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Genome-Wide Analysis of the Trehalose-6-Phosphate Synthase (*TPS*) Gene Family and Expression Profiling of *ScTPS* Genes in Sugarcane

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Abstract: The trehalose-6-phosphate synthase (*TPS*) gene family plays important roles in conferring plant stress resistance, but a comprehensive analysis of the gene family is lacking for sugarcane (*Saccharum* spp. hybrids). The objective of this study is to document functional classification, evolutionary characterization, and expression profiling of sugarcane *TPS* gene (*ScTPS*) family. Nine putative *ScTPS* genes were identified and assigned to two distinct classes based on gene structure and phylogeny. Phylogenetic analysis showed that 31 *TPS* genes from *Arabidopsis*, rice and sugarcane could be divided into five distinct clades, suggesting that there were at least five orthologous groups in monocot and dicot plants. Evolution analysis of *TPS* genes revealed that *TPS* family members appeared to have undergone strong negative selection. The strength of the selective pressure differed in most clades, especially Class I *TPS* genes, experienced significantly stronger negative selection pressure than Class II *TPS* genes. There were also *cis*-regulatory elements related to phytohormones and abiotic stresses. Additionally, *ScTPS* genes were found to exhibit divergent expression in response to simulated drought, salinity, and ABA stresses. Since *ScTPS* genes function in sugarcane adaptation to environmental stimuli, it might be used as a molecular marker in screening sugarcane germplasm for increased stress resistance.

Keywords: comprehensive analysis; expression profiling; *Saccharum*; trehalose-6-phosphate synthase

1. Introduction

Trehalose is a non-reducible disaccharide commonly found in many plant species. It is an energy carrier and an irreplaceable hydrophilic solute that protects cellular proteins and membranes from adverse environmental stresses such as drought, high salinity, and extreme temperatures. A high level of trehalose is thought to help *Selaginella lepidophylla* (Hook. & Grev.) Spring survive under extreme drought stress [1]. However, in *Arabidopsis thaliana* (L.) Heynh. and other drought-resistant species, only trace quantities of trehalose are detected despite the presence of numerous gene families that encode enzymes involved in trehalose biosynthesis [2]. Trehalose metabolism is an important target for genetic manipulation to enhance stress tolerance in plants [3].

Trehalose biosynthesis in plants is mainly through the trehalose-6-phosphate synthase (TPS)/trehalose-6-phosphate phosphatase (TPP) pathway, which involves two enzymatic reactions. First, UDP-glucose (UDPG) and glucose 6-phosphate (Glc6P) are catalyzed by TPS to form trehalose-6-phosphate (T6P), and then TPP dephosphorylates T6P to produce trehalose.

Trehalose-6-phosphate synthase is the key enzyme for trehalose synthesis as it regulates the levels of T6P, thereby affecting developmental and metabolic processes [4]. The TPS proteins typically have two domains: TPS (Pfam: Glyco-transf-20) and TPP (Pfam: Trehalose-PPase). A plant *TPS1* gene was first discovered in *A. thaliana* [5]. As an increasing number of genomes has been sequenced, more *TPS* or *TPS*-like genes have been found in plant species, including *Selaginella lepidophylla* [6], *Nicotiana tabacum* L. [7], *Oryza sativa* L. [8], *Glycine max* (L.) Merr. [9], *Triticum aestivum* L. [10], and *Solanum tuberosum* L. [11]. A study of the *TPS* gene family in *Selaginella lepidophylla* demonstrates that *SlTPS1* gene is involved in responses to heat and salinity response by enhanced T6P biosynthesis [6]. The overexpression of the *AtTPS1* gene is associated with drought tolerance in *A. thaliana* [12] and overexpression of *OsTPS1* significantly increases the tolerance to low temperature, high salinity, and drought in rice [13].

Sugarcane (*Saccharum* spp. hybrids) is a globally important sugar and energy crop. Relatively high temperature, high humidity, and sufficient light are beneficial to its growth and development. China is the third largest sugar producing country in the world; lately, its sugarcane production area has shifted to more stressful environments in higher altitudes with a lower amount of rainfall and poorer level of soil fertility. This trend also demands a shift in sugarcane breeding emphasis towards stress-resistant varieties [14,15]. Conventional sugarcane breeding program for stress resistant varieties was mainly screening sugarcane materials with great morphological traits, good physiological and biochemical characteristics under stress conditions. For instance, Deren et al. [16] screened for flood-tolerant varieties by a continuous, five-month flood. Hemaprabha et al. [17] assessed the performance of parents for drought resistance under water deficit stress. However, these efforts did not led to commercial successes. One reason is the narrow genetic base and limited resistant gene resources of sugarcane varieties, which are mostly developed by crossing between the noble canes (*Saccharum officinarum* L.) and the canes of China (*S. sinense* Roxb.) and India (*S. barberi* L.). In general, the genes for resistance to environmental stress and disease are mainly derived from wild species of *Saccharum*, such as *S. spontaneum* L. and *S. robustum* Brandes & Jesw. ex Grassl germplasm. The utilization of wild *Saccharum* species and intergeneric hybridization have been adopted to increase hybrid vigor in sugarcane breeding program. It was reported that *Erianthus arundinaceus* germplasm was utilized to breed new drought-resistant varieties of sugarcane [18]. However, the traditional selection of resistant varieties is a long-term and risky investment [19]. With the biotechnology development and sugarcane genome sequencing, it is possible to explore more resistance genes or traits to facilitate genetic manipulation via marker-assisted selection [20,21]. Trehalose-6-phosphate (T6P) could act as an signaling molecule in plants regulating plant growth and development [22]. Manipulation of T6P levels by chemical intervention strategy has shown extraordinary effects on crop yield and drought tolerance [23]. Since it has been reported that over-expression of the *Grifola frondosa* trehalose synthase gene led to high trehalose accumulation and enhanced tolerance to drought [24], a comprehensive analysis is needed for the sugarcane *TPS* gene (*ScTPS*) family to enhance yield and stress tolerance in sugarcane.

The objective of this study is to explore functional classification, evolutionary characterization, and expression profiling of the *ScTPS* family. The study of *ScTPS* genes may help sugarcane breeders utilize *ScTPS* marker-assisted selection of stress tolerant sugarcane germplasm and sustain sugarcane production under stressful field conditions.

2. Materials and Methods

2.1. Materials

The study was conducted on a drought tolerant sugarcane genotype YZ05-51 [25]. The genome database of sugarcane cultivar SP80-3280 (txid: 193079) was referred from the National Center for Biotechnology Information (NCBI). The *TPS* nucleotides and amino acid sequences of *Oryza sativa* Indica Group (txid: 39946) and *Arabidopsis thaliana* (L.) Heynh (txid: 3702) were obtained by querying reported *TPS* gene accession [8,26] from TIGR [27] and the Arabidopsis Information Resource (TAIR), respectively.

The accession number of *Arabidopsis TPS1-11* mRNA are HM050424–HM050434, and the accession number of rice *TPS1-11* mRNA are NM_001334832, NM_101559.2, NM_101560.5, NM_118890.2, NM_001341241.1, NM_202376.3, NM_100521.3, NM_105697.4, NM_102235.2, NM_001333885.1 and NM_127426.3.

2.2. Screening and Classification of TPS Gene Family Members in Sugarcane

Using the acquired amino acid sequences of TPS proteins in *Oryza sativa* Indica Group and *A. thaliana* as templates, TBLASTN was used to blast the sugarcane cultivar SP80-3280 whole-genome shotgun contigs (WGS) database (Accession:PRJNA431722) of NCBI to find all available sequences of sugarcane *TPS* genes. For BLAST searches, protein sequences of queries were used as inputs in the BLASTp tool, and the resulting hits were filtered by E-value ($1e^{-5}$). Only the longest sequence was retained if several results were found for the same gene. The open reading frames were sought using the Open Reading Frame Finder program. The TPS and TPP domains were further validated by NCBI-CDD [28] and PFAM database [29]. Pseudogenes that overlapped less than 50% of the complete TPS and TPP domains were discarded. The gene structure of all sugarcane *TPS* gene family members was analyzed using Gene Structure Prediction [30]. BioEdit v7.0 [31] was used to analyze the homology and structural characteristics of all *TPS* gene family members and to systematically classify and name these *TPS* gene family members in reference to the rice *TPS* gene family. All sugarcane TPS protein sequences were analyzed by ExPASy to obtain their basic physical and chemical properties, such as molecular weight (MW), isoelectric point (pI), amino acid composition, instability coefficient, liposolubility index, and total average hydrophilicity. The protein subcellular localization was predicted by CELLO v.2.5 [32].

2.3. Motif Analysis of Sugarcane TPS Proteins

The motif structure of sugarcane TPS family members was analyzed by the MEME program (Version 5.1.1), and there were no gaps in the protein sequences from MEME analysis. Letters that appeared in each position constituted a position-specific probability matrix (PSPM), which could be used to judge the possible motifs in the sequence group. In parameter design, the minimum length of a motif sequence was 10, the maximum was 60, and the maximum number of conservative sequence discoveries was six.

2.4. Phylogenetic Analysis of TPS Proteins

Multiple amino acid sequence alignment of selected 31 *TPS* subfamily members from *A. thaliana*, *O. sativa*, and sugarcane was performed using ClusterW. MEGA 7.0 was used to construct a phylogenetic tree using Neighbor-Joining method and repeated 1000 times to obtain a bootstrap value [32].

2.5. Selection Assessment and Testing

The program PAML version 4.9j was used to test the selection pressure by assessing the values of nonsynonymous substitutions (dN), synonymous substitutions (dS) and dN/dS ratio (ω). [33]. The branch model 2 (two ratios) and branch-site model A was used on CodeML module in PAML [34]. The branch model 2, allowing the foreground branch to evolve under a different rate, was compared to null model (model 0: one ratio) assuming that all branches had been evolving at the same rate. Branch-site model A was used to search for positive selection in codon sites, which allowed individual site and different setups of foreground lineages to be tested depending on the gene substitutions. The likelihood ratio test (LRT) was used to evaluate the fit of the alternative model comparing with the null model. The branch with a p value less than 0.05 and a higher ω value for the foreground branch than the background branch was considered as evolving with a significantly faster rate in the foreground branch. The sites under positive selection were further identified using naive empirical Bayes (NEB) method and Bayes empirical Bayes (BEB) method [35].

2.6. Cis-Regulatory Elements Analysis of Sugarcane TPS Gene Families

A 2000-bp upstream sequence of the coding region of each sugarcane *TPS* gene family was analyzed for the promoter region. The *cis*-acting elements were analyzed using online data analysis software Plantcare.

2.7. Plant Growth and Treatments

As described previously [36], drought stress, salt stress, and ABA treatments were simulated using PEG-6000, NaCl, or ABA, respectively. Sugarcane seedlings of YZ05-51 were grown hydroponically in Hoagland solution containing 10% (w/v) PEG-6000, 200-mM NaCl, or 30-nM abscisic acid, respectively. Control groups were collected right before treatment, and the exponential groups were collected at 6, 12, 24, and 48 h after the stress treatments were imposed. Two seedlings were mixed into one sample, and three biological replicates were adopted at each condition. Samples were frozen in liquid N_2 for further analysis.

2.8. Expression Analysis of ScTPSs

Total RNA was extracted using the Plant Total RNA Rapid Extraction Kit (Tiangen Technology Co., Ltd., Beijing, China), and the RNA was reverse transcribed into cDNA using the TIAN Script II first-chain synthesis kit (Tiangen Technology Co., Ltd.). Gene-specific primer synthesis and DNA sequencing were conducted by Beijing Genome Institute (Shenzhen, China). The specific nucleotide sequences of primers for quantifying every *ScTPS* expression were used, including q-ScTPS1 (F-5′ TG TGCCAACAAGAACTGACG R-5′ GCTCACAAGGTTCATCCCATC), q-ScTPS2 (F-5′ CGAGAAGG TCGTGGAGGTA R-5′ TGGTATTCAATTCCCAGCAT), q-ScTPS3 (F-5′ TGATGTTGCCTATTGGTCT AAG R-5′ GCTGTAATTGCCACTGTTCG), q-ScTPS4 (F-5′ AAGAGGTTGCGTCAC GATAAGC R-5′ TGAAGAGTGCGGCGGTCAT), q-ScTPS5 (F-5′ TCCGAGTTCGTCGGTTGCTC R-5′ CCCTGCTCCA CCTTGTGAAGTAA), q-ScTPS6 (F-5′ GTGGTAGTGACGGCTGTGAG R-5′ GCATGGCTTCCTAAA GTGATC), q-ScTPS7 (F-5′ TATGCTCGCCACTTCCTAT R-5′ CCTTTCAGCCACAGATACAG), q-ScT PS8 (F-5′ TTCCTCCACAGCCCGTTCC R-5′ TGTACCTCCTTCACATCCTTTCC) and q-ScTPS9 (F-5′ GACGTTGAGGAAGCGAAAT R-5′ CGTGAGACCTGACATAGCG). Gene expressions were normalized against an internal reference gene *GAPDH* using primers GAPDH-F (5′ CACGCCACTG AGCA) and GAPDH-R (5′ TCCAGTTCCATGCC). Three biological samples were evaluated and all reactions were performed in triplicate for the analysis of *TPS* gene expression on every condition. Independent gene expression experiments were repeated three times. The relative expression of *ScTPS* genes was calculated using the $2^{-\Delta\Delta c(t)}$ method of Meijerink et al. [37].

2.9. Statistical Analysis

Regarding the data distribution and the homogeneity of variance, the Kruskal–Wallis H test was used to analyze the difference of protein sequence identity in the pairwise comparisons of *TPS* gene members from *A. thaliana*, *O. sativa* and sugarcane at $p < 0.05$ (SPSS 10.0, Inc., Chicago, IL, USA). The Bonferroni method was further used for multiple comparison when there was statistical difference between groups. In the experiment of reverse transcription polymerase chain reaction analysis, the difference between relative gene expressions was analyzed using one-way ANOVA test, LSD-t method was used for further comparison between two groups at $p < 0.05$ (SPSS 10.0, Inc., Chicago, IL, USA). The data of each time points were independent by the chi-square test.

3. Results

3.1. Screening TPS Genes for Basic Physic-Chemical Properties of ScTPS Coding Proteins

Nine genes of the *TPS* family were identified from the sugarcane genome database. According to the homology of TPS proteins related to *O. sativa*, the nine sugarcane *TPS* genes were named *ScTPS1* to

ScTPS9 (Table 1). A protein domain analysis showed that the nine *ScTPS* gene-coded proteins typically contained two domains: TPS (Pfam: Glyco-transf-20) and TPP (Pfam: Trehalose-PPase). The nine ScTPS proteins had as few as 818 (ScTPS5) to as many as 976 (ScTPS1) amino acids. The molecular weights ranged from 91.05 (ScTPS5) to 108.39 (ScTPS1) kD. The isoelectric points ranged from 5.19 (ScTPS2) to 6.63 (ScTPS5) (Table 1). The ScTPS proteins were hydrophilic (hydrophobicity was <0). Except for ScTPS9, the other eight ScTPS proteins were unstable (instability coefficient > 40). Seven ScTPS proteins were localized in the cytoplasm, ScTPS2 was located in the cell membrane, and ScTPS7 in the nucleus.

Table 1. Attributes of the trehalose-6-phosphate synthase (TPS) protein family in sugarcane.

Protein	Gene Accession Number	Number of Amino Acid	MW KD	TPS Domain Location	TPP Domain Location	pI	GRAVY	Instability Index	Subcellular Location
ScTPS1	MN365026	976	108.4	129–594	653–852	6.23	−0.34	50.64	Cytoplasm
ScTPS2	MT406177	909	101.8	77–577	626–860	5.19	−0.233	54.21	Cytomembrane
ScTPS3	MT406178	875	98.6	73–558	607–842	5.68	−0.325	49.24	Cytoplasm
ScTPS4	MT406179	855	96.9	56–546	595–828	5.87	−0.228	46.14	Cytoplasm
ScTPS5	MT406180	818	91.1	18–502	551–787	6.63	−0.165	54.84	Cytoplasm
ScTPS6	MT406181	885	99.4	55–552	601–835	5.45	−0.261	49.28	Cytoplasm
ScTPS7	MT406182	865	98.0	58–544	593–829	6.09	−0.241	41.63	Nucleus
ScTPS8	MT406183	878	99.0	66–560	609–843	5.87	−0.2	45.82	Cytoplasm
ScTPS9	MT406184	861	97.5	57–544	593–827	6.22	−0.191	36.98	Cytoplasm

MW: molecular weight, GRAVY: Grand average of hydropathicity.

3.2. Gene Structures and Protein Domains Analysis of ScTPSs

The nine *ScTPS* genes could be divided into two subfamilies (I and II) based on gene structures and protein homology (Figure 1). Subfamily I consisted of *ScTPS1*, with 16 introns. Subfamily II consisted of the other eight *ScTPS* genes. Except for *ScTPS8* with three introns, the other *ScTPS* genes contained just two introns. Multiple sequence alignment of nine TPS protein sequences showed that the average amino acid sequence identity among nine full-length ScTPS proteins was 53.73%, with the highest identity between ScTPS2 and ScTPS6 (79.16%), and the lowest identity between ScTPS1 and ScTPS3 (32.40%). The average identities of amino acid sequence of TPS and TPP domains were 57.3% and 56.2%, respectively, with 35.30% of the sequences outside the domains. The amino acid identities between Class I (ScTPS1) with Class II (ScTPS2-9) were ranged from 32.40% to 35.27%, while the values were varied from 51.42% to 79.16% within Class II members.

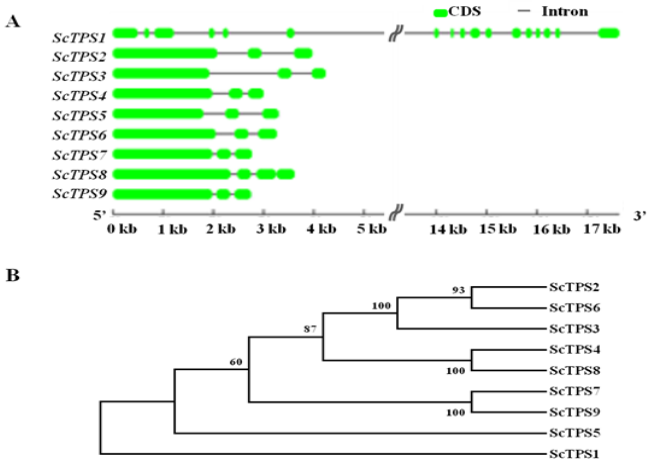

Figure 1. Gene structure of the trehalose-6-phosphate synthase (*TPS*) gene family (**A**) and phylogenetic tree (**B**) based on multiple sequence alignment of TPS protein in sugarcane. Part of nucleotide sequence in *ScTPS1* intron is omitted marked with double wave line. The bootstrap value in which the associated taxa clustered together are shown next to the branches.

Six motifs (Motifs 1 to 6) are found by motif analysis of ScTPS proteins (Figure 2). Motifs 1-5 are located in the TPS domain and Motif 6 is located in the TPP domain Figure 3. Eight TPS proteins contain all six motifs with a consistent arrangement order. However, ScTPS1 differs from the other proteins as it lacks Motif 2.

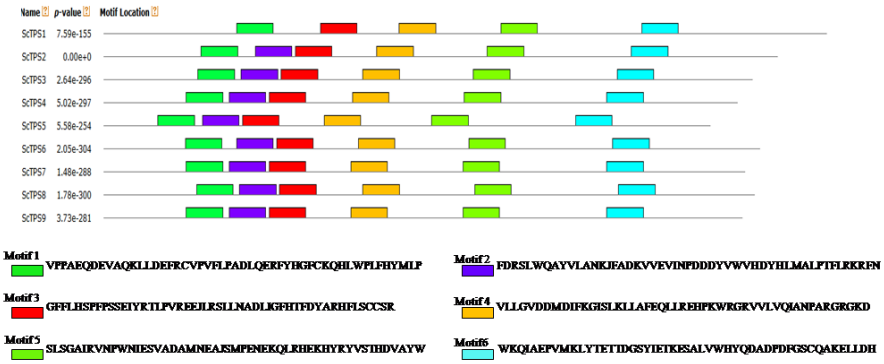

Figure 2. Motif analysis of the trehalose-6-phosphate synthasegene family in sugarcane. The different colored boxes represent different motifs and their positions in each TPS protein sequence.

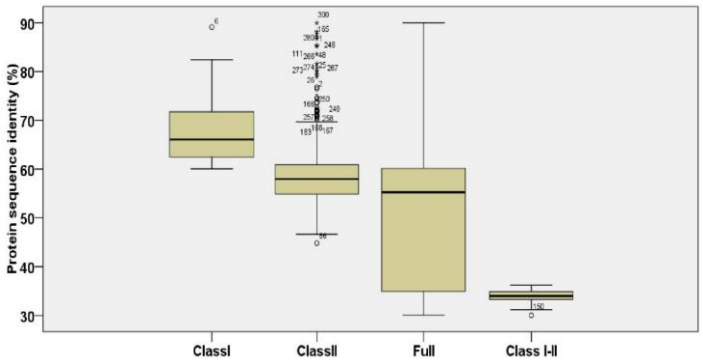

Figure 3. Pairwise protein sequence identity of ScTPS proteins. ClassI, ClassII, Full and Class I-II represent the protein sequence identities in Class I TPS proteins group, Class II TPS proteins group, full TPS proteins group and sequence identities between Class I and Class II TPS proteins. Outliers and extreme outside of the boxplot whiskers (labeled by data number) are shown as circles and asterisks, respectively.

3.3. Evolution Analysis of ScTPS Genes

Pairwise comparisons were analyzed among the 31 full-length TPS protein sequences from *Arabidopsis*, rice and sugarcane. The result revealed that the protein sequence identities were differed between different groups (Figure 3). The full-length of 31 TPS proteins share 30.03–89.99% identities, while the average pairwise sequence identities were 67.88%, 58.86% and 34.03% in class I, class II and the sequences between the two TPS classes (Class I–II), respectively. Pairwise comparisons in both Class I and Class II TPS proteins groups were significantly higher than that of Class I-II groups ($p < 0.0001$). It indicated that there was clearly an evolutionary divergence between class I and class II TPS subfamilies.

A phylogenetic tree of 31 *TPS* genes was further constructed for the characterization of their evolutionary relationships (Figure 4). These *TPS* genes were divided into two distinct subfamily with 100% bootstrap support, each contained 25 Class II and 6 Class I *TPS* genes, respectively. In order to analyze orthologous relations in each subfamily, the 25 Class II TPS genes could be further divided into four groups (A, B, C and D) with high bootstrap support. The number of *ScTPS* genes, *OsTPS* genes and *AtTPS* genes in each of groups were A (5, 5, 3), B (2, 2, 3), C (0, 0, 1), D (2, 2, 0) and E (1, 1, 4), respectively. Clades A, B and E all contained at least one *TPS* gene of the three species, while only

AtTPS gene was present in Clade C and no *AtTPS* genes belonged to Clade D. The six Class I *TPS* genes could be further assigned to two groups, the three *TPS1* genes (*AtTPS1*, *OsTPS1*, and *ScTPS1*) subclade I and three other *Arabidopsis* Class I *TPS* genes (*AtTPS2*, *AtTPS3*, and *AtTPS4*) subclade II. The quantity variance of Class I *TPS* orthologous may indicate that subclade II *TPS* genes had been lost from the rice and sugarcane genome. There was a consistent evolutionary relation between sugarcane and rice, and the branches identified in the purple line represented the most recent split between monocot and dicot plants. The nodes that divided into purple lines and black lines were designated as the monocot-dicot common ancestors. Therefore, it was presumed that there were at least five orthologous groups in monocot and dicot plants.

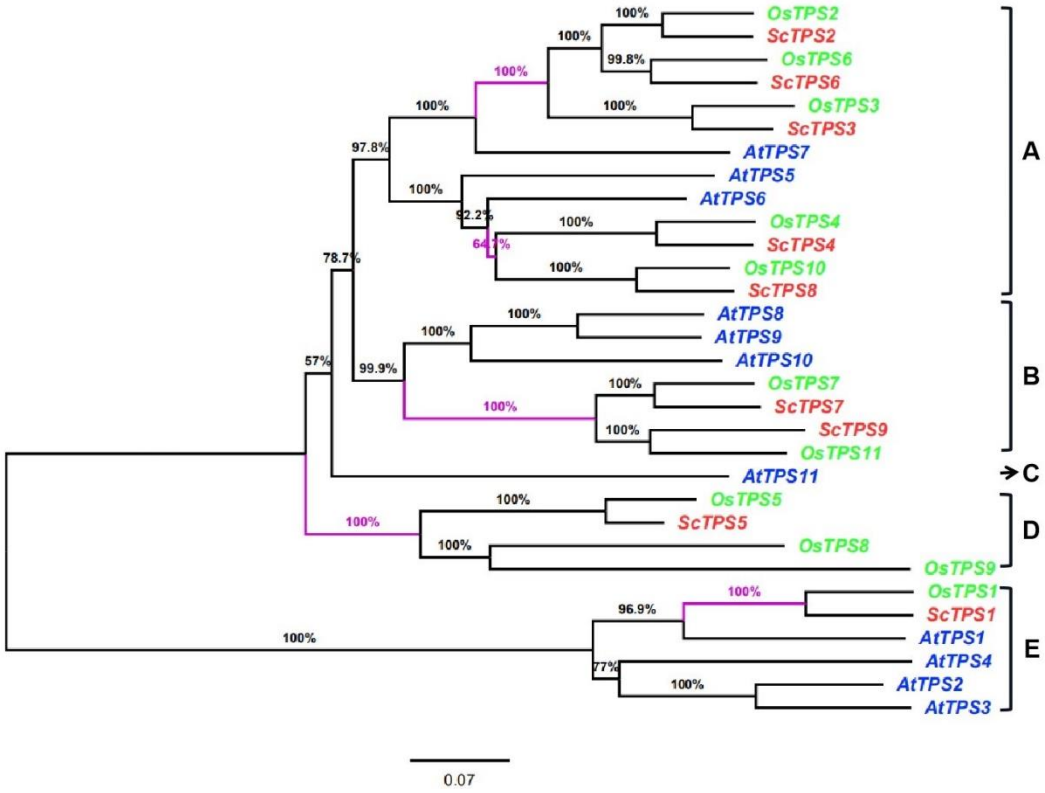

Figure 4. Phylogenetic analysis of the trehalose-6-phosphate synthase genes from sugarcane (*ScTPS*), *Arabidopsis* (*AtTPS*), and rice (*OsTPS*). The tree was constructed by the neighbor-joining method with 1000 bootstrap replicates, and all *TPS* genes were divided into five clades (Clade **A**–**E**). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The scale bar represents 0.07 units of amino acid substitutions per site.

Parameter estimates and likelihood ratio tests for the branch models were performed to evaluate the selective pressure on the five clades of *TPS* genes. The ω value of the one-ratio model (M0) was 0.10310, indicating a strong purifying selection pressure experienced by the *TPS* gene family (Table 2). Except for Clade A, there were significant diverse negative pressures on the other four clades by comparing M0 with M2. By comparing the values of background ω_0 and foreground ω_1, it was found that Clades B, C, D and E had clearly experienced stronger purifying selection. The tests also revealed that Class I *TPS* genes (all in Clade E) were under a stronger purifying selection than Class II *TPS* genes.

The branch-site model, which had the advantage of considering different branches with different selection pressures, was also applied to identify positive selective sites for five clades of *TPS* genes (Table 3). A subset of individual codon sites was significant under positive or diversifying selection pressures in Clades A, C, D and E by comparing to the corresponding null model. Estimated proportion of positive selection sites (proportion 2a + proportion 2b) of *TPS* genes were 2.9%, 7.0%, 4.3% and

32.3% in Clades A, C, D and E, respectively. There are three positive sites identified in both Clades A and C by NEB test at posterior probabilities (p) > 0.95 level. However, only one positive site in Clade A was significant in the BEB test at 0.95 cut-off, which was recommended by PAML [35]. This positively selected site identified in Clade A was 108R according to the references to first sequence of *AtTPS1*. Only a few positive selection sites were detected, further confirming that *TPS* genes were mainly affected by negative selection pressure.

Table 2. Parameter estimates and likelihood ratio tests for the branch models.

Model	p [a]	Estimates of Parameters	lnL	df	2|ΔlnL|	*p*
M0 (one ratio model)	1	$\omega_0 = 0.10310$	−37899.458	-	-	-
		Branch-specific model (Model2: two ratios)				
Estimate ω for A	2	$\omega_0 = 0.10296$, $\omega_1 = 0.15313$	−37899.349	1	0.218	0.640
Estimate ω for B	2	$\omega_0 = 0.10527$, $\omega_1 = 0.01488$	−37890.616	1	17.684	<0.001
Estimate ω for C	2	$\omega_0 = 0.10406$, $\omega_1 = 0.05586$	−37897.187	1	4.542	0.033
Estimate ω for D	2	$\omega_0 = 0.10741$, $\omega_1 = 0.00102$	−37865.102	1	68.712	<0.001
Estimate ω for E	2	$\omega_0 = 0.10346$, $\omega_1 = 0.00759$	−37895.919	1	7.078	0.008

[a] The number of estimated parameters for the ω ratios.

Table 3. Parameter estimates and likelihood ratio tests for the branch-site models.

Model	p [a]	Estimates of Parameters Site Class 0 1 2a 2b	lnL	df	2|ΔlnL|	*p*	Positively Selected Sites
		Branch-sites test for positive selection of Clade A					
null	3	proportion 0.90708 0.09292 0.00000 0.00000 background ω 0.09643 1.00000 0.09643 1.00000 foreground ω 0.09643 1.00000 1.00000 1.00000	−37564.649	-	-	-	-
alternative	3	proportion 0.88351 0.08743 0.02644 0.00262 background ω 0.09624 1.00000 0.09624 1.00000 foreground ω 0.09624 1.00000 19.25885 19.25885	−37557.107	1	15.084	0.0001	3(NEB:p > 0.95) 1(BEB:p > 0.95)
		Branch-sites test for positive selection of Clade B					
null	3	proportion 0.90708 0.09292 0.00000 0.00000 background ω 0.09643 1.00000 0.09643 1.00000 foreground ω 0.09643 1.00000 1.00000 1.00000	−37564.649	-	-	-	-
alternative	3	proportion 0.90708 0.09292 0.00000 0.00000 background ω 0.09643 1.00000 0.09643 1.00000 foreground ω 0.09643 1.00000 1.00000 1.00000	−37564.649	1	-2E-06	0.9989	none
		Branch-sites test for positive selection of Clade C					
null	3	proportion 0.81228 0.08171 0.09631 0.00969 background ω 0.09485 1.00000 0.09485 1.00000 foreground ω 0.09485 1.00000 1.00000 1.00000	−37551.627	-	-	-	-
alternative	3	proportion 0.84537 0.08458 0.06368 0.00637 background ω 0.09554 1.00000 0.09554 1.00000 foreground ω 0.09554 1.00000 70.65026 70.65026	−37545.377	1	12.499	0.0004	3(NEB:p > 0.95)
		Branch-sites test for positive selection of Clade D					
null	3	proportion 0.88381 0.09033 0.02346 0.00240 background ω 0.09606 1.00000 0.09606 1.00000 foreground ω 0.09606 1.00000 1.00000 1.00000	−37563.772	-	-	-	-
alternative	3	proportion 0.86913 0.08823 0.03871 0.00393 background ω 0.09603 1.00000 0.09603 1.00000 foreground ω 0.09603 1.00000 99.32701 99.32701	−37557.905	1	11.733	0.0006	none
		Branch-sites test for positive selection of Clade E					
null	3	proportion 0.58766 0.06196 0.31697 0.03342 background ω 0.09601 1.00000 0.09601 1.00000 foreground ω 0.09601 1.00000 1.00000 1.00000	−37534.060	-	-	-	-
alternative	3	proportion 0.61213 0.06467 0.29231 0.03088 background ω 0.09664 1.00000 0.09664 1.00000 foreground ω 0.09664 1.00000 34.71583 34.71583	−37531.563	1	4.994	0.0254	none

[a] The number of estimated parameters for the ω ratios.

3.4. Cis-Regulatory Element Analysis of ScTPS Genes

The promoter regions of the *ScTPS* gene family are rich in both plant hormone and abiotic stress-related *cis*-regulatory elements, and also contain an ABA response element (ABRE) (Figure 5). Eight *ScTPS* genes, except for *ScTPS9*, contained the methyl jasmonate reaction element (CGTCA-motif

and TGACG motif) and salicylic acid response element (as-1). The promoter of four *ScTPS* genes contain an auxin response element (TGA-motif).

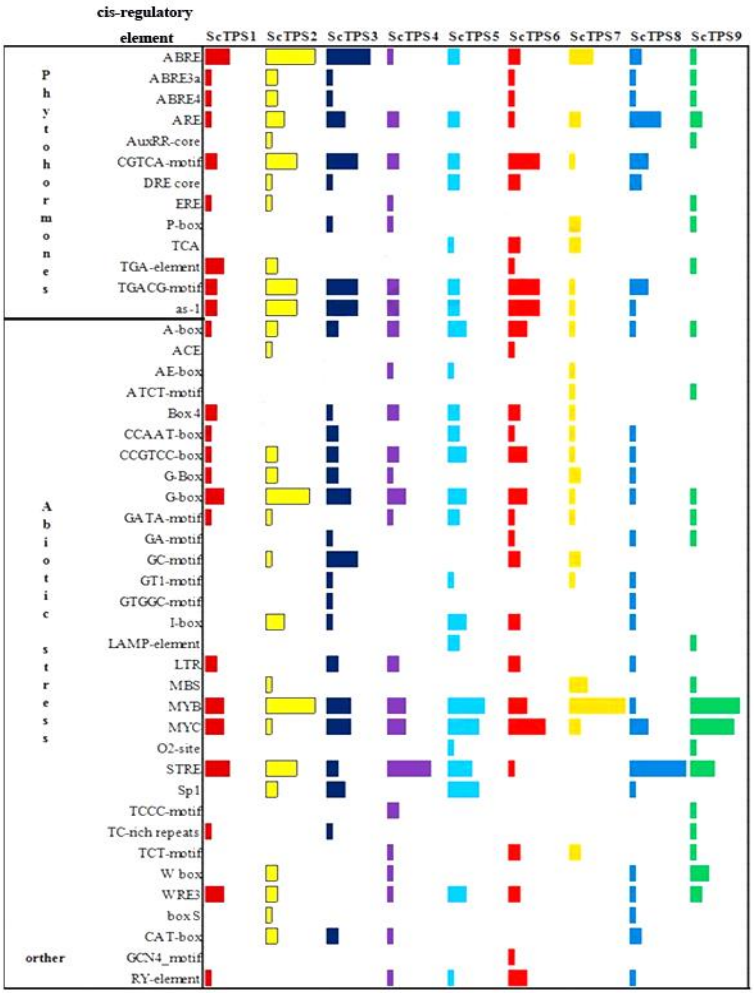

Figure 5. *Cis*-regulatory elements for the trehalose-6-phosphate synthase (*ScTPS*) gene family in sugarcane. The length of the bar represents the number of elements from 1 to 9. For reference, bars for *cis*-regulatory element MYB *ScTPS8* and *ScTPS7* represent 1 and 9 elements, respectively.

The promoter regions of the *TPS* gene also contain various *cis*-acting elements related to environmental and signal molecule responses. Within the promoter region, six *ScTPS* genes contain light response elements (G-box), seven *ScTPS* genes contain damage response elements (WRE3), eight *ScTPS* genes contain stress response elements (STRE), and nine *ScTPS* genes contain MYB and MYC transcription factor elements related to ABA or drought-induced expression regulation. Thus, the expression of *ScTPS* genes could be induced by various stresses, which might confer a homeostatic response to maintain normal growth and development under stress.

3.5. Analysis of ScTPSs Expression

It was found that *ScTPS* genes showed differential expression patterns under different treatments (Figure 6). Under salt treatment, *ScTPS1*, *ScTPS5*, *ScTPS7* and *ScTPS9* exhibited strong expression, with the highest expression level observed at 48 h post-treatment. In contrast, *ScTPS4* and *ScTPS8* were down-regulated with the lowest expression level observed at 24 h post-treatment. PEG-6000 treatment induced a decline in expression levels of most *ScTPS* genes, while *ScTPS1*, *ScTPS5* and *ScTPS7* exhibited obviously increased expressions at 24h post-treatment. Under ABA treatment, *ScTPS2*, *ScTPS4*, *ScTPS6* and *ScTPS8* were clearly down-regulated at both 24 and 48 h post-treatment,

whereas the expresion of *ScTPS1* and *ScTPS5* were induced in response to ABA. Some genes with a closer phylogenetic relationship frequently showed a similar expression pattern in different treatments, although the response to each stressor varied somewhat. For instance, high salt, simulated drought, and ABA treatments all induced the expression of *ScTPS1* and *ScTPS5*, and inhibited the expression of *ScTPS2* and *ScTPS6*.

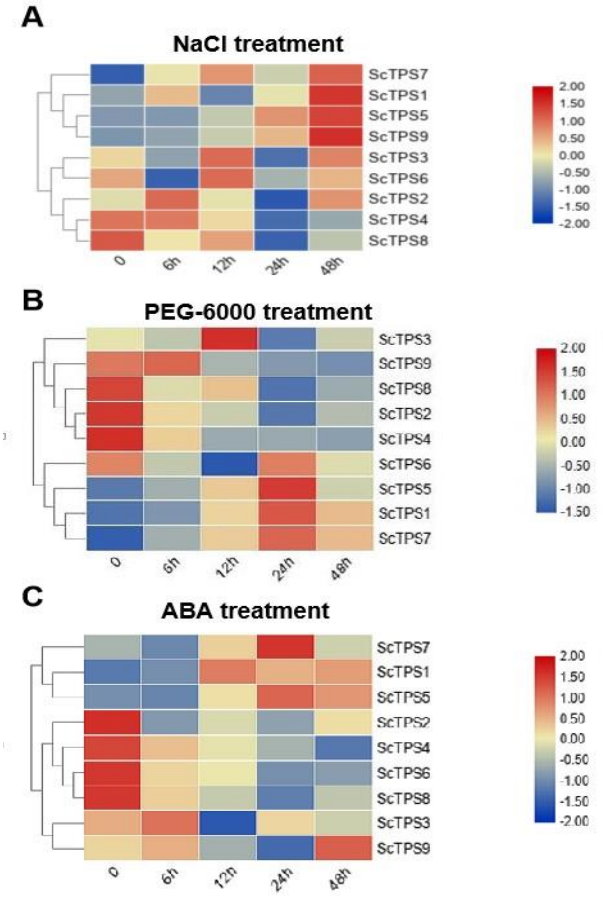

Figure 6. Expression pattern of *ScTPS* genes upon NaCl (**A**), PEG-6000 (**B**) and ABA (**C**) treatments. The stress conditions are salt (200 mM NaCl), 10% (w/v) PEG-6000 treatment and ABA treatment (30 nM). Control samples (setting of zero) are collected before treatment, and were compared with the test samples at 6, 12, 24 and 48 h post-treatment. Color scale represents fold changes (Treatment/Control) normalized log2 transformed data. Red indicates up-regulated and blue indicates down-regulated genes.

4. Discussion

We used bioinformatics methods to study the sugarcane *TPS* gene family in reference to *O. sativa* and *A. thaliana* genomes. In this study, nine *TPS* genes were identified in sugarcane, only *ScTPS1* belonged to Class I. The number of introns and the length of genes differ between Class I and Class II subfamilies. This is basically in accordance with previous studies that identified 11 *TPS* genes in each of the *Arabidopsis* and rice genomes, which contain four (*AtTPS1*, *AtTPS2*, *AtTPS3*, and *AtTPS4*) and one (*OsTPS1*) Class I *TPS* genes, respectively [8,26]. The amino acid sequences of *ScTPS* genes are conservative. The conservation of TPS protein sequences may be driven by the spatial conformation of a long N-terminal TPS domain (460–500 amino acids in size) and a C-terminal TPP domain (about 240 amino acids in size). This hypothesis is well supported in the results that the average identities of amino acid sequence of TPS and TPP domains are both much higher than that of the sequences outside domains. The average amino acid identity is much lower between Class I-Class II pairwise comparisons than that within Class II pairwise comparisons, suggesting a clear evolutionary divergence. It is further confirmed by the phylogeny analysis on 31 *TPS* genes from *Arabidopsis*, rice, and sugarcane.

The evolutionary fate of sugarcane *TPS* genes is basically the same with those of rice. Their difference mainly embodies in the subclade D, which contains three *OsTPS* gene, but only one *ScTPS* gene. The distribution of orthologous genes in each subclade differs largely between monocot and dicot plants, suggesting the ancestral origin of the genes as divergent evolution after the monocot–eudicot separation. These results are also noted by several previous reports [2,10,38–40]. It is noteworthy that four *Arabidopsis* Class I *TPS* genes are further divided into two subclades, thus one subclade is likely lost from the rice and sugarcane genome. This feature of the dicots is also found in cassava [38]. More detailed *TPS* phylogentic analysis is needed to confirm the evolutionary feature of *TPS* gene. The technologies of accurate genome assemblies and annotations are also imperatively demanded in plant genomics.

The evolution analysis of *TPS* genes reveals that *TPS* family members appear to have undergone strong negative selection implying functional conservation of these *TPSs*. The strength of the selective pressure differs in most Clades, especially Clade E (belong to Class I) that experienced significantly stronger negative selection pressure than most Class II *TPS* genes. It is generally considered that amino acid sequences with important biological functions are less variable across species due to selection pressure [41,42]. Indeed, it was presumed that only *TPS1* gene had TPS enzyme activity, until the discovery that both *TPS2* and *TPS4* of *A. thaliana* also had TPS enzyme activity [43]. Plant *TPS1* gene is closely related to the growth and stress resistance of plants. *Arabidopsis TPS1* was proven to be essential for embryogenic and vegetative growth [44]. Trehalose-6-phosphate, mainly synthesized by TPS synthase 1, is considered as a signaling molecule of carbohydrate status and is linked to the regulation of genes expression related to adverse environmental stresses [45,46]. The *TPS* class II gene has TPS domain, but has no obvious TPS enzyme activity, and its function is largely unknown. Thus it seems that most Class II gene is under relaxed purifying selection, but a positively selected site was identified in Clade A. This codon site may be functionally important, which may be a force directing the evolution of *TPS* gene in this Clade.

The distinct differences of expression pattern and function between Class I and Class II *TPS* genes might be related to wide variation in the exon—intron structures. It is found that certain introns are necessary for alternative splicing of mRNA, which could regulate the structure and function of gene-encoded proteins. Some may enhance the transcription and transport of mRNA, leading distinct tissue-specific patterns and diverse expression levels [47,48]. The loss of introns in Class II seems not to be related to selection pressure according to evolution analysis of *ScTPS* genes from our study. This could be due to gene mutation and other causations. Further research is needed to explore the introns of *TPS* genes on evolutionary reasons and their effects to TPS protein functions.

Plant *TPS* genes encode a key enzyme in trehalose metabolism pathway in response to diverse stressful conditions. The promoter region of *ScTPS* genes contains many *cis*-acting elements related to plant hormone and environmental stresses. Expression levels of *TPS1* and *TPS7* genes in *S. tuberosum* are significantly improved under high temperature, high salinity, and drought stress, suggesting that *TPS* genes might be involved in the signal transduction pathway of stress resistance [11]. An important feature of drought and salt stress is the osmotic effect on cells, which leads to ABA accumulation as an adaptive response [49]. The relationship between *ScTPS* and ABA signaling indicated that *ScTPS* might play an important role in plants counteracting drought stress.

Due to the protective effect of trehalose on abiotic stresses in plants, many researchers have attempted to introduce bacterial or yeast *TPS* genes to enhance plant stress tolerance, especially to drought. However, the over-expression of heterologous *TPS* genes is accompanied usually with abnormal phenotypes. It has been reported that *A. thaliana* and *N. tabacum* plants that overexpressed yeast *TPS* gene both exhibit obvious morphological defects [50,51]. One approach of enhancing stress resistance while avoiding undesirable phenotypic changes is through over-expression of endogenous *TPS* gene. For example, over-expression of *AtTPS1* in *A. thaliana* [12] and *OsTPS1* in *O. sativa* [13] improve their respective drought tolerance without increasing the frequency of aberrant phenotypes. The mechanism of phenotypic alterations caused by the expression of endogenous or heterologous

TPS gene remains unclear. Interestingly, Zhang et al. [24] found that transgenic sugarcane that over-expresses *Grifola frondosa* trehalose synthase gene has improved drought tolerance without obvious morphological changes and growth inhibition in the field. Recently, Nilson et al. have acquired two sugarcane clones with *STPS1* and *STPS2* EST sequences that are identical to the *ScTPS1* and *ScTPS6* genes in this study, respectively [52]. Their results showed that *STPS1* expression was up-regulated in drought-tolerant cultivars under water stress, while the expression of *STPS2* gene had no significant change under same treatments. In this study, it also found that *ScTPS1* gene expression was induced by salt, simulated drought, and ABA, suggesting that sugarcane plants increased trehalose-6-phosphate production to maintain stable intracellular osmotic pressure to mitigate simulated stress. Therefore, it is possible that genetic engineering of endogenous *ScTPS1* genes involved in trehalose biosynthesis might confer increased drought tolerance to sugarcane. More research is needed to better understand the molecular regulatory network of *ScTPS* in sugarcane. Nonetheless, *ScTPS* marker-assisted selection of stress tolerant sugarcane genotypes might help sustain sugarcane production under stressful field conditions.

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