RESEARCH ARTICLE

Identification of the trehalose-6-phosphate synthase gene family in winter wheat and expression analysis under conditions of freezing stress

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Abstract

Trehalose plays an important role in metabolic regulation and abiotic stress tolerance in plants. Trehalose contents are potentially modulated by trehalose-6-phosphate synthase (TPS), which is a key enzyme in the trehalose biosynthetic pathway. Using available wheat expressed sequence tag sequence information from NCBI and two wheat genome databases, we identified 12 wheat *TPS* genes and performed a comprehensive study on their structural, evolutionary and functional properties. The estimated divergence time of wheat *TPS* gene pairs and wheat–rice orthologues suggested that wheat and rice have a common ancestor. The number of *TPS* genes in the wheat genome was estimated to be at least 12, which is close to the number found in rice, *Arabidopsis* and soybean. Moreover, it has been reported earlier in other plants that *TPS* genes respond to abiotic stress, however, our study mainly analysed the *TPS* gene family under freezing conditions in winter wheat, and determined that most of the *TPS* gene expression in winter wheat was induced by freezing conditions, which further suggested that wheat *TPS* genes were involved in winter wheat freeze-resistance signal transduction pathways. Taken together, the current study represents the first comprehensive study of *TPS* genes in winter wheat and provides a foundation for future functional studies of this important gene family in Triticeae.

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Introduction

Trehalose (a-D-glucopyranosyl a-D-glucopyranoside) is a nonreducing disaccharide and is widely distributed in actinomycetes, fungi, insects, plants and invertebrates (Elbein *et al.* 2003). The prevalent pathway for trehalose synthesis includes two enzymatic reactions. *TPS* catalyses the synthesis of trehalose-6-phosphate (T6P) from UDP-glucose and glucose-6-phosphate. T6P is subsequently dephosphorylated to form trehalose via trehalose-6-phosphate phosphatase (*TPP*) (Goddijn and van Dun 1999). Trehalose has also been shown to efficiently stabilize dehydrated enzymes, proteins, and lipid membranes, and it promotes cellular integrity and protects biological structures against a variety of environmental stresses, which are associated with desiccation, heat and cold, among other factors (Elbein 1974; Redillas *et al.* 2012). Trehalose is involved in apoptotic cell death in plant (Arora and Singh 2006).

In plants, the *SlTPS1* gene from *Selaginella lepidophylla* can sustain trehalose biosynthesis and plays a major role in heat and salt tolerance (Zentella *et al.* 1999). The cultivated cotton *TPS* gene demonstrates high expression under drought stress (Kosmas *et al.* 2006), just as the maize *TPS* gene is upregulated in response to both salt and cold stress conditions (Jiang *et al.* 2010). *OsTPS1* may enhance the abiotic stress tolerance of rice by increasing the levels of trehalose and proline, and regulating the expression of stress-related genes (Li *et al.* 2011). The introduction of *AtTPS1* reveal a tolerance of tobacco against increases in several abiotic stresses, such as drought, desiccation and temperature stresses (Almeida *et al.* 2005). *AtTPS5* has also demonstrated a correlation with thermotolerance via an interaction with MBF1c, a transcriptional activator and *tps5* mutants are thermosensitive (Suzuki *et al.* 2008). Another *TPS* gene from *Arabidopsis*, *AtTPS6*, can alter cell morphology and plant architectural phenotype, and also functions in drought-tolerance (Chary *et al.* 2008). Iordachescu and Imai (2008)

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performed a comprehensive *in silico* analysis of *Arabidopsis TPS* gene expression in response to various abiotic stresses and their results reveal that most *TPS* genes are differentially regulated in response to abiotic stresses.

A large number of *TPS* genes have been identified and higher plants comprise a series of *TPS* gene families (Lunn 2007). For example, the *Arabidopsis TPS* gene family consists of 11 members (*AtTPS1-11*) (Vandesteene *et al.* 2010), rice consists of 11 members (*OsTPS1-11*) (Zang *et al.* 2011), populus consists of 12 members (*PtTPS1-12*) (Yang *et al.* 2012), and 28 genes for *TPS*s implicating in abiotic stress tolerance are found from pigeonpea (Singh *et al.* 2012). Plant TPS proteins contain both TPS and TPP domains, however, the activity of the TPP domain has been lost during evolution (Vandesteene *et al.* 2010). Wheat (*Triticum aestivum* L.) is one of the most important crop in the world. However, low temperature (LT) has been one of the most important factor limiting the growth and development of wheat (Akladious 2012).

Dongnongdongmai 1 is the only wheat cultivar that can survive winters in Heilongjiang province, winter survival rate in China is greater than 85% (Wang *et al.* 2009; Liu *et al.* 2013). In this study, we identified wheat *TPS* at the genomewide level. We also compared wheat TPS with the genes of members of *Arabidopsis*, rice and soybean, and analysed their phylogenetic relationships. Freeze-tolerant cultivar, Dongnongdongmai 1 and freeze-sensitive cultivar, Jimai 22 were used as an experimental model to analyse the expression patterns of wheat *TPS* genes under cold stress. To the best of our knowledge, this study is the first comprehensive study of *TPS* in wheat which provides a foundation for further functional characterization of this gene family.

Materials and methods

Database retrieval

The protein sequences of each of the 11 members of the *Arabidopsis* and rice *TPS* genes were used as query for TBLASTN searches against the database of expressed sequence tag (EST) (http://www.ncbi.nlm.nih.gov/dbEST/index.html), at an *e*-value of 1e-3 to avoid false positives. The searched *TPS* genes were subsequently assembled using the CAP3 sequence assembly program (http://silkworm.swu.edu.cn/silksoft/cap3.html) (Huang and Madan 1999). The wheat genome sequence was downloaded from the Triticum Aestivum Genome Project (http://mips.helmholtz-muenchen.de/plant/wheat/index.jsp) (Brenchley *et al.* 2012) and International Wheat Genome Sequencing Consortium (http://www.wheatgenome.org/) (Mayer *et al.* 2014). The genome sequence (removed redundant sequence) was used as local blast database. The gene sequences of 11 members of the *Arabidopsis* and rice *TPS* genes were used as query for blast searching against the local blast database. Open reading frames (ORFs) were identified using the ORF Finder at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). All obtained protein sequences of the *TPS* genes were confirmed using the Pfam (http://pfam.sanger.ac.uk/) and Smart (http://smart.embl-heidelberg.de/) databases, and genes without TPS domains were rejected. The cellular localization of each wheat TPS protein was predicted using the PSORT online program (http://psort.hgc.jp/).

Phylogenetic analysis

The protein sequences of wheat, rice, *Arabidopsis* and soybean TPS proteins were aligned using Clustal X 1.83 (Thompson *et al.* 1997). A phylogenetic tree was generated using MEGA 4.0 (Tamura *et al.* 2007) and the neighbour-joining (NJ) method (Saitou and Nei 1987). A bootstrap test was performed with 1000 replicates.

The Ka, Ks, Ka/Ks, and the average indel length were calculated using DnaSP ver. 5 (Librado and Rozas 2009) and the ORF sequences of the wheat and rice gene pairs were aligned using Clustal X 1.83. The divergence time of the duplicated pairs were calculated using the equation $T = Ks/2\lambda$ for rice, with $\lambda = 6.5 \times 10^{-9}$ (Yu *et al.* 2005).

Motif analysis

The conserved motifs of wheat TPS proteins were identified using the Multiple Em for Motif Elicitation (MEME) ver. 4.8.1 (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) (Timothy *et al.* 2009) with the following parameters: maximum number of motifs, 20; motif width, 6–50. The obtained motifs were annotated using the InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) search program.

Plant growth and stress treatment

The first variety analysed was Dongnongdongmai 1 (hexaploid, cultivated by Northeast Agriculture University), which was cultivated and validated by the Heilongjiang province (45°7′N∼126°6′E) Crop Variety Validation Committee in 2007. Dongnongdongmai 1 is the only winter wheat variety that can be cultivated in China's cold regions. Over the winters, the regrowth ratio average of this variety exceeded 70%, and even reached 90% in some years. The second variety analysed was Jimai 22 (hexaploid, cultivated by Shandong province Academy of Agriculture Science). In cold areas, the returning green ratio of Jimai 22 was 0% for six consecutive years (2007–2012). The plants were cultivated in plastic containers (length × width × height = 28 cm × 20 cm × 8 cm), and placed in a growth chamber with the temperature parameters set at 25 ± 1°C (15-h photoperiod) during the day and 20 ± 1°C during the night, while the relative humidity was 70% ± 5% and an illumination intensity 250 μmol/m^2/s^1. Under these conditions, the plants were grown to the third-leaf stage. Cold acclimation was performed by subjecting the germinated seedlings to a temperature of 6 ± 1°C during the day (10-h photoperiod and 175 μmol/m^2/s^1) and 4 ± 1°C during the night for

30 days, and all other conditions were kept constant. After cold acclimation seedlings were placed in a freezing chamber, and set to −20°C (12-h light / 12-h dark, 70 μmol/m^2/s^1 light intensity), then the roots, stems and leaves of two winter wheat varieties were harvested at 0, 1, 4, 8, 12 and 24 h. The seedlings were held at 4°C (12-h light / 12-h dark, 70 μmol/m^2/s^1 light intensity, 70% ± 5% relative humidity) in growth chambers as the control. A pool of roots/stems/leaves from 10 wheat seedlings were collected as one biological replicate, and the samples in each point time were performed thrice. They were flash-frozen in liquid nitrogen, and stored at −80°C. Two varieties were used for each time point.

RNA extraction

The frozen roots/stems/leaves tissue of winter wheat was ground in liquid nitrogen using a mortar and pestle. The total RNA was extracted using the RNA simple Total RNA kit (Tiangen Biotech, Beijing, China), according to the manufacturer's protocol. The RNA content was calculated using the Bioanalyzer 2100 algorithm (Agilent Technologies, Palo Alto, USA); high quality (RNA integrity number).

Real-time PCR analysis

Total RNA was extracted using the RNA Simple Total RNA kit (Tiangen Biotech) according to the manufacturer's instructions. DNaseI-treated total RNA was used to synthesize the first-strand cDNA using the Fermentas RevertAid First Strand cDNA Synthesis kit (Fermentas, Beijing, China). Real-time PCR was performed on the ABI Prism 7000 (ABI, Foster City, USA) with a 2× SYBR Green I PCR Master mix. The PCR reaction was performed as follows: 95°C for 5 min; 38 cycles of 95°C for 10 s, 55°C for 20 s and 72°C for 20 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. A pair of primers for each *TPS* gene was designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, USA). Sequences and other information of the specific primers are listed in table 1. The specificity of the primers was determined using melting curve analysis and agarose gel electrophoresis. The relative expression levels were calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{T,\ target} - C_{T,\ actin})_{time\ x} - (C_{T,\ target} - C_{T,\ actin})_{time\ 0}$ (Livak and Schmittgen 2001). Assay of RT-PCR was performed thrice.

Clustering analysis of differential gene expression patterns

Genes with similar expression patterns typically indicate a functional correlation. We performed cluster analysis for RT-PCR data of wheat *TPS* gene expression using the cluster software PermutMatrixEN, and the Pearson correlation method with the average linkage to generate a hierarchical clustering tree. Heat maps were used to describe the expression patterns of *TPS* genes in wheat under −20°C.

Results

Identification of TPS genes in wheat, rice, Arabidopsis and soybean

To identify all putative *TPS* genes in the wheat EST database, we used the *TPS* gene sequences from rice and *Arabidopsis* as bait sequences, and searched the wheat EST database and sequence assembly (table 2). After removing the redundant sequences, 12 *TaTPS* genes were identified. Thirteen *OsTPS* genes were identified by searching the Rice Genome Annotation Project (RGAP). In addition, 11 *AtTPS* genes were identified by searching the TAIR database to examine the expanded pattern of the *TPS* gene family after the monocot–dicot split, 13 *GmTPS* genes were identified by searching the phytozome and compared with the *TPS* genes in rice, *Arabidopsis* and soybean. Details of the gene name, full-length cDNA accession numbers, ORF, number of amino acids, phylogenetic group, N-terminal, subcellular localization, molecular weight (Mw), isoelectric point (pI), N-glycosylation site, and domain composition for each gene are listed in tables 2 and 3. The ORF of *TPS* were all longer than 2100 bp, except for *TaTPS2* and *TaTPS5*,

Table 1. Specific primers sequence list.

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Size (bp)
TaTPS1	GTGCCATTCTTGTAAATCC	GCCTGTGCCGCTTCTCT	99
TaTPS2	TTTACCGCACTCTGCCATCACG	CCAATAGGAAACGCTGCAACCC	190
TaTPS3	AAGTGGGAATTTCCGAGC	CCAGCCGACAAACATCAG	84
TaTPS4	CAAGAAATTCAACCGGGTCA	AATCAAGTCGGCATTCAGCA	124
TaTPS5	AGCTTGCTCTTAGCCTCATC	GTCACTTTCATTGCCACTTG	128
TaTPS6	CCGACAAGGTGCTGGAGGTGAT	CGAAGGTGTGGAAGCCGATGAG	228
TaTPS7	TGCGGCACGACAAGCACTAC	CAAGCGAGACCACCCTAAACC	156
TaTPS8	TTACTGTGGATTCTATTGTTGC	AGTTCGATTGATCGTAGTTTGT	104
TaTPS9	GCAGTGAGGGATGGGATG	ATGGCGAGCAACCTATGAA	136
TaTPS10	ACTACCACCTGCTCGCCCTCCC	GGATGGTGCGGAAGATCTCGGA	111
TaTPS11	GCTGAGGCACGAGAAGCACTAC	CTGAAACCAAATCCCAATCCAA	144
TaTPS12	TGGAGCGTGGAGTCCGTTGCC	GGTGCTCACATACTTGTAATGC	102
GAPDH	TAAGGGTGGTGCCAAGAAGGT	AGCAAGAGGAGCAAGGCAGTT	148

Table 2. The information of *TPS* genes in wheat.

Gene	Accession number cDNA/ gene locus	ORF (bp)	No. of amino acids	Phylogeny group	N-terminal (aa)	Subcellular localization	Mw (kDa)	pI	N-glycosylation site	Domain composition	Chromosome
TaTPS1	ACI16353	2580	859	I-1	MKQRLLUU	Mitochondrial	96.75	5.97	1:T319	Glyco_transf_20 Trehalose_PPase	1AL, 1BL, 1DL
TaTPS2	AK331389	1968	655	I-1	MSADAAGS	Chloroplast	72.48	6.55	2:S27, T447	Glyco_transf_20 Trehalose_PPase	1AL, 1BL, 1DL
TaTPS3	ND	2658	885	I-1	MVIFTKYG	Plasma membrane	98.34	6.19	3:K197, A594, T614	Glyco_transf_20 Trehalose_PPase	1AS, 1BS
TaTPS4	AK332212	2676	891	II-1	MASRSYSN	Cytoplasmic	100.99	5.05	0	Glyco_transf_20 Trehalose_PPase	5BL
TaTPS5	ND	1893	630	II-1	MEQLNAVL	Nuclear	70.73	5.24	0	Glyco_ transf_20 Trehalose_PPase	4AS, 4DL
TaTPS6	AK331757	2613	870	II-1	MUSSSYSN	Nuclear	97.97	5.45	1:S55	Glyco_transf_20 Trehalose_PPase	4AS, 4DL
TaTPS7	ND	2670	889	II-1	MASRSYSN	Cytoplasmic	100.54	7.34	0	Glyco_ transf_20 Trehalose_PPase	5BL
TaTPS8	ND	2961	986	II-2	MATGSAFV	Endoplasmic reticulum	111.75	6.75	4:R461, E654, P756, G760	Glyco_transf_20 Trehalose_PPase	5AS, 5BL, 5DL
TaTPS9	AK334843	2610	869	II-2	MFSRSYTN	Cytoplasmic	98.35	5.88	0	Glyco_transf_20 Trehalose_PPase	5AS, 5BS, 5DL
TaTPS10	ND	2232	743	II-2	MSRSYTNL	Chloroplast	83.45	5.84	0	Glyco_ transf_20 Trehalose_PPase	1AL, 1BL, 1DL
TaTPS11	AK333853	2739	912	II-2	MMSRSYTN	Nuclear	102.25	5.37	0	Glyco_transf_20 Trehalose_PPase	3AL, 3B
TaTPS12	ND	2547	848	II-4	MPSLSCHN	Cytoplasmic	93.88	6.43	3:S42, S44, T401	Glyco_transf_20 Trehalose_PPase	6DL

ND, no cDNA sequence available.

Table 3. *TPS* genes in rice, *Arabidopsis* and soybean.

Gene	Accession number cDNA/gene locus	ORF (bp)	No. of amino acids	Phylogeny group	N-terminal (aa)	Subcellular localization	Mw (kDa)	pI	N-glycosylation site	Domain composition
OsTPS1	Os05g44210	2958	985	I-1	MDTPAPSA	Nuclear	95.21	6.26	2:T3, T444	Glyco_transf_20 Trehalose_PPase
OsTPS2	Os01g54560	2745	914	II-2	MMSRSYTN	Nuclear	102.26	5.73	1:S907	Glyco_transf_20 Trehalose_PPase
OsTPS3	Os01g53000	2637	878	II-2	MFSRSYTN	Cytoplasmic	99.39	5.50	0	Glyco_transf_20 Trehalose_PPase
OsTPS4	Os03g12360	2583	860	II-1	MUSRSYSN	Nuclear	96.63	5.76	2:S54, S56	Glyco_transf_20 Trehalose_PPase
OsTPS5	Os02g54820	2253	750	II-4	MPSLSCHN	Nuclear	98.17	7.35	1:T403	Glyco_transf_20 Trehalose_PPase
OsTPS6	Os05g44100	2700	899	II-2	MMSRSYTN	Chloroplast	100.53	5.48	0	Glyco_transf_20 Trehalose_PPase
OsTPS7	Os08g31980	2589	862	II-3	MUSKSYSN	Nuclear	97.64	6.19	0	Glyco_ transf_20 Trehalose_PPase
OsTPS8	Os08g34580	2475	824	II-4	MPSLPNSG	Cytoplasmic	91.50	5.76	2:T17, S806	Glyco_transf_20 Trehalose_PPase
OsTPS9	Os09g25890	2661	886	II-4	MPHHRHLT	Cytoplasmic	96.28	7.38	4:S10, T18, S22, T24	Glyco_transf_20 Trehalose_PPase
OsTPS10	Os09g23350	2658	885	II-1	MUSRSYSN	Nuclear	99.44	5.83	0	Glyco_transf_20 Trehalose_PPase
OsTPS11	Os09g20990	2592	863	II-3	MUSKSYTN	Nuclear	97.81	5.96	0	Glyco_transf_20 Trehalose_PPase
AtTPS1	At1g78580	2829	942	I-1	MPGNKYNC	Chloroplast	105.97	6.70	3:T408, S890, S941	Glyco_transf_20 Trehalose_PPase
AtTPS2	At1g16980	2466	821	I-2	MDYDDARG	Chloroplast	92.87	5.61	0	Glyco_transf_20 Trehalose_PPase
AtTPS3	At1g17000	2352	783	I-2	MGYDNUCG	Cytoplasmic	89.46	7.34	0	Glyco_transf_20 Trehalose_PPase
AtTPS4	At4g27550	2388	795	I-2	MARPRLLU	Chloroplast	89.47	6.09	ND	Glyco_transf_20 Trehalose_PPase
AtTPS5	At4g17770	2589	862	II-1	MUSRSYSN	Chloroplast	97.45	5.76	ND	Glyco_transf_20 Trehalose_PPase
AtTPS6	At1g68020	2583	860	II-1	MUSRSYSN	Nuclear	97.70	5.90	2:S17, S814	Glyco_transf_20 Trehalose_PPase
AtTPS7	At1g06410	2556	851	II-2	MISRSYTN	Chloroplast	96.69	5.59	ND	Glyco_transf_20 Trehalose_PPase
AtTPS8	At1g70290	2571	856	II-3	MUSRSCAN	Nuclear	97.56	5.60	ND	Glyco_ transf_20 Trehalose_PPase
AtTPS9	At1g23870	2604	867	II-3	MUSRSCAN	Chloroplast	98.49	5.92	ND	Glyco_transf_20 Trehalose_PPase
AtTPS10	At1g60140	2586	861	II-3	MGSKSFGN	Nuclear	97.32	6.16	2:S459, S844	Glyco_transf_20 Trehalose_PPase
AtTPS11	At2g18700	2589	862	II-4	MSPESWKD	Nuclear	98.27	6.96	1:S823	Glyco_transf_20 Trehalose_PPase
GmTPS1	Gm08g12760	2760	919	I-1	MPGNNFNC	Peroxisomal	103.98	6.59	1:S806	Glyco_transf_20 Trehalose_PPase
GmTPS2	Gm05g29651	2763	920	I-1	MPGNNYNC	Mitochondrial	104.58	6.54	1:S806	Glyco_transf_20 Trehalose_PPase
GmTPS3	Gm13g33970	2802	933	I-1	MUGFQSDH	Cytoplasmic	105.38	7.90	1:T416	Glyco_transf_20 Trehalose_PPase
GmTPS4	Gm12g36280	2748	915	I-1	MUGFQSDH	Chloroplast	103.62	6.43	1:T146	Glyco_transf_20 Trehalose_PPase
GmTPS5	Gm02g09480	2241	746	II-1	MUSKSYSN	Nuclear	85.45	5.98	ND	Glyco_transf_20 Trehalose_PPase
GmTPS6	Gm12g15500	2589	862	II-2	MMSKSYTN	Chloroplast	97.93	5.85	ND	Glyco_transf_20 Trehalose_PPase
GmTPS7	Gm06g42820	2589	862	II-2	MMSRSYTN	Chloroplast	97.78	5.85	ND	Glyco_transf_20 Trehalose_PPase
GmTPS8	Gm02g03820	2583	860	II-3	MASRSYUN	Cytoplasmic	97.43	6.47	1:S612	Glyco_transf_20 Trehalose_PPase
GmTPS9	Gm08g39870	2586	861	II-3	MASRSYAN	Nuclear	96.85	5.75	1:S613	Glyco_transf_20 Trehalose_PPase
GmTPS10	Gm18g18590	2586	861	II-3	MASRSYAN	Nuclear	96.97	5.83	1:S613	Glyco_transf_20 Trehalose_PPase
GmTPS11	Gm06g19590	2598	865	II-3	MUARSCLN	Nuclear	98.02	5.71	2:S612, S774	Glyco_transf_20 Trehalose_PPase
GmTPS12	Gm13g01420	2217	738	II-4	MWHNKISP	Chloroplast	83.64	6.23	2:S496, S658	Glyco_transf_20 Trehalose_PPase
GmTPS13	Gm17g07530	2568	855	II-4	MLSRSCLG	Chloroplast	96.52	5.65	1:S776	Glyco_transf_20 Trehalose_PPase

ND, no cDNA sequence available.

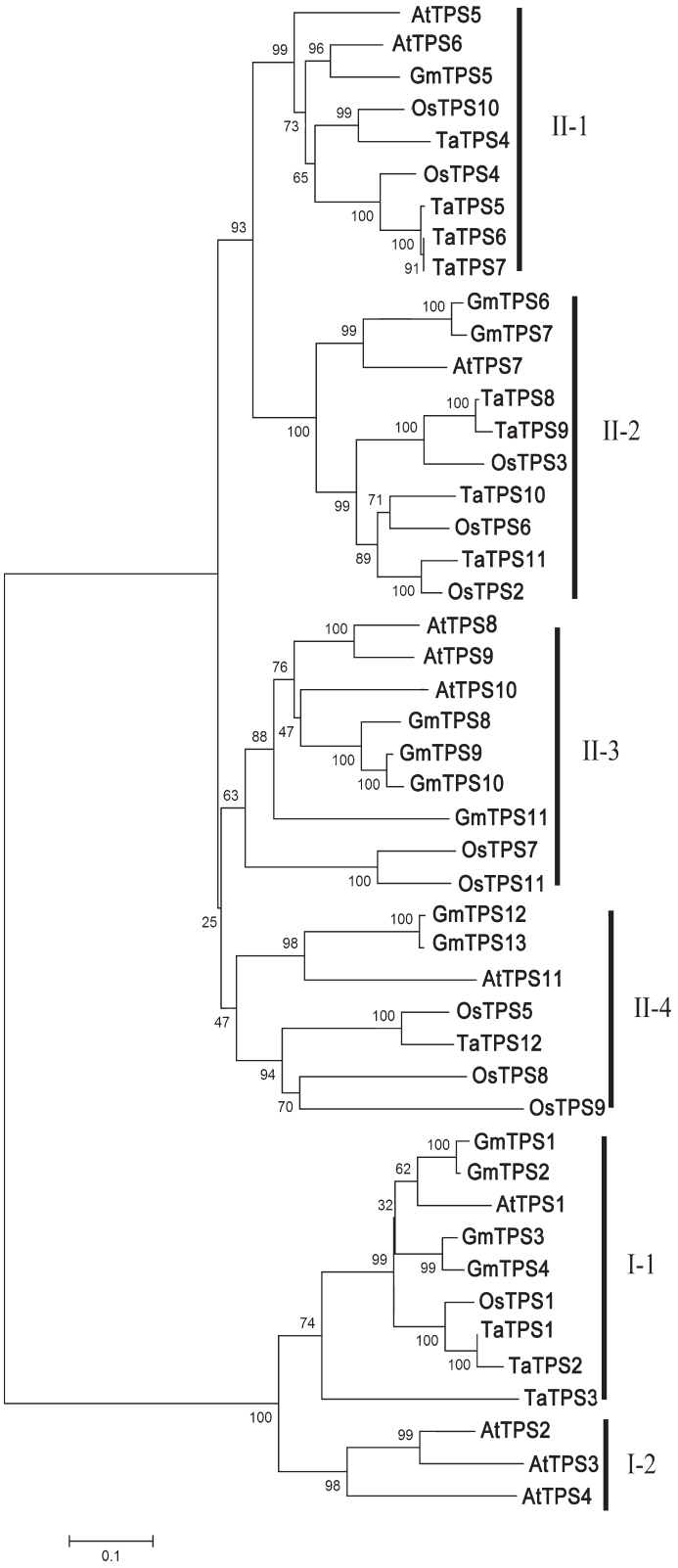

Figure 1. Phylogenetic analysis of the *TPS* gene family in wheat, rice, *Arabidopsis* and soybean. The joint unrooted phylogenetic tree containing 12 wheat, 11 rice, 11 *Arabidopsis* and 13 soybean *TPS* genes was constructed using neighbour-joining methods with bootstrap values from 1000 replicates. The scale bar represents 0.2 amino acid substitutions per site.

which corresponded to the *TPS* protein of >700 amino acids. *TPS* genes exhibited a variable N-terminal domain and several functional domains which mainly consisted of the Glyco_transf_20, while Trehalose_PPase domain was located in the C-terminal. In total, 47 *TPS* genes were analysed according to their subcellular localization using the online program. We found three *TPS* gene located in the peroxisome, plasma membrane and endoplasmic reticulum, respectively, two in the mitochondria, 10 in the cytoplasm, 14 in the chloroplast and 18 in the nucleus. Among these genes in wheat, there were three *TPS* genes that were located in the mitochondria, plasma membrane and endoplasmic reticulum, respectively, two in the chloroplast, four in the cytoplasm and three in the nucleus. Nine *TaTPS* genes (*TaTPS1*, *TaTPS2*, *TaTPS3*, *TaTPS5*, *TaTPS6*, *TaTPS8*, *TaTPS9*, *TaTPS10* and *TaTPS11*) had their homeologous genes, but were not found in *TaTPS4*, *TaTPS7* and *TaTPS12* (table 2).

Evolutionary analysis of TPS gene family in wheat, rice, Arabidopsis and soybean

A phylogenetic tree was constructed using the protein sequences of *TPS* genes in wheat, rice, *Arabidopsis* and soybean (figure 1). The phylogenetic tree revealed that the *TPS* genes in wheat, rice, *Arabidopsis* and soybean may be classified into two subfamilies (I and II). While subfamily I can be further classified into two subgroups I-1 and I-2, group II can be further classified into four subgroups II-1, II-2, II-3, and II-4. Subgroup I-2 consisted of three members (derived from *Arabidopsis*), subgroup I-1 consisted of nine members (one from rice, one from *Arabidopsis*, four from soybean and three from wheat); subgroup II-1 consisted of nine members (two from rice, four from wheat, two from *Arabidopsis* and one from soybean), subgroup II-2 consisted of 10 members (three from rice, four from wheat, two from soybean and one from *Arabidopsis*), subgroup II-3 consisted of nine members (three from *Arabidopsis*, two from rice and four from soybean), subgroup II-4 consisting of seven members (one from *Arabidopsis*, one from wheat, two from soybean and three from rice).

Except for subgroup I-2, the rest of the subgroups consisted of at least one monocot and one dicot member, indicating that the presence of *TPS* genes preceded the divergence of monocots and dicots. All members contained the Glyco_transf_20 and Trehalose_PPase domain. Thus, from the phylogenetic tree, we can infer that genes in the same group were evolutionarily close to each other on the basis of the higher bootstrap values, whereas members from different groups were evolutionarily further from one another. This finding indicated an extensive functional evolutionary divergence of the *TPS* gene family.

Evolution history of the wheat TPS gene family

Using phylogenetic analysis, we found that wheat *TPS* genes were similar to rice. To explicitly examine the evolutionary history of the wheat *TPS* gene family and to determine the divergence time of wheat and rice *TPS* genes, we calculated the nonsynonymous (Ka) and synonymous (Ks) mutation rates between wheat and rice orthologous *TPS* genes (Librado and Rozas 2009). Using a divergence rate of 6.5×10^{-9} mutations per synonymous site per year (Gaut *et al.* 1996), we determined the divergence time of 11 pairs of wheat–rice orthologues. Results shown in table 4 indicate that the speciation time for wheat–rice *TPS* orthologues was between 31 and 82 million years. The following calculation estimated the wheat–rice orthologues and determined that the average divergence time was not included in the divergence time of *TaTPS10* and *OsTPS6* owing to the estimated separation time of these two genes (82 million years) was much earlier than the known speciation time for wheat and rice (50–70 million years). Next, we used the remaining speciation time to calculate the wheat–rice orthologues and determined that the average divergence time was ~39 million years.

Table 4. Estimated divergence time between wheat–rice *TPS* orthologues.

Gene pair	Ka	Ks	Ka/Ks	Divergence time (million years)
TaTPS11 vs *OsTPS2*	0.0591	0.5491	0.107631	42
TaTPS10 vs *OsTPS6*	1.5802	1.0768	1.467496	82
TaTPS9 vs *OsTPS3*	0.0580	0.5167	0.112251	39
TaTPS8 vs *OsTPS3*	0.0932	0.5683	0.163997	39
TaTPS4 vs *OsTPS10*	0.1257	0.5829	0.215646	44
TaTPS6 vs *OsTPS4*	0.0783	0.4032	0.194196	31
TaTPS7 vs *OsTPS4*	0.1338	0.4060	0.329557	31
TaTPS5 vs *OsTPS4*	0.1125	0.4536	0.248016	31
TaTPS12 vs *OsTPS5*	0.0777	0.6779	0.114619	52
TaTPS1 vs *OsTPS1*	0.0374	0.5203	0.071882	40
TaTPS2 vs *OsTPS1*	0.0707	0.5331	0.132621	41

Ka, nonsynonymous substitution rate; Ks, synonymous substitution rate.

Table 5. Sequence of motifs identified and functions predicted from wheat TPS proteins.

Motif	Multilevel consensus sequence	Contain the motif of gene	Function
1	GFFLHSPFPSSEIYRTLPVREEILRALLNCDLIGFHTFDYARHFLSCCSR	TPS1,TPS2,TPS3,TPS4,TPS5,TPS6,TPS7,TPS8,TPS9,TPS10,TPS11,TPS12	Glyco_transf_20
2	AIRVNPWNIEAVAEAMNCALTMSEKEKNLRHEKHYRYVSTHDVQYWANSF	TPS1,TPS2,TPS3,TPS4,TPS5,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	Glyco_transf_20
3	VMLGVDDMDIFKGINLKILAFEKMLKQHPEWRGKAVLVQIANPARGKGKD	TPS1,TPS2,TPS3,TPS4,TPS5,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	Glyco_transf_20
4	WMQMAEPVMNLYTETTDGSYIEHKETALVWHHQDADPDFGSCQAKEMLDH	TPS1,TPS3, TPS4,TPS6,TPS7,TPS8,TPS9,TPS10,TPS11,TPS12	Trehalose_PPase
5	EVINPEDDYVWCHDYHLMALPTFLRKRFN	TPS1,TPS2,TPS3,TPS4,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	Glyco_ transf_20
6	NERFGTPGYSPIVLIDRPLQFHEKCAYYTIAECCVVTAVRDGMNLIPYEY	TPS1,TPS2,TPS3,TPS4,TPS5,TPS6,TPS7,TPS8,TPS9,TPS10,TPS11,TPS12	Glyco_transf_20
7	IPAEEQDQVAQNLYDRFRCVPVFLPEDLHHRFYHGFCKQHLWPLFHYMLP	TPS1,TPS2,TPS3,TPS4,TPS5,TPS6,TPS7,TPS9,TPS11,TPS12	Glyco_transf_20
8	MLGIEYQSKRGYIGLEYYGRTVTIKIMPVGIHMGQLQAVLCLPETQWKVA	TPS3,TPS4,TPS5,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	Glyco_transf_20
9	CWGIGFGFGFRVVALDPNFKKLAMEHIVMAYRKSKTRAILLDYDGTLMPQ	TPS3,TPS4,TPS5,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	HAD-like domain
10	ESVLANEPVSVKSGQHIVEVKPQGVSKGLVAEKMLSSMQEKGKQPDFVLC	TPS3,TPS4,TPS5,TPS6,TPS7,TPS8,TPS9,TPS10,TPS11,TPS12	Trehalose_PPase
11	NILNSLCQDQKNIVFICSGRGRCTLDEWF	TPS1,TPS3,TPS4,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	UDP-forming
12	PKTPVFACTVGQKPSKAKYYLDDTYDVVNMLQALAEVSEP	TPS1,TPS3,TPS4,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	UDP-forming
13	KSMLVVSEFIGCSPS	TPS1,TPS2,TPS3,TPS4,TPS5,TPS6,TPS7,TPS8,TPS9,TPS11,TPS12	UDP-forming
14	PCPNLGIAAEHGYFMR	TPS1,TPS3,TPS4,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	UDP-forming
15	GGRFDRSQWQAYVSANKMFAD	TPS1,TPS2,TPS3,TPS4,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	Trehalose_PPase
16	IQDLERTCKDHFNRR	TPS3,TPS4,TPS5,TPS6,TPS7,TPS9,TPS10,TPS11,TPS12	No hits found
17	IGDDRSDEDMFENIA	TPS3,TPS4,TPS6,TPS8,TPS9,TPS10,TPS11,TPS12	UDP-forming
18	RTIVVANQLPIRACRRADGQW	TPS1,TPS2,TPS3,TPS4,TPS6,TPS7,TPS9,TPS11,TPS12	UDP-forming

Table 6. Motif distribution in *TaTPS* genes.

Gene	Motif
TaTPS1	1, 2, 3, 4, 5, 6, 7, 11, 12, 13, 14, 15, 18
TaTPS2	1, 2, 3, 5, 6, 7, 13, 15, 18
TaTPS3	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18
TaTPS4	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18
TaTPS5	1, 2, 3, 6, 7, 8, 9, 10, 13, 16
TaTPS6	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18
TaTPS7	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18
TaTPS8	1, 4, 6, 10, 13, 17
TaTPS9	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18
TaTPS10	1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 14, 15, 16, 17
TaTPS11	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18
TaTPS12	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18

Motif analysis

A total of 18 putative conserved motifs of wheat TPS proteins with E-values $<1.00e^{-10}$ was identified using the MEME online program (tables 5 and 6). *TaTPS3*, *TaTPS4*, *TaTPS6*, *TaTPS9*, *TaTPS11* and *TaTPS12* contain all 18 motifs; *TaTPS1* does not contain motifs 8, 9, 10, 16 and 17; *TaTPS2* contain motifs 1, 2, 3, 5, 6, 7, 13, 15 and 18; *TaTPS5* contain motifs 1, 2, 3, 6, 7, 8, 9, 10, 13 and 16; only *TaTPS7* did not contain motif 17; *TaTPS8* contain motifs 1, 4, 6, 10, 13 and 17; and *TaTPS10* did not contain motifs 7, 13 and 18. Importantly, genes in the same group in the phylogenetic tree contained a similar number of motifs and categories. The distribution of the motifs also differed within each group, which indicated functional divergence among the groups during evolution. To investigate the relationships between motifs and protein function, we further analysed the conserved motifs, which are function-specific, by performing a search in the InterProScan database. We found that motif 9 exhibited a HAD-like domain (trehalose-6-phosphate phosphatase) function, and motifs 11–14, 17, and 18 have a UDP-forming ($\alpha.\alpha$-trehalose-phosphate synthase) function; however, no hit was found for motif 16. The other motifs were all found to be functionally associated with the Glyco_ transf_20 (Glycosyl transferase, family 20) or Trehalose_PPase (Trehalose-phosphatase) domain.

Expression analysis of the winter wheat TPS gene family under freezing stress conditions

To determine the expression pattern of wheat *TPS* genes in response to freezing stress conditions, real-time PCR analysis was performed using the total RNA of the winter wheat cultivar Dongnongdongmai 1 (freeze-resistance) and Jimai 22 (freeze-sensitive). Under normal growth conditions, we analysed their response to freezing conditions (−20°C) at 0, 1, 4, 8, 12 and 24 h. The expression of all *TPS* genes were detected in the roots, stems and leave of two varieties and showed altered expression patterns of either induction or suppression associated with at least one time node (figure 2).

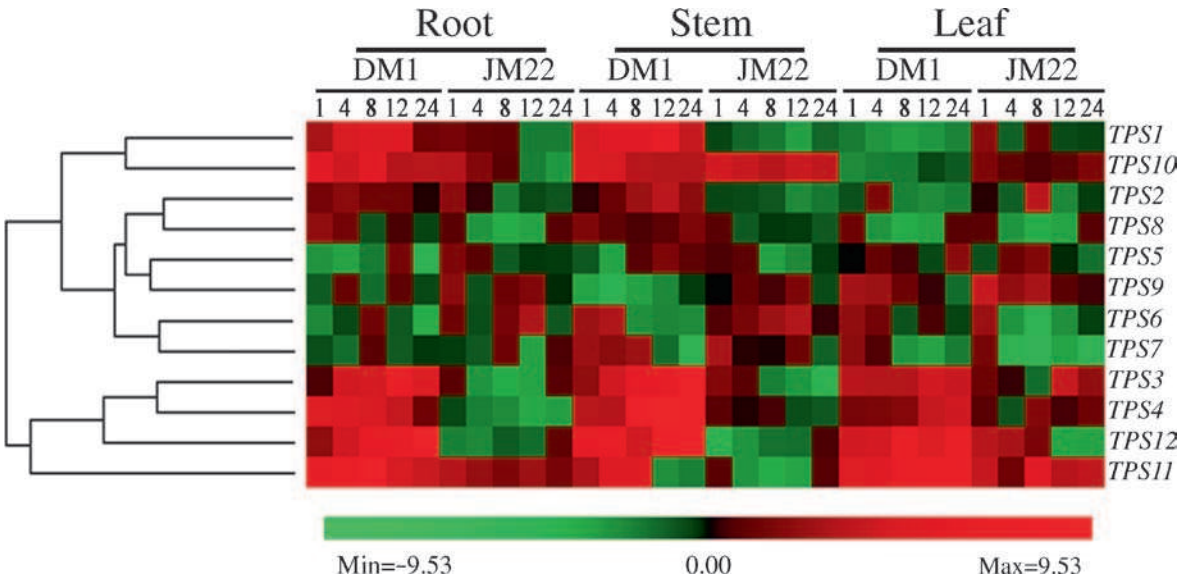

Figure 2. Expression patterns of *TaTPS* gene family under conditions of cold (−20°C) stress. 0, 1, 4, 8, 12 and 24 h indicate the sampling time after treatment. DM1, Dongnongdongmai 1; JM22, Jimai 22.

In the root of Dongnongdongmai 1, *TaTPS1*, *2*, *3*, *4*, *10*, *11* and *12* showed upregulated expression under −20°C treatment, but *TaTPS2* demonstrated no significant changes. However, all these genes had different degrees of downregulated expression in Jimai 22, except for *TaTPS11*. The expression patterns of *TaTPS5*, *6*, *7*, *8* and *9* were not induced by freezing conditions in the two varieties.

In the stem of Dongnongdongmai 1, *TaTPS1*, *2*, *3*, *4*, *8*, *10*, and *12* showed upregulated expression, and *TaTPS9* was detected as downregulated, whereas *TaTPS6*, *7* and *11* displayed upregulation in the earlier time points but subsequent downregulation following the extension of freeze treatment time. Nevertheless, in Jimai 22, *TaTPS6* and *TaTPS10* showed upregulated expression, and *TaTPS1* and *TaTPS2* showed downregulation. *TaTPS3*, *4*, *5*, *6*, *7* and *9* displayed initial upregulation and subsequent downregulation, but *TaTPS11* and *TaTPS12* had the opposite expression pattern for the six genes. *TaTPS1*, *2*, *3*, *4*, *8* and *12* all showed higher expression level in Dongnongdongmai 1 than Jimai 22.

In the leaf of Dongnongdongmai 1, *TaTPS3* and *TaTPS4* showed upregulated expression but freezing conditions induced no significant change. These two genes displayed varying degrees of downregulated expression in Jimai 22; *TaTPS11* and *TaTPS12* showed continued upregulation of expression in Dongnongdongmai 1 and even higher expression compared to Jimai 22. In contrast, *TaTPS10* demonstrated downregulated expression in Dongnongdongmai 1, and the opposite was observed in Jimai 22; *TaTPS5*, *7* and *9* showed an initially high and subsequent low expression pattern and this expression pattern was similar in two varieties. *TaTPS2* and *TaTPS6* expression pattern showed no regular change in two varieties.

Discussion

TPS are ubiquitous in actinomycetes, fungi, insects, plants and invertebrates, and are essential for many abiotic stresses and during growth and development via its participation in various signal transduction pathways (O'Hara *et al.* 2013; Lyu *et al.* 2013). In this study, we characterized this gene family in winter wheat. Using existing wheat EST information and two wheat genome databases, we obtained full-length cDNAs for 12 *TPS* genes. The main structure and functional domains of wheat *TPS* were similar to rice, *Arabidopsis* and soybean. We constructed a phylogenetic tree of the *TPS* gene in four species (include two monocots and two dicots). The *TPS* genes were divided into two subfamilies, which were further divided into six subgroups, and the bootstrap values of each group were low, which indicated that each group had a common ancestor. Remarkably, wheat and rice were included in every branch, which indicated that the *TPS* genes in wheat and rice had similar evolutionary patterns and divergence trends that occurred prior to the species split. Estimation of the divergence time according the phylogenetic tree revealed that the bootstrap of rice and wheat gene pairs was lower, and using wheat–rice gene pairs, the wheat *TPS* genes divergence time was estimated. Wheat–rice orthologues were used to calculate the average divergence time, which was estimated at ∼39 million years. This approximate dating provided an approximate time of divergence of wheat and rice *TPS* genes, which was similar to the divergence time of cereal plants. Indeed, the estimated divergence time of the majority of putative wheat and rice *TPS* gene orthologues were mostly consistent with the time when the two species diverged from their last common ancestor. When we used MEME to identify and

examine the conserved motifs in all 12 wheat *TPS* genes, we found a total of 18 conserved motifs, including trehalose-6-phosphate phosphatase (HAD-like domain), $\alpha.\alpha$-trehalose-phosphate synthase (UDP-forming), glycosyl transferase family (20Glyco_transf_20) and trehalose-phosphatase (Trehalose_PPase) domain. These four domains are all conserved in the *TPS* gene family. We obtained 12 wheat *TPS* genes, which contained all these domains, further confirming that the 12 genes were *TPS*.

Freezing are the major abiotic factors limiting plant area and yield in winter wheat. We performed an extensive study of the tissue-specific expression patterns of winter wheat *TPS* genes under −20°C stresses at the seedling stage, and clearly observed their responses to freeze stress. Our results showed that all *TPS* genes were expressed in both freeze-resistant and freeze-susceptible cultivars under freezing conditions. However, our data indicated that most of these *TPS* genes exhibited differentially expressed patterns among the roots, stems and leaves of the resistant and susceptible wheat cultivars (figure 2). The *TaTPS1*, *2*, *4* and *12* expression quantity showed all upregulation expression in the root and stem in Dongnongdongmai 1 and significantly higher than Jimai 22 under −20°C stress. Thus, we inferred that these genes were associated with the strong freeze hardiness of Dongnongdongmai 1.

Recently, *TPS* homologs have been detected in a variety of plant species (Wang *et al.* 2005; Kosmas *et al.* 2006; Jiang *et al.* 2010; Vandesteene *et al.* 2010; Zang *et al.* 2011). Some rice, *Arabidopsis* and soybean *TPS* gene have been previously characterized, and a phylogenetic tree has been constructed with members of rice, *Arabidopsis* and soybean. *TPS* genes could be further divided into two subfamilies; group I-2 of the phylogenetic tree contained three *Arabidopsis TPS* genes (*AtTPS2*, *3* and *4*), they do not exhibit *TPS* activity (Blazquez *et al.* 1998). Group I-1 contained one *Arabidopsis TPS* gene (*AtTPS1*), one rice *TPS* gene (*OsTPS1*), four soybean *TPS* genes (*GmTPS1*, *2*, *3* and *4*) and three wheat *TPS* genes (*TaTPS1*, *2* and *3*). Overexpression of the *AtTPS1* gene in *Arabidopsis* confers drought tolerance (Avonce *et al.* 2004) and *OsTPS1* overexpression improved the tolerance of rice seedling to cold, high salinity and drought treatments (Li *et al.* 2011). In our study, the induced significant expressions of *TaTPS1* and *TaTPS3* in the root, stem, leaf and *TaTPS2* in root of Dongnongdongmai 1 under −20°C stress were consistent with the biological function of *AtTPS1* and *OsTPS1*. Moreover, overexpression of *AtTPS1* genes in plants has also been attempted to improve stress tolerance (Avonce *et al.* 2004; Almeida *et al.* 2005). *OsTPS1* can enhance the abiotic stress tolerance of plants by increasing the amount of trehalose and proline, and regulating the expression of stress-related genes (Kim *et al*. 2005; Li *et al*. 2011). Previous studies have shown that *AtTPS1* and *OsTPS1* overexpression in plants will cause phenotypic changes in plant dwarf and delayed flowering (Dijken *et al*. 2004; Gomez *et al*. 2010). Taken together, the results of these studies and our present results suggest that *TaTPS1*, *2* and *3* may play a very important role in wheat stress resistance. We further speculate that these three genes may play a vital role in the strong freeze resistance of Dongnongdongmai 1. However, whether overexpression of these *TPS* genes in winter wheat will affect plant morphological requires further experiments. However, overexpression of some class II *TPS* genes including *OsTPS2*, *4*, *5*, *8* and *9* in transgenic rice have been shown to enhance rice tolerance under conditions of cold and salinity (Li *et al.* 2011). In this study, our phylogenetic tree analytical results showed that class II could be divided into four subgroups, among which *OsTPS2* vs. *TaTPS11*, *OsTPS4* vs. *TaTPS6*, *7*, *OsTPS5* vs. *TaTPS12* in the same subgroup and the same branch, respectively. Due to these wheat *TPS* genes have close relatives with correspond with rice *TPS* genes in class II, suggesting that the above rice and wheat gene pairs may have the same or similar biological functions.

In conclusion, the results obtained in this study provide a strong evidence for *TaTPS* gene family responding to freezing in winter wheat. For winter wheat, functional study of the *TPS* genes is just beginning. Extensive study of *TPS* genes is important for understanding the molecular mechanisms of winter wheat freezing responses, and their utilization in breeding could enhance the freezing resistance of winter wheat cultivars.

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