Genome-Wide Identification and Evolution Analysis of Trehalose-6-Phosphate Synthase Gene Family in *Nelumbo nucifera*

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Trehalose-6-phosphate synthase (TPS) plays a key role in plant carbohydrate metabolism and the perception of carbohydrate availability. In the present work, the publicly available *Nelumbo nucifera* (lotus) genome sequence database was analyzed which led to identification of nine lotus TPS genes (*NnTPS*). It was found that at least two introns are included in the coding sequences of *NnTPS* genes. When the motif compositions were analyzed we found that *NnTPS* generally shared the similar motifs, implying that they have similar functions. The \(d_N/d_S\) ratios were always less than 1 for different domains and regions outside domains, suggesting purifying selection on the lotus TPS gene family. The regions outside TPS domain evolved relatively faster than *NnTPS* domains. A phylogenetic tree was constructed using all predicted coding sequences of lotus TPS genes, together with those from *Arabidopsis*, poplar, soybean, and rice. The result indicated that those TPS genes could be clearly divided into two main subfamilies (I-II), where each subfamily could be further divided into 2 (I) and 5 (II) subgroups. Analyses of divergence and adaptive evolution show that purifying selection may have been the main force driving evolution of plant TPS genes. Some of the critical sites that contributed to divergence may have been under positive selection. Transcriptome data analysis revealed that most *NnTPS* genes were predominantly expressed in sink tissues. Expression pattern of *NnTPS* genes under copper and submergence stress indicated that NNU_014679 and NNU_022788 might play important roles in lotus energy metabolism and participate in stress response. Our results can facilitate further functional studies of TPS genes in lotus.

Keywords: trehalose-6-phosphate synthase, *Nelumbo nucifera*, protein properties, molecular evolution, expression pattern

INTRODUCTION

Trehalose, known as a non-reducing disaccharide of glucose, has been found in wide range of diverse organisms, for example, bacteria, fungi, invertebrates, and plants (Lyu et al., 2013). In higher plants, trehalose accumulates at quite low concentrations, with the exception of resurrection plants (Paul et al., 2008). It was found that in trehalose-rich organisms, trehalose functions both...
as a carbon source and as an osmoprotectant such in certain microorganisms and insects (Elbein et al., 2003; Zang et al., 2011). There is crucial evidence that trehalose helps prevent heat, osmotic, nutrient, dehydration stress, and toxic chemicals from damaging organisms (Chary et al., 2008). Trehalose synthesis in plants only occurs via the TPS/TPP pathway (Avonce et al., 2006; Jiang et al., 2010). In the first step of this pathway, trehalose-6-phosphate synthase (TPS) joins uridine diphosphoglucose with glucose 6-phosphate, thus forming trehalose 6-phosphate (T6P); (Jiang et al., 2010). Then, T6P is dephosphorylated as catalyzed by trehalose-6-phosphate phosphatase (TPP) to produce the disaccharide trehalose. TPS expression levels in cotton increased under drought stress (Mu et al., 2016). When maize was under salt and temperature stress, TPS genes were upregulated (Jiang et al., 2010). Genes which controlled the production of enzymes of trehalose synthesis, including the AtTPSI gene from Arabidopsis thaliana (Blázquez et al., 1998; Jiang et al., 2010). These findings made it practical to attain transgenic plants altered by a TPS gene of plant origin. Most transgenic plants with overexpressed TPS and/or TPP genes exhibited great tolerance to abiotic stress (Garg et al., 2002; Jang et al., 2003; Karim et al., 2007; Ge et al., 2008).

Although numerous studies found that over-expressing TPS and TPP genes could improve abiotic stress tolerance (Holmström et al., 1996; Jang et al., 2003; Schlupemann et al., 2004; Jiang et al., 2010), observed stress tolerance of transgenic lines did not correlate with trehalose amount (van Dijken et al., 2004). Researchers have investigated function of plant trehalose biosynthetic enzymes by identifying mutations in Arabidopsis TPSI gene and seeing which were still effective in recovering homologous gene function for yeast mutants deficient in TPS functions (Blázquez et al., 1998; Müller et al., 2001; Vogel et al., 2001). Beyond these stress response, recent intriguing evidence has implicated that TPS genes are important modulators in plant development and inflorescence architecture (Satoh-Nagasawa et al., 2006).

The plant TPS gene family belongs to a small gene family with multiple copies. Members of this gene family showed widespread functional diversification (Lunn, 2007; Chary et al., 2008; Vandesteene et al., 2010; Yang et al., 2012). The Arabidopsis TPS gene family contains 11 members (AtTPSI-11; Shima et al., 2007); rice also contains 11 members (OsTPSI-11; Tuskan, 2006; Vandesteene et al., 2010), and poplar contains 12 members (PitPSI-12; Vandesteene et al., 2010). The TPS family is divided into two distinct clades, class I and class II, and this dichotomy occurred early in the evolution of green plants (Jang et al., 2003; Shima et al., 2007; Suzuki et al., 2008; Ramon et al., 2009). Apart from polyploid and allopolyploid species, Arabidopsis is quite uncommon among all the angiosperms which have large number class I TPS genes (Delorge et al., 2015). Particularly, distinct characteristics of class I and class II TPS genes are displayed in gene expression patterns, copy number, physiological functions, and enzyme activity.

Sacred lotus (Nelumbo nucifera, Gaertn.) is a basal eudicot which is of significant cultural, agricultural, medicinal, as well as religious importance. TPS plays important role in plant response to environmental stimuli, and thus the study of TPS would be very important for lotus breeding and the research of stress-resistance mechanism in lotus. In this study, we investigated the lotus TPS genes from whole genome-wide studies and their evolutionary relationship with other TPS genes. We explored TPS gene family members’ expression patterns in various tissues and in response to various stresses. Having conducted a thorough analysis of gene sequences, molecular evolution, gene structures, and gene expression patterns, we offer an effective framework for further functional characterization of lotus TPS gene families.

**METHODS**

**Identification of Lotus TPS Genes**

The lotus genome DNA data (Ming et al., 2013) was downloaded from COGE (v2, id16885, https://www.genomevolution.org/coge/) and gene prediction was performed with hidden Markov model (HMM) program (Eddy, 1998). Hidden Markov models of TPP and TPS domain were obtained from PFAM (http://pfam.xfam.org/). Motif scanning in TPS was done by PROSITE scan (Hu et al., 2006). Gene structures of TPS genes were analyzed on the Gene Structure Display Server 2.0 (GSDS; http://gsds.cbi.pku.edu.cn/).

**Multiple Sequence Alignment and Phylogenetic Analysis**

Multiple sequence alignments in full-length protein sequences were executed by using MUSCLE 3.52, and this was followed by manual refinement and comparisons (Edgar, 2004; Cao et al., 2014). Phylogenetic analysis was performed with MEGA 6.0 using a maximum likelihood method with the JTT (Jones, Taylor, and Thornton) amino acid substitution model. Bootstrap support values were estimated using 1000 pseudo-replicates.

**Protein Properties and Conserved Motif Analysis**

Protein properties of TPS genes, e.g., the molecular weight (MW), isoelectric point (pI), and grand average of hydropathicity (GRAVY) were calculated using ProParam (http://web.expasy.org/protparam). Analysis for conserved motifs in TPS proteins was carried out using MEME (http://meme.sdsc.edu/cgi-bin/meme.cgo) with the default parameters.

**Functional Divergence Analysis**

To analyze the functional divergence between groups and predict the amino acid residues resulting in functional difference, program DIVERGE (Gu and Velden, 2002) was applied to calculate the coefficient of type I functional divergence θI as well as that of type II functional divergence θII. The likelihood test is approximated to a χ² distribution with 1 degree of freedom. After duplication, a significant θI means site-specific changed selective constraints (Xun, 2003; Gu, 2006; Yang et al., 2009). After duplication, a significant θII indicates amino acid physiochemical property’s obvious shifts (Xun, 2003; Gu, 2006; Yang et al., 2009).
Site-Specific Selection Assessment and Testing

The value of $d_N/d_S$ ratio (or $\omega$, the ratio of non-synonymous to synonymous distances) for each group was analyzed with the program codeml from PAML version 4 (Yang, 2007). A likelihood ratio test (LRT) was used to detect variation in $\omega$ among sites between M0 vs. M3 and M7 vs. M8, respectively. The $\chi^2$ statistics with degrees of freedom equal to the differences between parameters is twice the log likelihood difference between the two models in the LRT (3 for M0/M3 tests and 2 for M7/M8 tests; Mondragon-Palomino and Gaut, 2005). If the LRT was statistically significant and the evolution of genes below groups, then sites are assumed to be under positive selection pressure through Bayes methods (Yang et al., 2005).

In silico Expression Analysis

To identify expression pattern of TPS genes, expression level in different tissues and abiotic stress treatments, transcriptome datasets of lotus was analyzed. The data sets obtained from GenBank include: rhizome apical meristem (SRS413495), rhizome elongation zone (SRS413496), rhizome internode (SRS414455, SRS414456, SRS414457, SRS414458, SRS414459), leaf (SRS413488, SRS413489, SRS413490, SRS413491, SRS413492), petiole (SRS413498, SRS413499, SRS413500, SRS413501, SRS413502), root (SRS412272, SRS412273, SRS412278, SRS412279, SRS412280). The library preparation methods and statistics of sequencing data were detailed in Kim et al. (2013). To take advantage of our in house transcriptome data, we analyzed the expression pattern of TPS genes in response to submerged and 5 mm Copper-treated

### TABLE 1 | List of the NnTPS genes identified in this study.

| Gene accession number | Scaffold ID | Predicted position | CDS (bp) | Deduced polypeptide | Predicted subcellular localization |
|------------------------|-------------|-------------------|---------|---------------------|----------------------------------|
| NNU_020889             | Scaffold_3  | 36472545-36476497 | 2598    | 865                 | 97.58                          | Cytoplasmic                      |
| NNU_016707             | Scaffold_5  | 12364436-12368019 | 2580    | 859                 | 97.05                          | Plasma membrane                  |
| NNU_024672             | Scaffold_8  | 6741636-6749305   | 2535    | 845                 | 95.37                          | Cytoplasmic                      |
| NNU_014679             | Scaffold_20 | 449424-453667     | 2604    | 867                 | 97.60                          | Cytoplasmic                      |
| NNU_020115             | Scaffold_17 | 1846465-1854756   | 2616    | 871                 | 97.98                          | Cytoplasmic                      |
| NNU_004429             | Scaffold_4  | 32965362-32971524 | 2586    | 861                 | 97.13                          | Plasma membrane                  |
| NNU_000253             | Scaffold_4  | 13382224-13386331 | 2649    | 883                 | 100.39                         | Cytoplasmic                      |
| NNU_016432             | Scaffold_5  | 37054048-37058407 | 2814    | 937                 | 106.30                         | Plasma membrane                  |
| NNU_022788             | Scaffold_9  | 4294971-4298958   | 2547    | 848                 | 98.35                          | Cytoplasmic                      |

FIGURE 1 | Structure and phylogenetic analysis of NnTPS proteins. The unrooted phylogenetic tree resulting from the full-length amino acid alignment of all the NnTPS proteins is shown on the left side of the figure. Exon-intron structures of the identified NnTPS genes are shown on the right side. The graphic representation of the optimized gene model displayed using GSDS.
conditions. Three-month-old lotus seedlings were used for submergence (24 h) and 5 mm Copper-treatment (24 h). To achieve submergence, seedlings were transferred to a plastic tank and water was added into the tank to a water depth of 20 cm from top of lotus. The sample without copper or submergence treatment was the control (Con). After the treatments, total RNA was isolated using the CTAB-LiCl method. For mRNA-seq, three sets of total RNA were used for cDNA library construction and sequencing (without replicates) at the Beijing Genomics Institute (BGI, Shenzhen, China), following the manufacturer’s protocols.

All SRA format data were converted into FASTQ using the SRA toolkit. All raw reads were first filtered with NGS QC Toolkit (v2.3.3) to remove adapter sequences, reads containing poly-N, and low-quality sequences (Q < 20). An index of the lotus genome was built using Bowtie 2 and paired-end or single-end clean reads were aligned to the reference genome using TopHat. The expression levels (fragments per kb per million mapped read, FPKM) from the representative transcript were determined using cufflinks program (cufflinks v2.2.142) with default settings. We used a threshold of FPKM ≥ 1 to define a gene as “expressed.”

**Real-Time Quantitative PCR**

Two month-old lotus seedlings were used for submergence treatment and copper treatment. Seedlings were completely submerged or treated with 5 mm copper for 24 h (as described above) and then rhizomes were sampled. The sample without copper or submergence treatment was the control (Con). Meanwhile, different tissues of control seedlings were sampled. Tissues were homogenized with mortar and pestle in liquid nitrogen. Total RNA was isolated using CTAB-LiCl method. About 4 µg of total RNA was reverse-transcribed using an oligo(dT) primer and SuperScript Reverse Transcriptase (Invitrogen, USA). Real-time quantitative PCR was conducted using SYBR green (TaKaRa Biotechnology) on Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) with a final volume of 20 µl per reaction. The specific primers were listed in Table S1. The relative transcript abundance was normalized using lotus Actin gene. Gene expression in various tissues were represented by NnTPS/Actin. Fold changes of genes under copper stress and submergence treatment were

![FIGURE 2](attachment:figure2.png) **FIGURE 2 | Pairwise sequence identity of full-length TPS proteins.** Pairwise sequence identities between TPS domain, TPP domain, full length protein sequence, and sequence outside domain were calculated. The boxplot shows the median (black line), interquartile range (box), and maximum and minimum scores (whiskers) for each data set.

| Type | Q ≥ 0.80 | Q ≥ 0.95 | Type |
|------|---------|---------|------|
| ΘI ± SE | LRT | P | LRT | P |
| II-1/II-2 | 0.217 ± 0.101 | 4.607 | 0.032 | 1 | 0 | -0.014 ± 0.072 | 0.846 |
| II-1/II-3 | 0.352 ± 0.118 | 8.919 | 0.003 | 0 | 0 | 0.014 ± 0.08 | 0.861 |
| II-1/II-4 | 0.444 ± 0.107 | 17.262 | 0.000 | 4 | 1 | -0.019 ± 0.096 | 0.843 |
| II-1/II-5 | 0.525 ± 0.127 | 16.947 | 0.000 | 6 | 0 | -0.043 ± 0.107 | 0.688 |
| II-1/II-1 | 0.57 ± 0.123 | 21.432 | 0.000 | 14 | 0 | 0.477 ± 0.066 | 0 |
| II-2/II-3 | 0.143 ± 0.083 | 2.998 | 0.085 | 0 | 0 | -0.002 ± 0.087 | 0.982 |
| II-2/II-4 | 0.191 ± 0.075 | 6.425 | 0.011 | 1 | 0 | -0.043 ± 0.102 | 0.673 |
| II-2/II-5 | 0.268 ± 0.07 | 14.453 | 0.000 | 3 | 1 | -0.04 ± 0.113 | 0.723 |
| II-2/II-1 | 0.388 ± 0.092 | 17.500 | 0.000 | 6 | 0 | 0.425 ± 0.075 | 0 |
| II-3/II-4 | 0.153 ± 0.075 | 4.162 | 0.041 | 1 | 0 | -0.048 ± 0.106 | 0.664 |
| II-3/II-5 | 0.281 ± 0.079 | 12.664 | 0.000 | 2 | 0 | -0.068 ± 0.116 | 0.569 |
| II-3/II-1 | 0.434 ± 0.089 | 24.043 | 0.000 | 6 | 1 | 0.487 ± 0.074 | 0 |
| II-4/II-5 | 0.081 ± 0.047 | 2.979 | 0.085 | 0 | 0 | -0.101 ± 0.125 | 0.419 |
| II-4/II-1 | 0.304 ± 0.089 | 11.711 | 0.001 | 2 | 0 | 0.408 ± 0.088 | 0 |
| II-5/II-1 | 0.415 ± 0.093 | 19.781 | 0.000 | 4 | 1 | 0.432 ± 0.093 | 0 |

**TABLE 2 | Functional divergence between groups of the TPS gene family in plants.**

| Type | Q ≥ 0.80 | Q ≥ 0.95 | Type |
|------|---------|---------|------|
| ΘII ± SE | LRT | P | LRT | P |
| II-1/I-1 | 0.57 ± 0.123 | 21.432 | 0.000 | 14 | 0 | 0.477 ± 0.066 | 0 |
| II-2/I-1 | 0.143 ± 0.083 | 2.998 | 0.085 | 0 | 0 | -0.002 ± 0.087 | 0.982 |
| II-2/I-4 | 0.191 ± 0.075 | 6.425 | 0.011 | 1 | 0 | -0.043 ± 0.102 | 0.673 |
| II-2/I-5 | 0.268 ± 0.07 | 14.453 | 0.000 | 3 | 1 | -0.04 ± 0.113 | 0.723 |
| II-2/I-1 | 0.388 ± 0.092 | 17.500 | 0.000 | 6 | 0 | 0.425 ± 0.075 | 0 |
| II-3/I-1 | 0.434 ± 0.089 | 24.043 | 0.000 | 6 | 1 | 0.487 ± 0.074 | 0 |
| II-3/I-4 | 0.153 ± 0.075 | 4.162 | 0.041 | 1 | 0 | -0.048 ± 0.106 | 0.664 |
| II-3/I-5 | 0.281 ± 0.079 | 12.664 | 0.000 | 2 | 0 | -0.068 ± 0.116 | 0.569 |
| II-4/I-1 | 0.304 ± 0.089 | 11.711 | 0.001 | 2 | 0 | 0.408 ± 0.088 | 0 |
| II-5/I-1 | 0.415 ± 0.093 | 19.781 | 0.000 | 4 | 1 | 0.432 ± 0.093 | 0 |

The coefficient of functional divergence between two subgroups and its standard error.

The number of critical amino acid residues with posterior probability (Qk) > 0.80.

The number of critical amino acid residues with posterior probability (Qk) > 0.95.
values relative to control samples after normalization to Actin transcript levels. RT-qPCR data presented were the means ± SE of at least four independent experiments. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to multiple comparisons or t-test.

RESULTS AND DISCUSSION

Genome-Wide Identification of Lotus TPS Gene Family Members

Accumulating evidence showed that TPS genes play a vital role in modulating plants response to abiotic stresses, i.e., OsTPS1 could improve rice-seed tolerance to low temperature, salt, and drought (Jiang et al., 2010; Vandesteene et al., 2010; Li et al., 2011; Zang et al., 2011). However, identity and function of lotus TPS gene family was previously undescribed. The lotus genome was sequenced recently, which makes it possible to carry out genome-wide study on TPS genes in lotus. Plant TPS proteins contain a TPS as well as a trehalose-6-phosphate phosphatase (TPP) domain (Yang et al., 2012). Thus, HMM profile of two conserved TPS domains (TPS and TPP domain) was used to search local lotus protein data with HMMER program (Sonnhammer et al., 1998; Ross et al., 2007). Nine lotus TPSs (NnTPSs) gene family members were identified in lotus genome (Table 1). Taking advantage of these identified genes, we further conducted a homology search from genome and amino acid sequences of lotus. However, the search did not yield new potentially TPS encoding genes for lotus beyond the nine initially identified. The identified NnTPSs contained a similar number of amino acid from 845 (NNU_024672) to 937 (NNU_016432). The isoelectric point (PI) ranged from 5.60 (NNU_022788) to 6.33 (NNU_004429). The protein weight ranged from 95.37 (NNU_024672) to 106.21 (NNU_016432) kDa. Nine NnTPS scattered in 7 scaffolds. Scaffold 4 and 5 had 2 NnTPS genes, respectively. Previous studies have identified 11 TPS in Arabidopsis, 11 in rice, and 12 in poplar (Yang et al., 2012), suggesting that the number of genes remains stable in this family and that usually duplicated copies of TPS genes are not retained after a whole-genome duplication event (Hao et al., 2014).

Gene Structure Analysis of TPS in Lotus

To assess the evolutionary relationship of nine NnTPS genes, a small evolutionary tree was constructed using the nine NnTPS genes to exam NnTPS gene structures which are typical imprints of evolution within some gene families. The length of gene introns was larger and genetic structures were more complicated in NNU_024672, NNU_020115, NNU_000253, and NNU_016432 than the other NnTPS genes (Figure 1). All
**NnTPS** genes contain at least one intron in their CDSs: most of them have three introns, one (NNU_004429) has four introns and one (NNU_022788) has five introns. According to the previous reports (Nuruzzaman et al., 2010; Hu et al., 2016), the rate of intron gain is slower than that of intron loss after segmental duplication in rice. Thus, it can be concluded that NNU_022788 might be the most ancestral with the other **NnTPS** genes derived from it. Compared with similar CDS length (2535–2814 bp) among 9 **NnTPS** genes, their gene length is even more variable (3583–8291 bp).

**Conserved Sequences in TPS Proteins**

A homology analysis of nine full-length protein sequences indicated that nucleotide identity among them ranged from 63.66% (between NNU_016707 and NNU_022788) to 92.78% (between NNU_024672 and NNU_020115; Figure 2, Figure S1). Reflecting what was found in the protein sequences, a high similarity (ranging from 65.14 to 90.26%) was shared among nine **NnTPS** full-length sequences. Several residues in the catalytic center were highly conserved in all **NnTPS** genes (Figure S1) suggested that corresponding genes encoding active enzymes. Nevertheless, relatively high divergence was observed in some regions of amino acid sequences outside of the domain. It is likely that these un-conserved regions might contribute to functional distinction.

Subcellular localization of **NnTPS** isoforms was predicted using web subcellular localization predictor server (http://cello.life.nctu.edu.tw/; Table 1). Translated proteins of NNU_016707, NNU_004429, and NNU_016432 were predicted as being located on plasma membrane while most of **NnTPS**s were located in cytoplasm.

The identification of motifs for all **NnTPS** proteins were performed by software MEME (Bailey and Elkan, 1994) with default settings. We obtained sequences of 20 motifs and the distribution of these motifs in TPS proteins (Figure 3; Table S2). These motifs were conserved in all **NnTPS** genes except NNU_024672 which lacked motif12. According to Figure 3, motifs 1, 2, 3, 4, 6, 9, 10, 13, 14, 15, 17, and 20 were located in the TPS domain and motifs 8 and 19 were located in TPP domain. These features in conserved motifs of **NnTPS**s were also observed in other plants (Mu et al., 2016).

To explore whether selection pressure had affected **NnTPS** genes, we calculated ratio (ω) of the synonymous substitution rate (ds) vs. the non-synonymous substitution rate (dn) using the program codeml from PAML (version 4). Generally, ...= 1 indicates negative or purifying selection and a ratio = 1 indicates neutral evolution (Wang et al., 2005; Song et al., 2015). Results in Figure 5 showed that dn/ds ratios were always less than 1 for different domains and regions outside domain, suggesting purifying selection on lotus **TPS** gene family. The dn/ds ratios outside **NnTPS** domain were found to be much higher than the ratios inside the **NnTPS** domains (Figure 4). These results showed that **NnTPS** domains evolved faster than regions outside TPS domain. The relaxed purifying or positive selection in the regions outside TPS domain may lead to the above results.

**Relationships of Lotus TPS Family with Other Plant TPSs**

A phylogenetic tree was used to reveal homologous relationships and evolutionary roots of TPS from *Arabidopsis*, rice, poplar (Yang et al., 2012), and soybean (Yang et al., 2012). The amino acid sequence alignment of TPS in these plants was conducted

**TABLE 3 | Detection of positive selection under site-specific model.**

| Group | Na | dN/dS(ω) under M0 | 2Δ/M vs. M0 | 2Δ/M8 vs. M7 | M8 estimates | Positively selected sites |
|-------|----|------------------|-------------|-------------|--------------|-------------------------|
| II-1  | 7  | 0.425            | 72.153**    | 16.600**    | p1 = 0.031, ω = 19.426 | -                       |
| II-2  | 3  | 0.095            | 145.763**   | 1.774       | p1 = 0.018, ω = 1.328 | -                       |
| II-3  | 9  | 0.104            | 609.630**   | 0.008       | p1 = 0.00001, ω = 3.075 | -                       |
| II-4  | 13 | 0.149            | 981.750**   | 7.786**     | p1 = 0.015, ω = 1.263 | -                       |
| II-5  | 10 | 0.164            | 852.843**   | 0.621       | p1 = 0.002, ω = 4.772 | -                       |

*Number of the sequences in the group.

dN/dS(ω) is the average ratio over sites under a codon model with one-ratio.

Double asterisks represent P < 0.01 for chi-square (χ2) test, while single asterisk represents P < 0.05.

ω is estimated under model M8; p1 is the inferred proportion of positively selected sites; p and q are the parameters of the beta distribution.

The amino acid, which is listed in positively selected position, had a posterior probability > 0.95 under M8.

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for phylogenetic tree construction using MEGA 5.1 (Figure 5). According to the phylogenetic tree and previous division of *Arabidopsis* and rice TPS genes, these TPS genes are divided into two subfamilies (I and II; Vandesteene et al., 2010; Zang et al., 2011). *Arabidopsis*, rice, soybean, and poplar contain four (AtTPS1, 2, 3, and 4), one (OsTPS1), four (GmTPS1, 2, 3, and 4), and one (PtTPS1) subfamily I TPS, respectively. However, no lotus TPS subfamily I members was found. It should be noted that the absence of subfamily I TPS in lotus might be due to incomplete genome sequencing coverage (86.5%). However, we cannot exclude the possibility that these genes have been fatally lost in evolution, as the number of TPS genes in subfamily I is far less than TPS genes in subfamily II in previous reported species (Zang et al., 2011). In order to describe paralogous and orthologous relations among this family, the subfamily II TPS genes were further divided into five group (II-1, 2, 3, 4, and 5) with high bootstrap support, suggesting that genes in each subgroup might share a similar origin. In most cases, subgroups are duplicates of at least one lineage. For example, subgroup II-2 and II-3 were duplicated after the separation between dicots and monocots. We identified nodes that lead to dicots (*lotus, Arabidopsis*, poplar, and soybeans)-specific and monocots (rice)-specific subfamilies. Group II-2 only contained *lotus, Arabidopsis*, and poplar Class II TPS genes, indicating that genes might have been lost from the rice genome. Subfamily I-1 only contained three *Arabidopsis* Class I TPS. Moreover, most proteins in lotus TPS family were contained in paralogous pairs, including 4 pairs of NnTPS accounting for 89% NnTPSs. This result indicated that most lotus TPS genes expanded in a species-specific manner.

Functional divergence of the duplicate genes has been recognized as an important source of evolutionary novelty (Yang et al., 2012). To evaluate potential functional divergence, type I and type II functional divergence between groups of the TPS family were estimated by posterior analysis (Gu and Velden, 2002). For this analysis, the collected plant TPS proteins were used and estimation was based on multiple alignments of proteins for any two groups (Table 2). Previous work proposed several possible evolutionary fates of duplicate genes (Hughes, 1994; Force et al., 1999). Our results showed that most type I coefficients ($\theta_1$) of functional divergence were significantly greater than zero ($P < 0.01$), while few of the type II coefficients ($\theta_{II}$) was statistically greater than zero. These results suggested that type I functional divergence was the dominant pattern for evolution of TPS family in these plants, and significantly site-specific altered selective constraints should contribute to most of the TPS members which would, after the diversification, cause a group-specific functional evolution.

Further, the calculation of site-specific profiles on the basis of posterior analysis for all group pairs with functional divergence would predict the critical amino acid residues, which are...
accountable for the functional divergence. Among aligned sites, most of them had low posterior probabilities. \( Q_k \geq 0.80 \) and \( Q_k \geq 0.95 \) were utilized empirically as cutoffs to determine type I functional divergence-related residues existing between groups, so as to greatly reduce false positive (Wang et al., 2011). For most of pairs of groups, at least one site had the posterior probability higher than 0.8, and four pairs of groups had one site with posterior probability higher than 0.95 (Figure 6). A cut-off value \( Q(k) \geq 0.95 \) was used for estimated key amino acid residues. The number of amino acid residues that presumably contributed to alter functional constraints was small between TPS groups. Three amino acid residues (669, 704, and 753) in all comparisons were identified as being most important for the functional divergence. These amino acids are all localized in the TPP domain.

To examine if selective pressure between the seven groups of TPS genes would change, the likelihood ratio tests of positive selection were used via the ML methods as well as codon substitution models (Yang, 1998; Yang and Bielawski, 2000; Yang et al., 2012). Two tests were performed during the analysis process. First, models M0 and M3 were compared in order to assess whether \( d_N/d_S \) ratio variations existed among codon positions below each group. On the whole, the maximum likelihood estimates of \( d_N/d_S \) values as for groups under model M0 were close to zero, with three exceptions of Arabidopsis TPS genes in group I-2. This implied that the principal force which acting on the evolution of TPS family was purifying selection (Table 3). It is obvious that although the majority of protein residues are heavily dependent on constant purifying selection, some sites are also being impacted by positive selection. Positive selection is an important adaption mechanism; thus, sites under positive selection pressure may have sped up functional divergence of TPS; in this case it would allow the plants to adjust to its environment. The results we get are in accordance with this conclusion. As the statistical differences of log likelihood between model M0 and M3 were significant for all groups, it suggests that the overall selective constraint levels varied across the TPS family group lineages. Secondly, the LRT which is applied to compare data fit to models M7 vs. M8 was used to determine whether positive selection promoted divergence of TPS family below groups or not. Two groups (group I-1 and II-2) of the 7 groups

![Figure 6](image)

**FIGURE 6** | Type I functional divergence among the plant TPS gene members. Posterior probability (PP) profiles of the site-specific type I functional divergence. The line indicates cutoff = 0.95.

![Figure 7](image)

**FIGURE 7** | Expression profiles of the nine NnTPS genes in different tissues (A) and upon submergence and copper stresses (B). The color scale represents PPKM normalized log2 transformed data. Red indicates high expression level and blue indicate low expression level. OL, leaf; RTZ, Combined Rhiz Tip Zone; Rhiz, Rhizome interinode; R, Root; P, Petiole; T, Rhizome apical tip; Z, Rhizome elongation zone.
analyzed, were believed to have undergone positive selection, because they satisfied that (1) an estimate of $\omega > 1$ under M8, (2) sites identified to be under positive selection, and (3) a significant LRT. This result suggests that positive selection contributed to the evolution of genes in these groups. When the LRT suggested positive selective action had occurred, positively selected sites are identified under model M8 using Bayesian method (Nielsen and Yang, 1998; Yang and Bielawski, 2000). We found 31 and 4 positively selected sites in subgroup I-1 and II-2, respectively.

**Gene Expression Analysis**

TPS genes are known to be involved in development and stress responses. In this study, expression pattern of TPS genes in different lotus tissues has been studied using transcriptome data set of lotus (available at NCBI; Kim et al., 2013) as well as our in house transcriptome dataset of lotus under submergence and copper stress. NnTPS genes showed differential expression level in various lotus tissues (Figure 7A). No tissue-specific expressed NnTPS genes were found. Among them, NNU_016432 showed the highest expression level while

![Figure 7A](image_url)

**FIGURE 7A** - Validation of expression of selected NnTPS genes in various tissues and in rhizome upon exogenous stimuli. Tissues including leaf, petiole, and rhizome were sampled from 2 month old lotus seedlings. Meanwhile, lotus seedlings were treated with submergence stress or copper stress for 24h and then rhizome was sampled. The sample without 5 mm copper treatment or submergence treatment was the control (Con). Gene expression was analyzed by real-time RT-PCR. The relative transcript abundance was normalized using lotus actin gene. Values are means ± SE of at least four independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Turkey’s multiple range test. Fold changes of genes under copper stress and submergence treatment were values relative to control samples. Asterisks indicate significant differences compared with control sample at $P < 0.05$ levels according to t-test.
NNU_004429 showed the lowest transcript level in most examined tissues, in comparisons with other NnTPS genes. Genes from the same group frequently showed similar expression pattern within specific tissues. For example, both NNU_000253 and NNU_016432 had relatively low expression in leaf and rhizome internode, and high expression in root, rhizome apical tip, and rhizome elongation zone. Based on FPKM calculations, the total transcript abundance of NnTPS genes were obviously low in leaf and petiole and high in root and rhizome. These results indicated that NnTPS genes might primarily have functions in sink tissues.

In response to submergence and copper stress, the total transcript abundance of NnTPS did not show apparent difference (**Figure 7B**). Two NnTPS genes (NNU_0044229 and NNU_016432) were highly induced by submergence treatment. In contrast, most NnTPS genes (i.e., NNU_024672, NNU_014679, and NNU_022788) were down-regulated. The abundance of NNU_024672 decreased over 80%. Submergence leads to lower concentration of oxygen in a plant as a result of a lower diffusion rate of molecular oxygen in water as opposed to air, which would ultimately influence energy metabolism. The reaction of NNU_024672 to low oxygen demonstrates possible involvement in energy metabolism in the seedlings and that highly expressed NnTPS genes could play vital roles during this complicated biological process. Some plant metabolic processes contain copper as an essential micronutrient, functioning as a constituent of enzymes and other proteins, and in photosynthetic reactions (Maksymiec, 1998; Hall, 2002; Yruela, 2009; Martins et al., 2014). Excessive copper can lead to oxidative stress and be phytotoxic (Mithöfer et al., 2004). It can inhibit growth, influence plant metabolism and affect the function of several enzymes (Martins and Mourato, 2006; Mourato et al., 2009). Under copper stress, the transcript abundance of most NnTPS genes was not obviously changed, except two NnTPS genes (NNU_014679 and NNU_022788). It is interesting that abundance of two genes were apparently decreased in response to submergence while increased upon copper stress. We speculate that under copper stress more energy was needed to strengthen antioxidant system, while more energy was needed to be preserved under submergence as photosynthesis is inhibited. The contrasting expression pattern under submergence and copper stress indicated that NNU_014679 and NNU_022788 might play important roles in lotus energy metabolism and participate in stress response. Similar to previous work, TPS has been reported to play an important role in the regulation of carbohydrate metabolism and especially the perception of carbohydrate availability (Müller et al., 2001).

*In silico* analysis showed that NnTPS genes exhibited differential abundance in various tissues and some of them are obviously regulated by environmental stimuli. Some of these obviously changed genes were then validated by RT-qPCR. As shown in **Figure 8**, RT-qPCR results of genes under various tissues and stresses were similar in magnitude to those obtained by deep sequencing. NNU_020889, NNU_014679, and NNU_022788 were the most abundant genes in rhizome. It is interesting, that while under submergence, NNU_020889 was significantly induced while NNU_000788 was significantly down-regulated. Moreover, NNU_024672, which has the minimum transcript level were further down-regulated upon submergence.

**CONCLUSION**

Collectively, our results characterize the structure, evolutionary, and some functional features of the lotus TPS gene family. Based on systematic analysis of lotus genomes, we identified 9 TPS genes. Gene structure analysis showed that NNU_022788 might be the ancestral member, from which other genes were derived. Regions outside the TPS domain in nine NnTPS genes evolved faster than regions within the TPS domains. Based on phylogenetic tree constructed using TPS genes from lotus, *Arabidopsis*, polar, soybean, and rice, TPS genes could be classified into two main subfamilies (I-II). Lotus TPS genes showed species-specific expanded manner. Three amino acid residues (669, 704, and 753) in TPP domain were identified as the most important residues for the functional divergence of these TPS genes. Moreover, 2 groups (group I-1 and II-2) were believed to have undergone positive selection based on likelihood ratio test. Expression pattern analysis showed that NnTPS genes might mainly have function in sink tissues and two NnTPS genes (including NNU_014679 and NNU_022788) might play important roles in lotus energy metabolism and stress responses.

**AUTHOR CONTRIBUTIONS**

QJ and XH conceived the study; QJ, BW, and XL did data analysis and drafted the manuscript; NM, YW, HJ, and YX revised the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01445

**Figure S1** | Amino acid sequence alignment of three lotus TPS proteins. Strictly conserved sequence is in white on black background; similar amino acids are in black on green background. Residues involved in the catalytic center are placed in boxes.

**Table S1** | List of primers used in this study.

**Table S2** | Detail information of identified motifs.
REFERENCES

Avonce, N., Mendoza-Vargas, A., Morett, E., and Iturriaga, G. (2006). Insights on the evolution of trehalose biosynthesis. BMC Evol. Biol. 6, 109. doi: 10.1186/1471-2148-6-109

Bailey, T. L., and Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc. Int. Conf. Intell. Syst. Mol. Biol. 2, 28–36.

Blazquez, M. A., Santos, E., Flores, C. L., Salinas, J., and Gancedo, C. (1998). Isolation and molecular characterization of the Arabidopsis TPS1 gene, encoding trehalose-6-phosphate synthase. Plant J. 13, 685–689. doi: 10.1046/j.1365-313X.1998.00065.x

Cao, J., Han, X., Zhang, T. C., Yang, Y. P., Huang, J. L., and Hu, X. Y. (2014). Genome-wide and molecular evolution analysis of the subtilase gene family in Vitis vinifera. BMC Genomics 15, 1116. doi: 10.1186/1471-2164-15-1116

Chary, S. N., Hicks, G. R., Choi, Y. G., Carter, D., and Raikhel, N. V. (2004). Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in Arabidopsis. Plant Physiol. 146, 97–107. doi: 10.1104/pp.107.107744

Delorge, I., Figueroa, C. M., Fei, R., Lunn, J. E., and Van Dijck, P. (2015). Trehalose-6-phosphate synthase 1 is not the only active TPS in Arabidopsis thaliana. Biochem. J. 466, 283–290. doi: 10.1042/Bj20141322

Eddy, S. R. (1998). Profile hidden Markov models. Bioinformatics 14, 755–763. doi: 10.1093/bioinformatics/14.9.755

Elbein, A. D., Pan, Y. T., Pastuszak, I., and Carroll, D. (2003). New insights on trehalose: a multifunctional molecule. Glycobiology 13, 17R–27R. doi: 10.1093/glycob/cwg047

Force, A., Lynch, M., Pickett, E. B., Amores, A., Yan, Y. L., Postlethwait, J., et al. (1999). Preservation of duplicate gene by complementary, degenerative mutations. Genetics 151, 1531–1545.

Garg, A. K., Kim, J. K., Owens, T. G., Ranwala, A. P., Choi, Y. D., Kochian, L. V., Hughes, A. L. (1994). The evolution of functionally novel proteins after gene duplication. Bioinformatics 10, 136–145. doi: 10.1093/bioinformatics/10.2.136

Hu, W., Yang, H. B., Yan, Y., Wei, Y. X., Tie, W. W., Ding, Z. H., et al. (2010). Overexpression of a trehalose-6-phosphate synthase/phosphatase fusion gene enhances tolerance and photosynthesis during drought and salt stress without growth aberrations in tomato. Plant Cell Tiss. Org. 112, 257–262. doi: 10.1007/s12229-011-0225-7

Maksymiec, W. (1998). Effect of copper on cellular processes in higher plants. Photosynthetic Res. 53, 321–342. doi: 10.1023/A:1006818815528

Makšić, M., Dong, X. W., and Wang, X. P. (2017). Analysis of the Arabidopsis TP51 gene reveals tissue-specific expression patterns in Arabidopsis thaliana. Acta Physiol. Plant. 39, 3195–3207. doi: 10.1007/s10457-017-2007-z

Müller, J., Aeschbacher, R. A., Wingler, A., Boller, T., and Wiemken, A. (2001). Biotic and heavy metal stress response in plants: evidence for common signals. FEBS Lett. 519, 1–5. doi: 10.1016/S0014-5793(01)01949-4

Nelumbo nucifera—The long-living sacred lotus (Nelumbo nucifera, Gaertn.). Genome Biol. 14:R41. doi: 10.1186/gb-2013-14-5-r41

Nielsen, R., and Yang, Z. (1998). Likelihood models for detecting positively selected sites and counting evolutionary changes at amino acid sites. J. Mol. Evol. 47, 140–150. doi: 10.1007/s002390050432

Oremland, R. S., and Wolk, J. (1983). Trehalose and trehalase in the long-living sacred lotus (Nelumbo nucifera, Gaertn.). Ann. Rev. Plant Physiol. 34, D227–D230. doi: 10.1146/annurev.pl.34.050183.001215

Planta 228, 191–201. doi: 10.1007/s00425-011-00729-x

Trehalose and trehalase in the long-living sacred lotus (Nelumbo nucifera, Gaertn.). Genome Biol. 14:R41. doi: 10.1186/gb-2013-14-5-r41

Mourato, M. P., Martins, L. L., and Campos-Andrade, M. P. (2009). Physiological responses of Lupinus luteus to different copper concentrations. Biol. Plant. 53, 105–111. doi: 10.1007/s10535-009-0014-2

Mühlhoffel, A., Schulze, B., and Boland, W. (2004). Biotic and heavy metal stress response in plants: evidence for common signals. FEBS Lett. 566, 1–5. doi: 10.1016/j.febslet.2004.04.011

Mondragon-Palomino, M., and Gau, B. S. (2005). Gene conversion and the evolution of three leucine-rich repeat gene families in Arabidopsis thaliana. Mol. Biol. Evol. 22, 2444–2456. doi: 10.1093/molbev/msa241

Mortarato, M. P., Martins, L. L., and Campos-Andrade, M. P. (2009). Physiological responses of Lupinus luteus to different copper concentrations. Biol. Plant. 53, 105–111. doi: 10.1007/s10535-009-0014-2

Müller, J., Aeschbacher, R. A., Wingler, A., Boller, T., and Wiemken, A. (2001). Trehalose and trehalase in Arabidopsis. Plant Physiol. 125, 1086–1093. doi: 10.1104/pp.125.2.1086

Mu, M., Lu, X.-K., Wang, J.-J., Wang, D.-L., Yin, Z.-J., Wang, S., et al. (2016). Genome-wide identification and analysis of the stress-resistance function of the TPS (Trehalose-6-Phosphate Synthase) gene family in cotton. BMC Genet. 17:54. doi: 10.1186/s12863-016-0360-y

Nielsen, R., and Yang, Z. (1998). Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. Genetics 148, 929–936.

Nuruzzaman, M., Manimekalai, R., Sharomi, A. M., Satoh, K., Kondoh, H., Ooka, H., et al. (2010). Genome-wide analysis of NAC transcription factor family in rice. Genes 465, 30–51. doi: 10.1104/pp.109.109322

Paul, M. J., Primavera, L. F., Ihurretza, D., and Zhang, Y. (2008). Trehalose metabolism and signaling. Annu. Rev. Plant Biol. 59, 417–441. doi: 10.1146/annurev.arplant.59.032607.092945

Ramón, M., De Smet, I., Vaesteneere, L., Naudts, M., Laymen, B., Dijck, P. V., et al. (2009). Extensive expression regulation and lack of heterologous enzymatic activity of the Class II trehalose metabolism proteins from Arabidopsis thaliana. Plant Cell Environ. 32, 1015–1032. doi: 10.1111/j.1365-3040.2009.01985.x

Ross, C. A., Liu, Y., and Shen, Q. X. J. (2007). The WRKY gene family in rice (Oryza sativa). J. Integr. Plant Biol. 49, 827–842. doi: 10.1111/j.1744-7909.2007.00500.x
Suzuki, N., Bajad, S., Shuman, J., Shulaev, V., and Mittler, R. (2008). A trehalose metabolic enzyme controls inflorescence architecture in maize. *Nature* 441, 227–230. doi: 10.1038/nature04725

Schlupekmann, H., Van, D. A., Aghdasi, M., Wobbes, B., Paul, M., and Smeekens, S. (2004). Trehalose mediated growth inhibition of Arabidopsis seedlings is due to trehalose-6-phosphate accumulation. *Plant Physiol.* 135, 879–890. doi: 10.1104/pp.104.039503

Shima, S., Matsui, H., Tahara, S., and Imai, R. (2007). Biochemical characterization of rice trehalose-6-phosphate phosphatases supports distinctive functions of these plant enzymes. *FEBS J.* 274, 1192–1201. doi: 10.1111/j.1742-4682.2007.05658.x

Song, J., Gao, Z. H., Huo, X. M., Sun, H. L., Xu, Y. S., Shi, T., et al. (2015). Genome-wide identification of the auxin response factor (ARF) gene family and expression analysis of its role associated with pistil development in Japanese apricot (*Prunus mume* Sieb. et Zucc.). *Acta Physiol. Plant.* 37:145. doi: 10.1007/s11738-015-1882-z

Sonnhhammer, E., Eddy, S., Birney, E., Bateman, A., and Durbin, R. (1998). Pfam: multiple sequence alignments and HMM-profiles of protein domains. *Nucleic Acids Res.* 26, 322–325. doi: 10.1093/nar/26.1.320

Suzuki, N., Bajad, S., Shuman, J., Shulaev, V., and Mittler, R. (2008). The transcriptional co-activator MBF1c is a key regulator of thermotolerance in *Arabidopsis thaliana*. *J. Biol. Chem.* 283, 9269–9275. doi: 10.1074/jbc.M709187200

Tuskan, G. A. (2006). The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313, 1596–1604. doi: 10.1126/science.1128691

Vandesteene, L., Ramon, M., Le Roy, K., Van Dijck, P., and Vandamme, E. (2010). A trehalose metabolic enzyme controls inflorescence architecture in *Arabidopsis thaliana*. *FEBS J.* 274, 322–325. doi: 10.1111/j.1742-4682.2007.05658.x

van Dijken, A. J., Schluepmann, H., and Smeekens, S. C. (2004). Trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. *Plant Physiol.* 135, 969–977. doi: 10.1104/pp.104.039743

Vogel, G., Fiehn, O., Jean-Richard-dit-Bressel, L., Boller, T., Wienken, A., Aeschbacher, R. A., et al. (2001). Trehalose metabolism in *Arabidopsis*: occurrence of trehalase and molecular cloning and characterization of trehalose-6-phosphate synthase homologues. *J. Exp. Bot.* 52, 1817–1826. doi: 10.1093/jxb/52.362.1817

Wang, Q., Wang, M., Zhang, X., Hao, B., Kaushik, S. K., and Pan, Y. (2011). Wrky gene family evolution in *Arabidopsis thaliana*. *Genetica* 139, 973–983. doi: 10.1007/s10709-011-9599-4

Wang, X., Shi, X., Hao, B., Ge, S., and Luo, J. (2005). Duplication and DNA segmental loss in the rice genome: implications for diploidization. *New Phytol.* 165, 937–946. doi: 10.1111/j.1469-8137.2004.01293.x

Xun, G. (2003). Functional divergence in protein (family) sequence evolution. *Genetica* 118, 133–141. doi: 10.1007/978-94-010-0229-5_4

Yang, H. L., Liu, Y. J., Wang, C. L., and Zeng, Q. Y. (2012). Molecular evolution of trehalose-6-phosphate synthase (TPS) gene family in Populus, Arabidopsis and rice. *PLoS ONE* 7:e42438. doi: 10.1371/journal.pone.0042438

Yang, Z. (1998). Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. *Mol. Biol. Evol.* 15, 568–573. doi: 10.1093/oxfordjournals.molbev.a025957

Yang, Z. (2007). PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* 24, 1586–1591. doi: 10.1093/molbev/msm088

Yang, Z., and Bielasawski, J. P. (2000). Statistical methods for detecting molecular adaptation. *Trends Ecol Evol.* 15, 496–503. doi: 10.1016/S0169-5347(00)01994-7

Yang, Z., Gao, Q., Sun, C., Li, W., Gu, S., and Xu, C. (2009). Molecular evolution and functional divergence of HAK potassium transporter gene family in rice (*Oryza sativa* L.). *J. Genet. Genomics* 36, 161–172. doi: 10.1016/S1673-8527(08)60103-4

Yang, Z., Wong, W. S., and Nielsen, R. (2005). Bayes empirical bayes inference of amino acid sites under positive selection. *Mol. Biol. Evol.* 22, 1107–1118. doi: 10.1093/molbev/msi097

Yruela, I. (2009). Copper in plants: acquisition, transport and interactions. *Funct. Plant Biol.* 36, 409–430. doi: 10.1071/FP08288

Zang, B., Li, H., Li, W., Deng, X. W., and Wang, X. (2011). Analysis of trehalose-6-phosphatase synthase (TPS) gene family suggests the formation of TPS complexes in rice. *Plant Mol. Biol.* 76, 507–522. doi: 10.1007/s11103-011-9781-1

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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