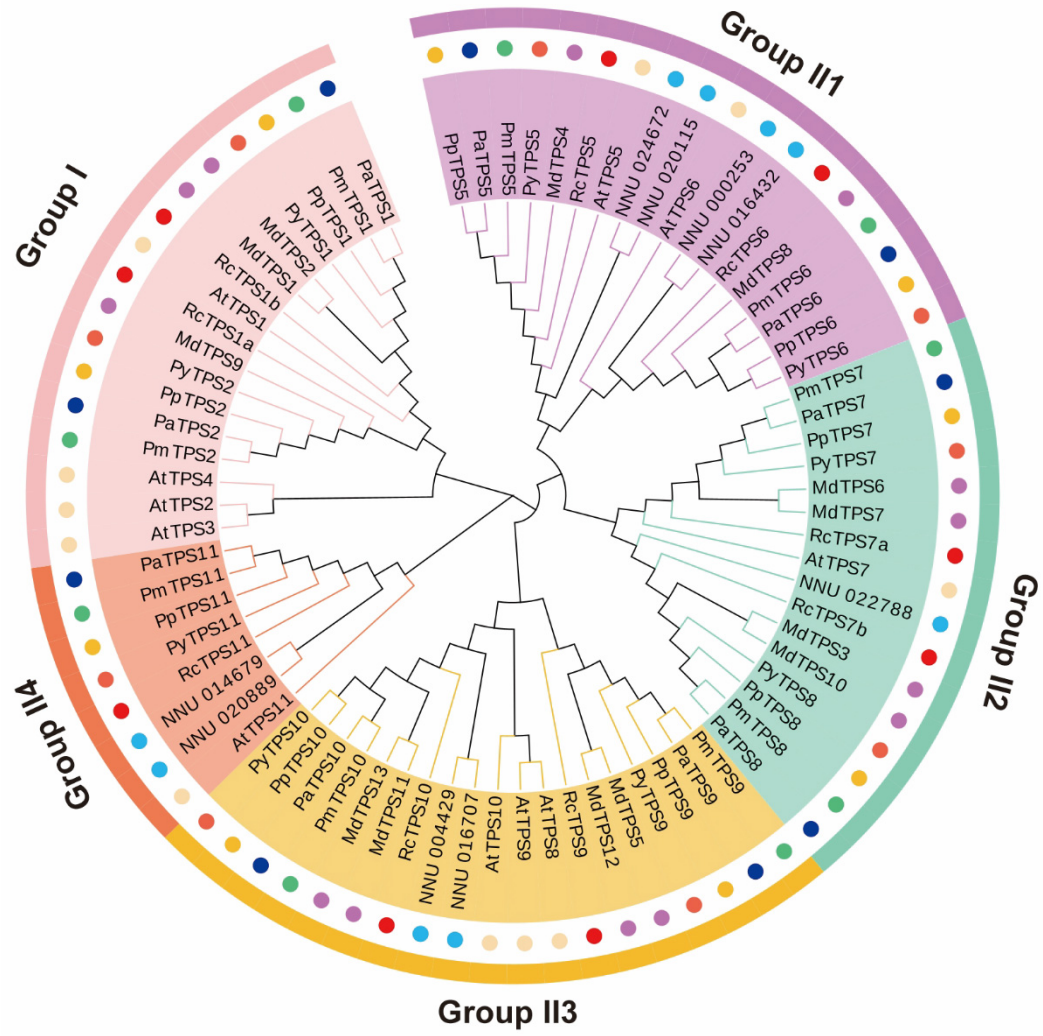

Figure 5. The phylogenetic tree of TPS family members from *R. chinensis* (RcTPS), *Prunus persica* (PpTPS), *Prunus yedoensis* (PyTPS), *Prunus armeniaca* (PaTPS), *Prunus mume* (PmTPS), *Malus domestica* (MdTPS), *Nelumbo nucifera* (NnTPS), and *Arabidopsis thaliana* (AtTPS). The light pink, violet, olive green, light yellow, and dark pink colored regions represent groups I, II1, II2, II3, and II4, respectively. TPSs from 8 plants are labeled by circles of red, orange, tangerine, mazarine, green, purple, baby blue, and light yellow colors, respectively.

The evolutionary relationship among the 78 *TPS* gene family members indicated that the TPS orthologues in the same cluster may have originated from a common ancestor and may have a similar function. Notably, most *RcTPS* genes were clustered with the TPSs of *M. domestica*, indicating that the *RcTPS* family is more closely related to the *MdTPS* family.

The results of the selection pressure analysis of the eight *TPS* family members in the eight plants is shown in Table 2. In the M0 model, the estimated ω values of the five clusters were <1, indicating that purity selection is the main selection mode acting on the *TPS* family. However, there were significant differences between the M3 and M0 chi-square tests in the *TPS* family, indicating that some specific sites were still affected by positive selection forces. Therefore, we compared model M8 and model M7 to test whether some positive selection of specific codon substitutions contributes to an effect on *TPS* family gene divergences. By comparison and estimation, 11, 2, 13, 5, and 25 codon-positive selection sites were found in group I, group II1, group II2, group II3, and group II4, respectively, but only 2 positive selection sites were found at the 95% cut-off points in group II1 and group II3. Therefore, purity selection is the main selection mode acting on the *TPS* family

members of these eight plants, indicating that most *TPS* family members in the same group were relatively conserved during evolution.

Table 2. Selection force analysis of the *TPS* family under the site models.

Group	N [a]	dN/dS(ω) under M0	2Δ/M3 vs. M0 [b]	2Δ/M8 vs. M7	M8 Estimates	Selective Position
I	17	0.09691	295.922 **	44.089	p1 = 0.02814 ω = 2.49539 (p = 0.59741 q = 5.21453)	137L, 255S, 388D, 401I, 435P, 503E, 562D *, 632N, 637S, 639Q, 679S
II1	18	0.05352	475.124 **	17.556	p1 = 0.01043 ω = 7.57672 (p = 0.26744 q = 3.43511)	300K, 426A
II2	16	0.08810	339.810 **	25.457	p1 = 0.03229 ω = 3.83594 (p = 0.36190 q = 3.30447)	3L, 54S, 166S, 228R, 409L, 476A, 482R, 506A, 552Q, 682L, 685I, 691S, 716N
II3	19	0.11694	712.521 **	34.111	p1 = 0.02958 ω = 9.36648 (p = 0.44335 q = 2.88651)	25A, 44Y, 622T, 771I, 772M *
II4	8	0.09783	318.212 **	28.595	p1 = 0.05407 ω = 2.40780 (p = 0.44632 q = 4.10918)	20N, 39V, 40P, 46S, 65A, 88K, 113S, 128M, 307E, 315Q, 458D, 459R, 504A, 542R, 630L, 656T, 752Q, 780S, 781S, 786V, 825S, 826T, 828P, 829K, 830L

[a] The number of sequences in the group. [b] Significance test was conducted using the chi-square test, in which double asterisks (**) represent $p < 0.01$ and one asterisk (*) represents $p < 0.05$.

3.6. Expression Pattern Analysis of RcTPS Genes under Heat Stress

To investigate the functions of *RcTPS* genes in heat tolerance, the expression patterns of the nine *RcTPS* genes in the roots, stems, and leaves were detected. The results suggest that the expression profiles of the nine *RcTPS* genes differed, except for *RcTPS1b*, while the other members of the *RcTPS* family were upregulated in at least one tissue under heat stress.

RcTPS1a showed significantly increased expression after heat treatment, which peaked at 1 and 2 h in root and leaf tissues, respectively (Figure 6A,C). However, *RcTPS1a* was not upregulated in stem tissues under heat stress (Figure 6B). In addition, the other group I *TPS* family member *RcTPS1b* was not upregulated in all the roots, stems, and leaves under heat stress (Figure 6).

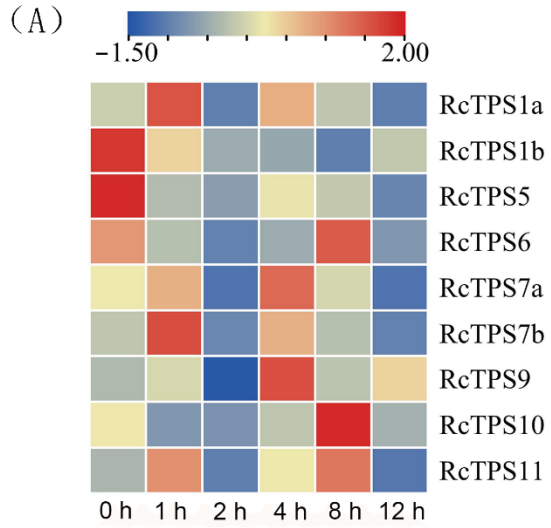

Figure 6. *Cont.*

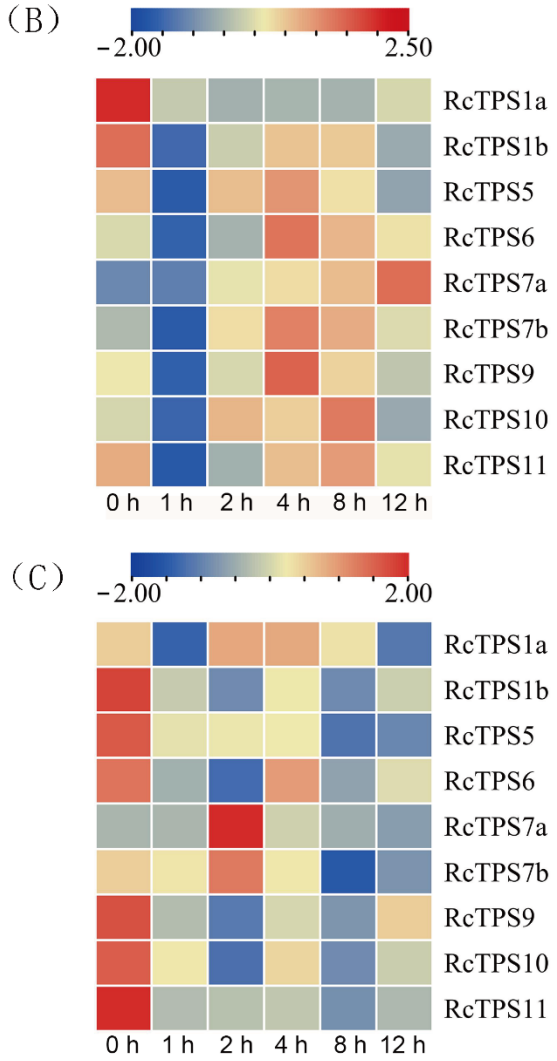

Figure 6. Expression patterns of *RcTPS* genes in *R. chinensis* roots, stems, and leaves under heat stress. (**A**–**C**) represent roots, stems, and leaves, respectively. The number under the heat map represents the heat treatment time for 0 (control), 1, 2, 4, 8, and 12 h. The color scale represents fold changes (heat treatment/control) normalized log2 transformed data.

Regarding the group II *TPS* family members, except for *RcTPS5*, the expression of the other six *RcTPS* members in the root tissue increased at different time points after heat stress (Figure 6A). However, in the stem tissue, all seven group II *TPS* members were upregulated at different time points after heat stress (Figure 6B). In the leaf tissue, only *RcTPS7a* and *RcTPS7b* tended to show significantly increased expression at 2 h after heat stress (Figure 6C). In summary, most *RcTPS* members showed upregulated expression in the roots and stems, and *RcTPS7a* and *RcTPS7b* especially showed significantly increased expression after heat treatment in all root, stem, and leaf tissues.

4. Discussion

Trehalose is a nonreducing sugar that is used as a carbon source and a pressure protector in various organisms. This compound can withstand heating at 100 °C for 24 h and protects protein and membrane invariance [3,31]. Some drought-resistant plants, such as *Selaginella lepidophylla*, accumulate large amounts of trehalose under drought conditions and can maintain metabolic dormancy for several years until the environmental humidity increases [4].

In this study, the trehalose content in the root and stem tissues of *R. chinensis* significantly increased by about 1.4-fold compared to control plants after 8 and 24 h, respectively, of heat stress. The trehalose content in the leaf tissue showed little change before 24 h of heat stress but rapidly decreased after 24 h. In this study, the trehalose content in the leaf tissue of *R. chinensis* was the highest compared to the root and stem tissues. In general, the results of this experiment show that the trehalose content in *R. chinensis* increased after heat stress, but the increased amount was not very large, and slowly decreased after 24 h. These results are consistent with those of other species. According to some studies, trehalose is present in low concentrations in most cultivated plants [3]. Trehalose contents are slightly increased in wheat, cotton, cassava, and *P. mume* grown under water-deficit conditions [3,16,32]. In addition, due to the low content of trehalose synthesized in higher plants, current studies have confirmed that trehalose is mainly involved in abiotic stress as a signal molecule [7]. Therefore, a small increase in the trehalose content in plants after abiotic stress may activate the metabolic regulatory pathways related to abiotic stress [3,7].

Transcriptome and metabonomics analyses of plants exposed to cold and heat stress have revealed changes in the expression levels of *TPS* genes [33,34]. Heat treatment upregulates the expression of *AtTPS5* and enhances the heat tolerance of *A. thaliana* while it decreases the heat tolerance of *tps5* mutants [26]. When the mycelia of *Pleurotus tuoliensis* were exposed to heat stress, the expression of the partial *TPS* gene increased and trehalose was accumulated [35]. The yeast *TPS1-TPS2* fusion gene was transferred to *A. thaliana* and the heat tolerance of the transgenic plants was improved [36]. The *Escherichia coli* TPS/TPP fusion gene (*TPSP*), when overexpressed in *Lycopersicon esculentum*, increased the trehalose content in transgenic tomato seeds. Moreover, the germination rate of *TPSP* transgenic tomato seeds significantly increased as the expression of various heat stress-responsive genes was increased, and the heat resistance of seeds was improved [37].

In this study, under heat stress, seven of the nine *RcTPS* genes were highly expressed in the root tissue in response to heat stress, the transcription of seven *RcTPS* members increased in the stem tissue, and only three *RcTPS* members showed increased expression in the leaf tissue. This result was consistent with the increasing trend of the trehalose content in different tissues under heat stress. Among the nine *RcTPS* members, the most important group I *TPS* member, *RcTPS1a*, was highly expressed in root and leaf tissues, and the group II TPS members *RcTPS7a* and *RcTPS7b* showed high transcript accumulation in the leaf, stem, and root tissues. In addition, all group II *TPS* members were highly expressed in the stem tissue. Thus, *RcTPS7a* and *RcTPS7b* may play important roles in the molecular mechanism of the *R. chinensis* heat stress response. However, the exact functions of *RcTPS1a*, *RcTPS7a*, and *RcTPS7b* in *R. chinensis* need to be further explored.

In higher plants, the TPS enzyme is a key synthase in the trehalose synthesis pathway [12]. In recent years, the *TPS* gene families of many plants have been identified. For example, *A. thaliana* [17], *O. sativa* [13], *T. aestivum* [14], *Zea mays* [38], *N. nucifera* [39], *S. tuberosum* [15], *Saccharum officinarum* [40], *Cucumis sativus* [41], *Populus trichocarpa* [42], *M. domestica* [43], and *Gossypium arboretum* [44] have 11, 11, 12, 9, 8, 9, 7, 12, 13, and 14 *TPS* genes, respectively. *TPS* gene family members differ between different plants, including monocotyledons and dicotyledons.

Initially, researchers believed that only the protein encoded by *AtTPS1* among the 11 *TPS* genes in *Arabidopsis* has TPS enzyme catalytic activity [17]. However, studies have shown that in addition to *AtTPS3*, *AtTPS2* and *AtTPS4*, which belong to group I, also have TPS enzyme catalytic activity [18]. In group II genes, *AtTPS6* proved to be complementary to yeast *tps1*Δ mutants, possibly possessing TPS enzyme catalytic activity [45,46]. Studies have also shown that group II *TPS* genes are related to heat resistance, growth, and development in *Arabidopsis*. For example, *AtTPS5* is involved in heat resistance while *AtTPS6* can control cell morphogenesis [26]. There are 11 *OsTPS* genes in rice, of which only *OsTPS1*, a group I *TPS* gene, can encode the TPS protein with enzyme catalytic activity [27]. The group I *TPS* gene *MoTPS1* in *Moringa oleifera* also has enzymatic activity [47]. Of the 12 members of the maize *TPS* gene family, *ZmTPS1* belongs to group I and *ZmTPS2–ZmTPS12* belong

to group II. *ZmTPS1* and *ZmTPS3* have TPS enzyme catalytic activity [28]. In our study, according to the phylogenetic analysis, *RcTPS1a* and *RcTPS1b* were most closely related to *AtTPS1* in *Arabidopsis*; however, more experiments are needed to verify which *RcTPS* gene family members have TPS enzyme catalytic activity.

The ABA metabolism pathway is one of the important pathways in plant resistance to abiotic stress [48]. Abiotic stress causes changes in the osmotic pressure of cells, which accumulates ABA as a stress response [49]. Studies have shown that plants that overexpress *TPS* genes induce a high expression of stress-related genes, including genes involved in ABA synthesis signaling pathways [50,51]. In this study, the promoter sequence of *RcTPS7b* contained the largest number of ABA responsive elements, and *RcTPS7b* expression significantly improved under heat stress in the roots, stems, and leaves. These results indicate that *RcTPS7b* genes might be involved in the ABA signal transduction pathway of heat stress resistance, but more studies need to be implemented in the future.

According to the results of the collinearity analysis, the type II subfamily gene pairs *RcTPS9–RcTPS10* and *RcTPS7a–RcTPS7b* were created by segmental duplication events. The same segmental duplication events were also identified in rice and populous *TPS* family members [42]. Thus, segmental duplications may significantly contribute to the expansion and gene functional diversity of these *TPS* gene families. In this study, 78 *TPS* gene family members were identified in *R. chinensis*, *A. thaliana*, *N. nucifera*, *P. yedoensis*, *P. persica*, *P. mume*, *P. armeniaca*, and *Malus domestica*. The number of TPS family members of these species ranged from 9 to 13, indicating that they experience different gene duplication events. The 78 genes were divided into 2 main subfamilies (groups I and II). To explore homologous relationships, group II subfamilies were further classified into four subgroups: II1, II2, II3, and II4, similar to the classification in sugarcane [36] and *P. mume* [14]. To explore the division mechanism of these *TPS* family members, selection force analysis was implemented among the five clusters. The results showed that most members of the *TPS* family experienced strong purity selection, indicating that the functions of the members in the same group were mostly conservative. However, two significant positive selection sites were identified in groups I and group II3, suggesting that these members may have some specific gene functions. These two codon sites may have important functions and may be a guiding force in the evolution of *TPS* genes in the group.

5. Conclusions

Our study showed that the trehalose content in the different tissues of *R. chinensis* under heat stress was higher than that under normal conditions, which indicates that trehalose has an important effect on the heat tolerance of *R. chinensis*. We identified nine members of the *RcTPS* gene family and analyzed their gene structure, phylogenetic relationships, and selection force. Most *cis*-acting elements in the promoters of *RcTPS* members were related to plant hormones, especially ABA hormones. Segmental duplications contributed to the expansion of the *RcTPS* family, and *RcTPS9–RcTPS10* and *RcTPS7a–RcTPS7b* were created by segmental duplication events. Phylogenetic trees of 78 TPS orthologous proteins of *R. chinensis* and 7 other species were constructed, which could be divided into 5 groups. Purity selection is the main selection mode acting on the 78 *TPS* members of these 8 plants, thus these *TPS* orthologous proteins were relatively conserved during evolution. Under heat stress, eight of the nine members of *RcTPS* could respond to heat treatment in the root, stem, or leaf tissues, among which *RcTPS7a* and *RcTPS7b* showed significantly high expression levels in all three tissues. Therefore, the results show that *RcTPS* genes play an important role in the heat stress of rose. This study provides valuable information that reveals the roles of trehalose and *RcTPS* family members in the heat tolerance of *R. chinensis*, and will be helpful for heat tolerance function and molecular mechanism analysis of *RcTPS7a* and *RcTPS7b* in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8050429/s1. Table S1: The TPS protein sequences

used to reconstruct phylogenetic trees; Figure S1: Phylogenetic tree of 40 TPS protein sequences from *Arabidopsis*, *Oryza sativa*, *Prunus mume* and *Rosa chinensis*; Table S2: Specific primers of *TPS* family members and reference gene in *Rosa chinensis*; Figure S2: Multiple sequences alignment of 9 *TPS* family members in *Rosa chinensis*; Figure S3: The phylogenetic tree of TPS family members from *R. chinensis* (RcTPS), *Prunus persica* (PpTPS), *Prunus yedoensis* (PyTPS), *Prunus armeniaca* (PaTPS), *Prunus mume* (PmTPS), *Malus domestica* (MdTPS), *Nelumbo nucifera* (NnTPS) and *Arabidopsis thaliana* (AtTPS).

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