Analysis of Expression and Bioinformatics of Trehalose-6-phosphate Phosphatases (TPP) Gene Family in Wheat Indicates a Role in Plant Development and Stress Response

Linying Du

Northwest A&F University

Shumin Li

Northwest A&F University

Li Ding

Northwest A&F University

Xinxu Cheng

Northwest A&F University

Zhensheng Kang

Northwest A&F University

Hude Mao (✉ mao_dehu@nwsuaf.edu.cn)

Northwest A&F University

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Abstract

Background: Trehalose-6-phosphate phosphatases genes (*TPPs*) are involved in the development and stress response of plants by regulating the biosynthesis of trehalose, though little is currently known about *TPPs* in common wheat (*Triticum aestivum* L.).

Results: In this study, we performed a genome-wide identification of the *TPP* gene family in common wheat, and identified a total of 31 *TPP* genes. These were subdivided into six subfamilies based on the phylogenetic relationships and the conservation of protein in six monocot and eudicot plants. The majority of *TPP* genes were represented by 2-3 wheat homoalleles (named *TaTPPX_ZA*, *TaTPPX_ZB*, or *TaTPPX_ZD*), where Z is the location on the wheat chromosome of the gene number (X). We also analyzed the chromosomal location, exon-intron structure, orthologous genes, and protein motifs of the *TaTPPs*. The RNA-seq data was used to perform an expression analysis, which found 26 *TaTPP* genes to be differentially expressed based on spatial and temporal characteristics, indicating they have varied functions in the growth and development of wheat. Additionally, we assessed how the promoter regulatory elements were organized and used qRT-PCR in the leaves to observe how they were expressed following ABA, salt, low temperature, and drought stress treatments. All of these genes exhibited differential expression against one or more stress treatments. Furthermore, overexpressing *TaTPP11* in *Arabidopsis* results delayed plant development and enhanced drought tolerance, but not affect seed morphology.

Conclusions: *TaTPPs* could serve important roles in the development and stress response in wheat. These results provide a basis for subsequent research into the function of *TaTPPs*.

Background

Improving crop yields under both positive and negative field conditions is needed to increase worldwide food security. However, increasing the resilience and potential of crop yields at the same time is difficult, since the factors that responsible for stress tolerance and productivity are typically at odds with each other [1]. Carbon allocation and use can improve crop yields under variable conditions. Trehalose, a non-reducing disaccharide, is found in algae, invertebrates, bacteria, plants, fungi, and invertebrates [2]. The fact that trehalose is present in such a variety of life forms, coupled with the various biosynthetic pathways in each, indicates that trehalose metabolism serves an important evolutionary role by guarding the structure of cells and bioactive materials (including nucleic acids, membranes, and proteins) under environmental stressors (including freezing, oxidative, low-temperature, high-temperature, high-saline, and drought conditions) [3–5].

Plants typically only accumulate small levels of trehalose and its intermediates, making it unlikely to play an osmoprotective role [4]. Instead, the trehalose metabolic pathway and its related intermediates detect and regulate energetic status [2, 6]. For example, when it is exogenously applied, trehalose changes the physiology of the organism as well as its gene expression (including the induction of *AGPase* genes found in *Arabidopsis* [7]), and increases the drought tolerance and biomass yield [8–10]. The *ramosa3* mutant of *Zea mays* have significantly reduced trehalose and results excessive branching [11, 12]. Trehalose-6-phosphate (T6P) is an intermediate of the trehalose metabolic pathway and serves an important roles during the signaling of plant sugars, assists in the regulation of the use and allotment of sucrose, and regulates the growth and development of crops [2, 6, 13–15]. Inducing an increase in T6P decreases the degradation of starch in *Arabidopsis*, while T6P alterations regulate flowering patterns and the photoperiod [16]. Recent studies have demonstrated that the relationship between SnRK1 (SNF1-related/AMPK protein kinases) and T6P can significantly alter how carbon is used and allotted in plants. Raising the levels of T6P can induce flux via the biosynthetic pathways responsible for yield and growth, while lowering the levels of T6P can mobilize carbon stores and induce the transport of carbon related to stress response [1, 2, 14, 17]. As such, T6P can be targeted at particular types of tissues and cells during specific developmental stages to increase the resilience and potential of crop yields.

Plants synthesize trehalose by way of a conserved, two-step metabolic pathway. The first step entails the catalyzation of glucose from UDP-glucose to glucose 6-phosphate (G6P) via trehalose-6-phosphate synthase (TPS), resulting in trehalose-6-phosphate (T6P). The T6P is then dephosphorylated into trehalose via trehalose-6-phosphate phosphatase (TPP) [18]. The *TPP* and *TPS* genes have been found in species from all major plant taxa, suggesting that the metabolism of trehalose is likely found throughout the plant kingdom [7, 19–21]. Eleven *TPS* genes are encoded by the rice and *Arabidopsis* genomes, while 10 and 13 *TPP* genes are encoded by the rice and *Arabidopsis* genomes, respectively. There are two kinds of *TPS* genes: Class I *TPS* genes are typically single-copies and encode catalytically active TPS enzymes with both TPP and TPS domains possessing inactive phosphatase boxes; Class II *TPS* genes possess TPP and TPS domains but lack the TPS domain residue necessary to relate to the substrate. The majority of Class II TPS genes possess conserved phosphatase domains, but do not have any TPP or TPS activity [23]. *TPP* genes in all plants are comprised of a particular TPP domain that has conserved phosphatase domains, while all encode functional TPP enzymes in *Arabidopsis*. *TPP* genes share similar activities but differ in their patterns of differential expression, suggesting they could have a function related to specific tissues, stages, or processes [24].

A few *TPP* genes have recently been associated with abiotic stress responses. *AtTPPD* is a plastidial isoform regulated by redox reactions and associated with oxidative stress and salt resistance in *Arabidopsis* [22], while *AtTPPF* and *AtTPPI* are two isoforms associates with drought response [25, 26]. *OsTPP1* and *OsTPP2* are induced by cold stress in rice [27, 28], while the *OsTPP7* gene helps resist anaerobiosis during the germination stage in rice. This trait has been lost from many kinds of commercial varieties, along with the *OsTPP7* gene [29]. The MADS6 promoter

is active during the flowering of reproductive tissue and contributes to the expression of the *OsTPP1* gene. This allows for significant improvements in both grain set and yield during the flowering stage under various drought conditions [13]. A number of TPP proteins serve important roles during plant development. For example, losing maize *RAMOSA3* and *ZmTPP4* reduces the determination of the meristem and increases inflorescence branching [11, 30].

Bread wheat (*Triticum aestivum*; $2n = 6x = 42$; AABBDD) is widely grown and eaten around the globe [31]. The *TPP* genes play important roles in the development and stress response in plants. As such, we conducted a genome-wide analysis of the identification and expression of *TPP* genes in wheat. We produced a phylogenetic tree to assess the evolutionary relationships of TPP with wheat and other species of plants. We analyzed the conserved motifs, gene structures, and expression patterns in the stems, leaves, flag leaves, roots, spikes and grains across various developmental stages, as well as the *cis*-regulatory elements of the promoter sequences and ABA, salt, low temperature, and drought stress responses in wheat. Additionally, we found that *TaTPP11* transgenic overexpressing *Arabidopsis* slowed plant development and increased transgenic drought tolerance. This study provides a basis for subsequent research on the function of *TaTPP* genes.

Results

Identification of the TPP gene family in wheat

We obtained the wheat genome data used by this study from the Chinese Spring IWGSC RefSeq v1.1 reference genome assembly (<https://wheat-urgi.versailles.inra.fr/>). First, we used a UNIX pipeline to convert the wheat genome to a local BLAST database. We then used 23 TPP protein sequences from *Arabidopsis* and rice to execute a BLAST search (BLASTP) with the local blast database, using a cut-off *E*-value $< 1e^{-10}$. After filtering redundant sequences, we analyzed the remaining protein sequences and used the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1). to identify the TPP domain. Lastly, we identified 31 TPP domain containing proteins in the most recent wheat genome (Additional file 1: Table S1). Of these, we assigned 11 clusters to various A, B, or D sub-genomes, which we considered to be homoeologous copies of a single *TPP* gene. Wheat *TPP* genes were labeled as *TaTPPX_ZA*, *TaTPPX_ZB*, or *TaTPPX_ZD*, and *Z* denote the location on the wheat chromosome where the gene number (X) is located. The detailed information of *TaTPP* genes in wheat was listed in Table 1. As shown in Table 1, the identified *TaTPP* genes in wheat encode proteins ranging from 249 (TaTPP5-2A) to 584 (TaTPP7-3D) amino acids (aa) in length with an average of 386 aa. Furthermore, the computed molecular weights of these TaTPP proteins ranged from 28.66 (TaTPP5-2A) to 96.02 (TaTPP7-3D) kDa. The theoretical pI of the deduced TaTPP proteins ranged from 5.53 (TaTPP1-1B and TaTPP1-1D) to 9.26 (TaTPP10-6A).

Table 1
Information on wheat *TaTPP* genes

Gene name	Locus_ID	Chr.	Position (bp)		ORF	Introns	Deduced polypeptide			Trehalose_Ppase	
			Start	End			Length (aa)	MW (Da)	pI	Start (aa)	End (aa)
TaTPP1-1A	TraesCS1A02G210400	1A	372639121	372643307	1146	9	381	42614.46	5.61	119	364
TaTPP1-1B	TraesCS1B02G224300	1B	402147526	402150391	1146	12	381	42660.51	5.53	119	364
TaTPP1-1D	TraesCS1D02G213700	1D	298692275	298696295	1146	9	381	42625.49	5.53	119	364
TaTPP2-2A	TraesCS2A02G161000	2A	111921839	111925164	1074	8	357	39430.21	6.11	106	339
TaTPP2-2B	TraesCS2B02G187000	2B	161722263	161725704	1077	8	358	39621.52	6.04	107	340
TaTPP2-2D	TraesCS2D02G168100	2D	111588533	111591881	1077	8	358	39664.41	5.77	107	340
TaTPP3-2A	TraesCS2A02G161100	2A	112744717	112747766	1077	8	358	39625.56	7.14	107	340
TaTPP3-2B	TraesCS2B02G187100	2B	162007594	162010969	1077	8	358	39606.47	6.57	107	340
TaTPP3-2D	TraesCS2D02G168200	2D	112099169	112102442	1077	8	358	39503.35	6.84	107	340
TaTPP4-2A	TraesCS2A02G161200	2A	113309228	113312068	1077	8	358	39632.62	6.77	107	340
TaTPP4-2B	TraesCS2B02G187200	2B	162445597	162448929	1077	8	358	39653.69	7.12	107	340
TaTPP4-2D	TraesCS2D02G168300	2D	112177996	112181524	1179	9	392	43583.32	6.44	141	374
TaTPP5-2A	TraesCS2A02G167100	2A	119307539	119314162	750	8	249	28665.56	8.28	2	230
TaTPP5-2B	TraesCS2B02G193300	2B	168831609	168853380	1680	11	559	62281.71	8.61	312	540
TaTPP6-2A	TraesCS2A02G412100	2A	669749666	669753186	1113	9	370	41228.87	5.70	112	347
TaTPP6-2B	TraesCS2B02G430700	2B	619679522	619682850	1143	9	380	42099.78	5.75	112	348
TaTPP6-2D	TraesCS2D02G409300	2D	524105415	524108680	1113	7	370	41112.88	5.58	112	347
TaTPP7-3A	TraesCS3A02G085700	3A	55223622	55255982	1662	9	553	61554.27	8.06	306	533
TaTPP7-3D	TraesCS3D02G085800	3D	43259299	43283531	1755	9	584	65022.10	8.86	337	564
TaTPP8-5A	TraesCS5A02G190000	5A	394181080	394183400	1122	5	373	40852.40	8.95	114	345
TaTPP8-5B	TraesCS5B02G193100	5B	348448002	348450302	1122	4	373	40926.44	8.97	114	345
TaTPP8-5D	TraesCS5D02G200800	5D	303758772	303761166	1122	5	373	40860.33	8.96	114	345
TaTPP9-6A	TraesCS6A02G248400	6A	461143866	461147635	1119	8	372	41114.13	5.68	119	355
TaTPP9-6B	TraesCS6B02G276300	6B	500209451	500213722	1119	8	372	41281.38	5.89	119	355

Gene name	Locus_ID	Chr.	Position (bp)		ORF	Introns	Deduced polypeptide			Trehalose_Ppase	
TaTPP9-6D	TraesCS6D02G230500	6D	323712099	323716021	1119	8	372	41047.03	5.56	119	355
TaTPP10-6A	TraesCS6A02G301800	6A	535151913	535154867	1251	8	416	45367.17	9.26	114	341
TaTPP10-6B	TraesCS6B02G330900	6B	581079293	581082545	1224	8	407	44291.96	8.64	154	381
TaTPP10-6D	TraesCS6D02G281100	6D	388537685	388540648	1110	8	369	40252.37	8.79	114	341
TaTPP11-7A	TraesCS7A02G180800	7A	135006112	135008690	1086	9	361	39542.13	8.41	108	335
TaTPP11-7B	TraesCS7B02G085800	7B	97972425	97975038	1095	9	364	40160.77	8.11	108	335
TaTPP11-7D	TraesCS7D02G182600	7D	136013159	136015620	1092	9	363	39907.51	8.60	108	335

The phylogenetic tree of TPP proteins from wheat, maize, rice, *Populus*, *Arabidopsis*, and *B. distachyum*. AMEGA 6.0 was used to build a neighbor-joining tree, which was visualized with the online tool Evolvew. TPPs from one plant species are marked with leaf labels. I-VII denotes the seven plant TPP protein subfamilies. The percentage bootstrap scores were calculated from 1000 replications.

Phylogenetic and syteny analysis of the wheat TPP gene family

We assessed *TPP* genes from various plant species (with fully sequenced whole genomes) to assess the phylogenetic relationship of the *TPP* gene families in both wheat and other species, including monocotyledonous angiosperms maize (*Zea mays*), *Brachypodium distachyon*, and rice (*Oryza sativa*), and the dicotyledonous angiosperms *Arabidopsis thaliana* and poplar (*Populus trichocarpa*). We used 86 TPP proteins to produce a phylogenetic tree, which categorized the TPPs into six subfamilies (I-VII) (Fig. 1; Additional file 2: Figure S1). Subfamily II was comprised of *TPP* genes from six different species, while subfamilies III and IV were comprised only of *TPP* genes from the dicotyledonous angiosperms *Arabidopsis thaliana* and poplar. Subfamilies V, VI, and VII were comprised of *TPP* genes from monocotyledonous angiosperms maize, rice, wheat, and *Brachypodium distachyon*. Analysis of the phylogenetic tree indicates the presence of 4, 9, 6, 3, and 9 TaTPPs in the TPP subfamilies I, II, V, VI, and VII, respectively (Fig. 1).

Genomic comparison is a quick and easy method of transporting genomic information from a well-studied species to a newly-studied species. We used the genomic position information to locate 31 *TaTPP* genes over 17 wheat chromosomes, which ranged from 1 to 5 members per chromosome (Table 1; Fig. 2). We used Holub’s method [32] to identify nine tandem duplication events (*TaTPP2-2A/ TaTPP3-2A*, *TaTPP2-2A/ TaTPP4-2A*, *TaTPP3-2A/ TaTPP4-2A*, *TaTPP2-2B/ TaTPP3-2B*, *TaTPP2-2B/ TaTPP4-2B*, *TaTPP3-2B/ TaTPP4-2B*, *TaTPP2-2D/ TaTPP3-2D*, *TaTPP2-2D/ TaTPP4-2D*, and *TaTPP3-2D/ TaTPP4-2D*) in wheat *TPP* genes, suggesting that certain *TaTPP* genes could be produced via gene duplication (Fig. 2). *Brachypodium distachyon* has a close phylogenetic relationship with wheat, and is considered a model for monocotyledonous angiosperm plants. As such, we performed a syteny analysis between wheat and *Brachypodium distachyon* *TPP* genes to explore their relationship. This analysis of wheat and *Brachypodium distachyon* *TPP* genes identified 14 pairs of sytenic *TPP* genes between *Brachypodium distachyon* and wheat (with *E*-value < 1e⁻⁵), including 17 *TaTPP* genes (*TaTPP1-1A*, *TaTPP1-1D*, *TaTPP3-2A*, *TaTPP3-2D*, *TaTPP6-2A*, *TaTPP6-2D*, *TaTPP7-3A*, *TaTPP7-3D*, *TaTPP8-5A*, *TaTPP8-5D*, *TaTPP9-6A*, *TaTPP9-6D*, *TaTPP10-6A*, and *TaTPP10-6D*) and seven *BdTPP* genes (*BdTPP1*, *BdTPP2*, *BdTPP3*, *BdTPP4*, *BdTPP5*, *BdTPP7*, and *BdTPP8*) (Fig. 2). This suggests that most *TPP* genes existed before *Brachypodium distachyon* and wheat diverged.

Analysis of gene structure and motif composition

The structural divergence of exons and introns served an important role as several families of genes have evolved [33]. We generated a different phylogenetic tree using 31 full-length wheat TaTPP protein sequences to better understand the diversity of the structure of wheat *TaTPP* genes. We divided the wheat TaTPP proteins into five separate subfamilies, according to the above description (Fig. 3A). We next mapped the locations of the exons/introns to the coding regions of each *TaTPP* gene. We found that *TaTPP8-5B* had four introns, *TaTPP8-5A* and *TaTPP8-5D* had five introns, *TaTPP6-2D* had seven introns, *TaTPP5-2B* had eleven introns, and *TaTPP1-1B* had twelve introns. Of the remaining proteins, there were 15 *TaTPP* genes had 8 introns and 10 *TaTPPs* had 9 introns (Fig. 3B). The gene structure of orthologous genes is typically highly conserved, which helps to determine their evolutionary relationships [33]. Wheat *TaTPP* genes in the same subfamily typically have similar gene structures (intron number and exon length), particularly those of subfamily VII, which all had eight introns (Fig. 3B). The hexaploid bread wheat genome was generated by the merging of the *T. urartu* (subgenome A), *Aegilops speltoides* (subgenome B), and *A. tauschii* (subgenome D) genomes hundreds of thousands of years ago. Most of the genes in the A, B, and D sub-genomes (60.1–61.3%) have orthologs in all related diploid genomes. Analysis of the related

intron/exon gene structures based on the phylogenetic tree provided intron gain/loss information for all *TaTPP* genes in the A, B, and D sub-genome. Of these, four clusters altered the structure of their introns/exons, such as *TaTPP1-1A/B/D*, *TaTPP5-2A/B*, *TaTPP6-2A/B/D*, *TaTPP8-5A/B/D* (Fig. 3B). Due to the high number of orthologs in the wheat A, B, and D sub-genomes, the gain or loss of introns in these orthologs complicates the transcriptomes and proteomes found in wheat.

We next used the online MEME tool to identify the conserved motifs and assess the diverse structures of wheat TPP proteins (Fig. 4; Additional file 3: Figure S2), and found 20 conserved protein domains (with $E\text{-value} \leq 1e^{-30}$) across 31 wheat TPP proteins. The TPP domain consisted of motif 5, 6, 7, 8, 9, and 10, which is a common conserved domain located in the C-terminal of all the TaTPP proteins (Additional file 4: Figure S3). Most motifs share orders within the same subfamily, and motifs with similar compositions shared by TaTPP proteins are clustered closely (Fig. 4). This suggests that those members of a particular group have similar functional characteristics.

Subcellular localization of TaTPP proteins in different subfamilies

We further characterized the subcellular localization of four TaTPPs (TaTPP6, TaTPP7, TaTPP9, TaTPP11) that belong to the distinct cluster in the phylogenetic tree shown in Fig. 1. In order to confirm the subcellular localization of these TaTPPs, we developed the 35S::*TaTPP6-GFP*, 35S::*TaTPP7-GFP*, 35S::*TaTPP9-GFP*, and 35S::*TaTPP11-GFP* transient expression vectors to express *TaTPP6-GFP*, *TaTPP7-GFP*, *TaTPP9-GFP*, and *TaTPP11-GFP* fusion proteins in wheat protoplasts, with 35S::*GFP* as positive control. The result was as expected, all four TaTPPs-GFP fusion proteins were located in both cytoplasm and the nucleus (Fig. 5).

Cis -acting regulatory elements in TaTPP promoters

Specific gene expression is primarily regulated by certain promoters, the action of which is mediated by transcription factors via directly binding to *cis*-acting regulatory elements [34]. As such, analyzing upstream regulatory sequences will contribute to a better understanding of how target genes are regulated, allowing us to assess potential functions [35]. We extracted and scanned ~2000 bp of non-coding sequences upstream from the predicted translation start site of each *TaTPP* gene to fully identify the putative *cis*-acting regulatory elements. We used the online software tools PlantCARE and PlantRegMap to locate the abundant regulatory cores associated with responses to hormones, stress, and development (Fig. 6; Additional file 5: Table S2).

We observed significantly enriched hormone-related motifs in the majority of the regulatory regions of the *TaTPP* genes we tested, including abscisic acid (ABRE-element), auxin (TGA-element, AuxRE-core), gibberellin (P-box, GARE-motif and TATC-box), salicylic acid (TCA-element), and methyl jasmonate (TGACG- and CGTCA-motif). Statistical analysis indicated that two kinds of stress-related motifs are involved in abscisic acid and MeJA (methyl jasmonate), which were the most common *cis*-acting hormone-responsive elements. These elements were found in the promoters of most *TaTPP* genes, except *TaTPP5-2B*, *TaTPP6-2D*, *TaTPP7-3D* and *TaTPP10-6D* for ABA response and *TaTPP5-2A*, *TaTPP7-3A*, *TaTPP8-5B*, *TaTPP8-5D*, *TaTPP9-6D*, and *TaTPP11-7D* for MeJA response. Of the 31 *TaTPP* genes, 17 contained both gibberellin-response elements (P-box, GARE-motif and TATC-box) and auxin-response elements (TGA-element or AuxRE-core). We also found the salicylic acid-responsive TCA-element in the promoters of 11 *TaTPP* genes (Fig. 6; Additional file 5: Table S2).

Along with hormone-related motifs, we observed stress elements in the *TaTPP* gene promoters. In particular, elements pertaining to light response were found in all *TaTPP* gene promoters, including G-box, TCT-motif, I-box, Sp1, and MRE. Regarding drought response, seven *TaTPP* gene promoters possessed DRE (dehydration-responsive element) or MBS (MYB binding site involved in drought-inducibility) elements. LTR is a low-temperature response element and is a primary component of the motifs related to stress observed in 16 *TaTPPs* promoters. A WUN-motif wound response element was found in 10 *TaTPP* genes, while the other seven *TaTPPs* genes possessed TC-rich repeats, which are *cis*-acting elements associated with defense and stress responses. Certain *cis*-elements are involved in the specific expression in organs and tissues or with metabolism, including the role of MBS I in flavonoid biosynthetic genes regulation, the role of motif I in root-specific expression, the role of CAT-box in meristem expression, the role of GCN4 motif in endosperm expression, the role of the RY-element in seed-specific regulation, and the role of O2-site in zein metabolism regulation, and the role of MSA-like in cell cycle regulation (Fig. 6; Additional file 5: Table S2). These results indicated that *TaTPP* genes might be involved in plant development, multiple hormone and stress responses.

Tissue-specific expression profiles of TaTPP genes

Gene expression is required for the normal growth and development of healthy plants, and, as a result, is highly regulated. Specific patterns of expression of candidate genes indicate potential roles in both growth and development. We used publicly available RNA-seq data to observe these expression patterns in seedling stems, seedling roots, seedling leaves, flag leaves, and during two stages of spike development (5 days and 15 days after head sprouting) and four stages of grain development (5 days, 10 days, 15 days, and 20 days after pollination), allowing us to assess the possible role of *TaTPP* genes during the growth and development of wheat. We obtained 26 *TaTPP* gene transcripts (Fig. 7), and could not locate five other *TaTPP* genes due to low levels of expression or the fact that they could be pseudogenes. Levels of expression vary widely in different tissues of wheat *TaTPP* genes, and between different tissues in individual *TaTPP* genes. We observed three homologous genes *TaTPP8-5A/B/D*

that demonstrated widespread expression patterns that were higher in almost all tissues and stages. There are high levels of *TaTPP1-1A/B/D* expression in both seedling stems and young spikes, while there are high levels of *TaTPP2-2A* and *TaTPP4-2A/D* expression in seedling leaves, seedling stems, and grains. Compared with the seedling roots, *TaTPP3-2A/D* display relatively higher expression in other tissues and stages. There are high levels of *TaTPP9-6A/D* expression in seedling leaves, roots, spikes, and stems. There are higher levels of *TaTPP10-6A/D* expression in seedling stems, leaves, and mature spikes, while there is a strong and particular expression of *TaTPP2-2D*, *TaTPP4-2B*, and *TaTPP11-7A/B/D* during grain development, suggesting that these genes could play significant roles during this stage (Fig. 7).

Most homologous genes demonstrate similar patterns of expression during developmental stages, though several clustered expression profiles do not have similar genes, including copies of individual kinds of *TaTPP* genes from their sub-genomes; some demonstrate opposite expression patterns. For example, *TaTPP2-2A* is found on chromosome 2A and is preferentially expressed in the seedling leaves and stems, while the homologous *TaTPP2-2D* gene (located on chromosome 2D) is expressed in these tissues at a lower point. *TaTPP10-6A* is located on 6A and displays higher levels of expression in mature spikes and seedling stems. The homologous *TaTPP10-6B*, found on 6B, is expressed preferentially in the seedling leaves, stems, and mature spikes, while homologous genes from 6D are expressed only in the seedling leaves (Fig. 7). This difference in expression profiles between homologous genes from different subgenomes demonstrates that some have acquired new functions or lost old functions following polyploidization during wheat's evolutionary history.

Expression analysis of TaTPP genes respond to abiotic stresses

Environmental stresses significantly affect the productivity of wheat, making it important to study the wheat genes responsible for stress response in order to increase yields. We used quantitative real-time PCR (qRT-PCR) to assess how *TaTPP* gene expression responds to continuous ABA, low temperature, and salt stress, allowing us to analyze the role of *TaTPP* genes that could be associated with plant defense to abiotic stresses. We designed allele pairs from A-, B- and D-subgenomes and tested them together, since the products of their transcription share similar sequences. Each gene we analyzed had a different expression when responding to a minimum of one abiotic stress (Fig. 8). In response to ABA, there were eight up-regulated *TaTPPs* (*TaTPP1*, *TaTPP3*, *TaTPP4*, *TaTPP6*, *TaTPP7*, *TaTPP8*, *TaTPP9*, and *TaTPP11*) and three down-regulated *TaTPPs* (*TaTPP2*, *TaTPP5*, and *TaTPP10*) in seedling leaves at a minimum of one time point. In response to low-temperature conditions, there were seven up-regulated *TaTPPs* (*TaTPP1*, *TaTPP3*, *TaTPP4*, *TaTPP7*, *TaTPP8*, *TaTPP9*, and *TaTPP11*) and four down-regulated *TaTPPs* (*TaTPP2*, *TaTPP5*, *TaTPP6*, and *TaTPP10*). In response to salt stress, there were eight up-regulated *TaTPPs* (*TaTPP1*, *TaTPP3*, *TaTPP4*, *TaTPP6*, *TaTPP7*, *TaTPP8*, *TaTPP9*, and *TaTPP11*) and three down-regulated *TaTPPs* (*TaTPP2*, *TaTPP5*, and *TaTPP10*) (Fig. 8).

To better understand the functions of *TaTPP* genes in regulating wheat drought response, the expression patterns of 11 *TaTPPs* were experimentally examined in leaves and roots of 3-week-old drought-stressed wheat seedlings. As illustrated in Fig. 9, a dramatic upregulation of 8 *TaTPP* genes (*TaTPP1*, *TaTPP2*, *TaTPP3*, *TaTPP4*, *TaTPP5*, *TaTPP9*, *TaTPP10*, and *TaTPP11*) were observed in response to drought stress, especially in the leaves. *TaTPP7* also showed a slightly up-regulation in leaves and roots after drought stress. The changed expression levels of *TaTPP2*, *TaTPP3* and *TaTPP4* in leaves after drought stress were very sharp, with more than 60 folds, indicating that these genes are extremely susceptible to drought stress. Some genes showed very similar expression profiles after drought stress, such as *TaTPP3* and *TaTPP4* pairs. Some *TaTPPs* were significantly upregulated after light/early drought stress, such as *TaTPP2*, *TaTPP3*, *TaTPP4* and *TaTPP9* in leaves and *TaTPP9* and *TaTPP11* in roots, suggesting positive roles of these genes in early drought stress response. Some genes were significantly upregulated after severe stress, such as *TaTPP1*, *TaTPP10* and *TaTPP11* in leaves and *TaTPP6* and *TaTPP8* in roots, suggesting that these genes are important for the plant response to drought stress at a severe level. Upon drought stress, *TaTPP5* and *TaTPP10* were upregulated in leaves but downregulated in roots (Fig. 9). These data show the potential of some *TaTPP* genes for enhancing adversity resistant capacity, especially for wheat drought improvement.

Ectopic expression of TaTPP11 in Arabidopsis delayed plant development and enhanced drought tolerance

Alignment of the protein sequences determined the presence of three *TaTPP11* homeologs sharing a sequence similarity of approximately 95% (Additional file 6: Figure S4). Additional information regarding the spatiotemporal profile of *TaTPP11* expression could contribute to a better understanding of how *TaTPP11* functions biologically. In this case, we observed *TaTPP11* expression across various tissues and organs of wheat at different stages of development, such as the roots and leaves of seedlings, young panicles, flag leaves, and seeds. Our results demonstrated high levels of *TaTPP11* expression in seedling leaves and developing seeds, and low levels of *TaTPP11* expression in developing panicles (Fig. 10A). This indicates that *TaTPP11* could serve an important purpose as wheat seeds develop.

To better understand how *TaTPP11* relates to the development of wheat, we produced 35S::*TaTPP11-7D* transgenic *Arabidopsis* lines and assessed their levels of *TaTPP11-7D* expression to select three independent transgenic lines (*OE1*, *OE2*, and *OE3*) for subsequent analysis (Fig. 10B; Additional file 7: Figure S5). First, we observed the germination of the seeds and found there were no significant differences between transgenic and wild type lines (Fig. 10C, D). Next, we detected the phenotypes of the 35S::*TaTPP11-7D* transgenic *Arabidopsis* lines and that of the wild-type over

various developmental stages. The *TaTPP11-7D* transgenic *Arabidopsis* seedlings grew vegetation for much longer, bolted and flowered later, and had a lower plant height compared to the wild type (Fig. 10E). We also analyzed the organs from both the *Arabidopsis* wild-type plants and transgenic lines, and did not find any significant differences in the organs of the transgenic and wild type varieties, including in the seeds, flowers, and siliques (Fig. 10E, F).

We next assessed the drought tolerance of the wild type and transgenic plants to identify the role of *TaTPP11* in plant drought stress. Both 35S:: *TaTPP11-7D* transgenic plants and wild type plants were grown for three weeks in soil, after which they were not watered for 14 days. The plants were then watered for six days. Afterward, approximately 30% of wild type plants survived, while approximately 80–90% of the transgenic plants survived (Fig. 11A, B). Next, the trehalose contents of the wild type plants and the 35S:: *TaTPP11-7D* transgenic plants were assayed, demonstrating that the trehalose levels in transgenic lines were significantly higher than in wild type plants (Fig. 11C). Additionally, we assessed other cellular processes impacted by drought stress conditions, paying particular attention to substances regulating osmosis. We observed increased levels of proline and soluble sugar, and decreased levels of malonaldehyde under drought stress conditions in transgenic lines (Fig. 11D–I). These results indicate that *TaTPP11* overexpression in *Arabidopsis* might delay the development of plants, but could also increase the drought tolerance of transgenic varieties.

Discussion

There is a significant body of evidence indicating that T6P is an important signal metabolite in plants that affects metabolism, growth, and development. Therefore, there is a pressing need to better understand the processes and function of several plant TPP proteins. Whole-genome sequencing and the widespread nature of worldwide genomic databases have allowed researchers to closely analyze complex genomes, such as wheat [35]. Identifying *TPP* genes in wheat is needed to additionally characterize these genes. While the family of *TPP* genes has been studied in both monocots and dicots, their specific functions are still unclear, particularly in wheat. To better understand this function, we comprehensively identified and analyzed *TPP* genes in wheat.

We identified 31 candidate genes in the family of *TPP* genes in wheat (Table 1). There were three times as many *TPPs* in wheat as in *Brachypodium distachyon* and rice, which is evidenced by the fact that hexaploid wheat descended from a hybridization of A-, B-, and D-genome ancestors approximately one or two million years ago [37, 38]. This study assessed certain traits found in wheat *TPP* genes. The majority of *TaTPP* genes encode proteins with a predicted molecular mass of 39–65 kDa (with the exception of TaTPP5-2A) as well as an isoelectric point of approximately 5.5–9.2, which is similar to the *TPPs* found in other species of plants, while the majority of *TaTPP* genes possessed 8–9 introns (Table 1). We performed multiple sequence alignment and analyzed the phylogenetics of the 31 TaTPP protein sequences. We observed significant sequence divergence, particularly at the C-terminus, during the multiple sequence alignment of wheat TaTPP proteins (Additional file 4: Figure S3). This demonstrates the variable role that *TaTPP* genes serve during the growth and development of plants [2, 30]. We classified the wheat TaTPP protein family into five subfamilies (I, II, V, VI, VII) by assessing how they relate to homologous *TPPs* in other species (Fig. 1; Fig. 3A), gene structures (Fig. 3), and motif arrangements (Fig. 4). The smallest subfamily was the TaTPP VI subfamily (Fig. 1; Fig. 3A). Analysis of the gene structure demonstrated that genes from each TaTPP subfamily have similar numbers and positions of their exon-intron structures (Fig. 3B), though the TaTPP I subfamily diverged more (Fig. 3B). This suggests that the TaTPP I subfamily genes could perform different roles during the development of wheat. Additionally, wheat TaTPP proteins all possess particular conserved *TPP* domains, consisting of six conserved motifs (Fig. 4). With the exception of *TPP* domain-based conserved motifs, there are specific motifs for each TaTPP subfamily. For example, motifs 1 and 2 exist only in TaTPP VII subfamily proteins, while motif 11 only exists in the TaTPP VI subfamily. Motifs 18, 19, and 20 only exist in the TaTPP I subfamily (Fig. 4).

Duplicate genes located on different chromosomes are considered segregation duplication events. It is common for gene duplication events to occur in wheat, which will assist in the future analysis of the function and evolution of genes [31]. Whole genome duplications are common in angiosperms [39], and typically expand the gene family [40]. Gene duplication can result in the emergence of novel functions for certain plant genes. Syntenic relations and segregation duplication events between *Brachypodium distachyon* and wheat suggest that certain *TaTPP* genes were produced via gene duplication, indicating their similar origins (Fig. 2).

We analyzed the expression of publicly available RNA-seq data from 10 organs/tissues at various stages of development to assess the role play by *TPP* genes across wheat's life cycle. Wheat *TaTPP* genes are similar to *Arabidopsis* in that they are differentially transcribed, depending on the tissue, stage, and cell [23]. High levels of *TaTPP* genes expression were detected in the leaves, such as *TaTPP2-2A*, *TaTPP3-2A/D*, *TaTPP4-2A/D*, *TaTPP8-5A/B/D*, *TaTPP9-6A/D* and *TaTPP10-6B/D* (Fig. 7). This is similar to prior results, which found higher levels of expression of *TPPA*, *TPPB*, and *TPPG* in the leaves compared to other organs [23]. There were higher levels of *TaTPP9-6A/D* and *TaTPP8-5A/B/D* expression in the roots, which is similar to *TPPA*, while there were higher levels of *TPPD*, *TPPG*, and *TPPI* in the root caps and protoderms [23], indicating that they could be involved in the development of roots. In wheat, *TaTPP2-2A/D*, *TaTPP3-2A/D*, *TaTPP4-2A/B/D*, *TaTPP8-5A/B/D*, and *TaTPP11-7A/B/D* all demonstrated higher levels of expression in the grains at various stages of development, indicating they could be involved in grain development (Fig. 7). These results indicate that *TaTPP* genes could serve a variety of roles during the development of wheat. Additional ectopic *TaTPP11* expression in *Arabidopsis* displayed delays in development and flowering (Fig. 10), highlighting the importance of *TaTPP11* during plant development.

The expression or transcription of genes begins with an upstream regulatory promoter region, which is the combination of several cis-acting regulatory components joined with a minimal basic start element. Different regulatory cores provide the promoters with strength, time-space specificity, and stimuli response. As such, analyzing a target gene promoter's regulatory elements can allow us to predict how its expression will respond to different stimuli. A review of the promoters of the *TaTPP* gene family demonstrated that several phytohormone-, stress- and development-related regulatory elements were present (Fig. 6; Additional file 5: Table S2). Significant amounts of hormone-related cis-acting regulatory elements in most *TaTPP* promoters suggest that they play important roles as regulators of the core in various hormone-signaling pathways. Members of the *TaTPP* gene family play roles in response to biotic and abiotic stresses, which is consistent with enriching several cis-elements related to stress. Certain *TaTPP* promoters possess regulatory cores related to stress, such as ABA-responsive elements ABRE, SA-responsive elements (TCA-elements), defense and stress-responsive elements (TC-rich repeats and MBS), low-temperature-related elements (LTR), MeJA-responsive elements (CGTCA- and TGACG-motif), and wound-responsive elements (WUN-motif) (Additional file 5: Table S2). Of these, CGTCA- and TGACG-motifs are typical MeJA-responsive cis-acting elements, and were found in 20 of 31 promoters of the *TaTPP* genes. Jasmonic acid, a primary growth hormone, regulates resistance to plant diseases and responses to abiotic stresses [41]. We also observed certain elements specific to organs or tissues related to the development of roots, seeds, or endosperms (Fig. 6; Additional file 5: Table S2). Promoters of *TaTPP2-2B*, *TaTPP4-2D*, *TaTPP7-3A*, *TaTPP8-5A*, *TaTPP8-5B*, and *TaTPP10-6B* possess RY-element, a seed-specific regulation core, which indicates that they could play a role in seed development. The *TaTPP6-2A* and *TaTPP6-2B* genes should be further studied, since they possess the root-specific motif I element and could play a role in the development of roots (Fig. 6; Additional file 5: Table S2).

Abiotic stresses are primarily responsible for reducing crop quality and yield. Recent research has sought to better understand how plants respond to abiotic stresses, which would contribute to improved crop tolerance. While the overexpression of *E. coli* *TPS* and *TPP* fusion proteins can increase the tolerance of abiotic stress in rice [3], there is little known about how endogenous *TPP* functions in plants. We analyzed the cis-acting regulatory elements and found high levels of regulatory cores in the *TaTPP* gene family promoters. We performed qRT-PCR analysis to observe how *TaTPP* genes found in the seedling leaves respond to salt, low-temperature, ABA, and drought stresses (Fig. 8; Fig. 9). Our results demonstrated that the majority of *TaTPP* genes were up-regulated after ABA treatment, while some decreased. We saw the same expression profiles after drought, salt, and low-temperature treatments, however, the intensity of induction and the whether the expression was suppressed or up-regulated was different between all stressors. All four abiotic stresses regulated *TaTPP* gene expression positively and negatively in wheat (Fig. 8; Fig. 9), though the leaves and roots responded differently to drought stress conditions (Fig. 9). The fact that *TaTPP2*, *TaTPP5*, and *TaTPP10* were all down-regulated under low-temperature, ABA, and salt stress treatments suggests they are suitable candidates for using CRISPR-Cas9 gene editing to improve the abiotic tolerance of wheat plants. *TaTPP* genes respond to stress in a manner that indicates they assist in adapting to variable environmental conditions.

Conclusions

We identified 31 *TPP* family genes in wheat, all of which have at least one conserved *TPP* domain. The *TPP* family in wheat can be classified into five subfamilies based on their similar exon/intron structures and motifs. Additionally, there are four *TaTPP* protein found in the cytoplasm and nucleus (*TaTPP6*, *TaTPP7*, *TaTPP9*, and *TaTPP11*). A pattern analysis of tissue-specific expression demonstrated that *TaTPP* genes in wheat are expressed differentially, indicating that they play different roles in the growth and development of wheat. Analysis of the promoter cis-elements and expression patterns when subjected to abiotic and biotic stresses demonstrate that *TaTPP* genes in wheat respond to different stimuli. Additionally, the ectopic expression of *TaTPP11* in *Arabidopsis* demonstrated that plant development is delayed and drought tolerance is enhanced, highlighting the importance of *TaTPP11* during both plant development and in response to stress. This study provides significant information on the wheat *TPP* gene family, which will allow for the future study of their functional divergence and how they can be manipulated in the future.

Methods

Stress treatment and plant material

We used the wheat cultivar *Chinese spring* for this study, which was acquired from Northwest A&F University. However, we also could have obtained this variety from Chinese Crop Germplasm Resources Information System (<http://www.cgris.net/zhongzhidinggou/index.php>). The cultivar was surface-sterilized using 75% ethanol, after which it was washed using deionized water and germinated on wet filter paper for three days at 25°C. We then placed the germinated seeds in a nutrient solution (0.1 mM KCl, 0.75 mM K₂SO₄, 0.65 mM MgSO₄, 0.25 mM KH₂PO₄, 1.0 mM MnSO₄, 1.0 mM ZnSO₄, 0.1 mM EDTA-Fe, 2.0 mM Ca(NO₃)₂, 0.005 mM (NH₄)₆Mo₇O₂₄, 0.1 mM CuSO₄) and hydroponically cultivated them in a 16°C growth chamber under a 16/8 h light/dark cycle.

For ABA and salt treatments, we immersed seedlings at the three-leaf stage into hydroponic solutions with 200 mmol NaCl and 100 µmol ABA, and obtained samples at 0, 1, 3, 6, 12, and 24 h after treatment. For low-temperature treatments, we immersed seedlings at the three-leaf stage into hydroponic solutions at 4°C for 0, 1, 3, 6, 12, and 24 h and obtained samples. For drought treatment, we placed seedlings at the three-leaf stage onto a clean bench and subjected them to drought conditions (25°C, relative humidity 40-60%) and collected the roots and leaves from three seedlings at 0, 1, 3, 6, 12 and 24 h. We quickly froze all samples in liquid nitrogen and stored them at -80 °C to isolate the RNA.

Quantitative real-time PCR and RNA extraction

We used a Total RNA Rapid Extraction Kit for Polysaccharides Polyphenol Plant (BioTeke), according to the instructions of the manufacturer, to isolate and purify the total RNA. We then treated the resulting purified RNA with RNase-free DNase I (TaKaRa, China) to remove traces of DNA and ensure the sample was not contaminated. We then synthesized first-strand cDNA from 1 µg of total RNA with Recombinant M-MLV reverse transcriptase (Promega, USA) and used an ABI7300 Thermo-cycler (Applied Biosystems, USA) to conduct quantitative real time-PCR (qRT-PCR) in optical 96-well plates. All reactions were performed in 10 µl volume, with 1 µl diluted cDNA, 200 nM gene-specific primers, and 5 µl SYBR Premix Ex Taq II (TaKaRa) according to the following: 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 30 s at 60°C. We verified the specificity of each primer's amplicon via melting curve analysis and used the wheat *Actin* (Gene ID: 542814) as an internal control for analyzing the expression of *TaTPP11* in wheat. We calculated relative levels of gene expression levels using the $2^{-\Delta\Delta Ct}$ method [42], while expression variation was estimated from three biological replicates. Additional file 6: Table S3 outlines the primer pairs used in qRT-PCR analysis.

Genome-wide identification and annotation of *TPP* genes in wheat

We used 23 TPP protein sequences from *Arabidopsis* and rice to identify the genes from the Chinese Spring IWGSC RefSeq v1.1 reference genome assembly (Ensembl Plants; https://plants.ensembl.org/Triticum_aestivum/Info/Index) with the local blast program (E -value < 1e-10). After removing duplicate searches using the CD-hit program, we identified the rest of the protein sequences with the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1). We performed a phylogenetic analysis to filter the genes from TPP proteins previously identified from *Brachypodium distachyon*, *Populus trichocarpa* (poplar), *Arabidopsis thaliana*, *Oryza sativa* (rice), and *Zea mays* (maize). We also used phylogenetic analysis to sort the various TPP subfamilies and named the *TPP* genes from each of three wheat subgenomes (A, B, and D genomes) *TaTPPX_ZA*, *TaTPPX_ZB*, or *TaTPPX_ZD*, respectively, where X is the gene number and Z indicates its location on the wheat chromosome. We calculated the theoretical pI (isoelectric point) and Mw (molecular weight) of each putative wheat TPP protein using ExPasy (http://web.expasy.org/compute_pi/).

Systematic analysis of the bioinformatics of the wheat TPP family

In order to perform a phylogenetic analysis of the wheat TPP family along with other species of plants, we obtained proteomes of *Populus trichocarpa* (poplar), *Brachypodium distachyon*, *Arabidopsis thaliana*, *Zea mays* (maize), and *sativa* (rice) from JGI (<https://phytozome.jgi.doe.gov/pz/portal.html>). We acquired all of the TPP protein sequences either directly from the supplemental materials or from the proteomes based on the gene locus, as indicated by other papers. We used the ClustalW program (default settings) to produce the multiple sequence alignments [43], and produced unrooted phylogenetic trees using the neighbor-joining (NJ) method and the MEGA6.0 software, using the full-length of the TPP protein sequences [44]. We used 10,000 replications to estimate the bootstrap probability of each branch. We acquired information on the gene structure of *TaTPP* genes from the Chinese Spring IWGSC RefSeq v1.1 reference genome, which were analyzed with the Gene Structure Display Server 2.0 (GSDS; <http://gsds.cbi.pku.edu.cn/>). We identified the conserved TaTPPs motifs with the online MEME program (<http://meme-suite.org/>), while we used the circIz package in R to assess the chromosomal distribution and draw the collinearity map. We analyzed the *cis*-acting regulatory elements using plantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Using RNA-seq data to analyze gene expression

We obtained RNA-seq data from ten different tissues, including the leaves, roots, and stems of five-leaf stage wheat seedlings, spikes at the heading stage, young spikes at early booting stage, flag leaves at the heading stage, and the grains of 5, 10, 15 and 20 DPA in order to analyze how *TaTPP* genes were expressed in different tissues (http://genedenovoweb.ticp.net:81/Wheat_GDR1246/index.php?m=index&f=index). We used Cufflinks and TopHat to assess gene expression, based on the RNA-seq data [45, 46] and calculated the FPKM value (fragments per kilobase of transcript per million fragments mapped) for each *TaTPP* gene. To produce the heat map, we used the log₁₀-transformed (FPKM +1) values of the *TaTPP* genes.

Subcellular localization

We generated green fluorescent protein (GFP) expression vectors (CaMV35S-*GFP-NOS*) to analyze the subcellular localization of the TaTPP proteins. PCR and gene-specific primers were used to amplify the coding regions of *TaTPP6*, *TaTPP7*, *TaTPP9*, and *TaTPP11*, which were independently connected to the N-terminus of GFP in the expression vector. We isolated the wheat protoplasts from the mesophyll tissue of 2-week-old wheat seedlings, and used the PEG transfection method, along with the plasmid DNA of 35S::*TaTPP6-GFP*, 35S::*TaTPP7-GFP*, 35S::*TaTPP9-GFP*, 35S::*TaTPP11-GFP*, and 35S::*GFP* control, as described previously, for transformation [47]. Following PEG transfection, we incubated the wheat protoplasts in W5 solution (2 mM MES, 154 mM NaCl, 125 mM CaCl₂, and 5 mM KCl, pH = 5.7) in a dark chamber for 18 h at 23°C, and observed GFP fluorescence using a laser-scanning confocal microscope (A1, Nikon Corporation, Tokyo, Japan).

***Arabidopsis* transformation and *TaTPP11* isolation**

We obtained the *Arabidopsis* ecotype Columbia from Professor Zhensheng Kang's Lab (Northwest A&F University, China), which we used to transform *TaTPP11*. The full-length opening reading frame of *TaTPP11* was amplified from the wheat variety Chinese Spring with gene-specific primers that were closed using the cauliflower mosaic virus (CaMV) 35S promoter, into the pGreen0029-GFP vector. The recombinant vector (35S::*TaTPP11-7D*) was then introduced into *Agrobacterium tumefaciens*, after which the floral dip method [48] was used to turn it into *Arabidopsis* (*Arabidopsis thaliana*; ecotype Columbia). We placed the T₁ seeds in an MS medium with 2% sucrose and 50 mg/mL kanamycin, allowing us to identify the transformants. Homozygous T₃ plants were used to analyze the phenotype.

Drought tolerance assay

Seven-day-old seedlings from the WT and 35S::*TaTPP11-7D* transgenic lines were transferred from the MS medium to pot that had 230 g of a 2:1 ratio of nutrient soil and vermiculite (with 16 plants in each pot) for the drought tolerance assays. The 21-day-old plants that had been grown in a suitable environment (a relative humidity of 60%, a photoperiod of 16-h/8-h light/dark, and a temperature of 22°C) were then exposed to drought stress conditions, where we did not water the plants for approximately 14 days. They were then watered and allowed to recover. We observed the number of plants that survived six days later, and compared a minimum of 48 transgenic plants with WT plants in each data. We performed four independent experiments and used the data from each for our statistical analysis, while a Student's *t*-test was used to assess the differences between the WT and transgenic plants.

Phylogenetic analysis and multiple sequence alignments

A [phylogenetic analysis](#) was performed on the full-length protein sequences from the TPP proteins of different plant species. We used MEGA (v6.0) software and the Neighbor-Joining (NJ) algorithm with 1,000 bootstrap re-samplings to construct the phylogenetic tree, while the ClustalW software was used to conduct multiple [sequence alignments](#), which were manually edited with BioEdit (v7.1).

Statistical analyses

Each experiment was performed in triplicate, and data is presented and was analyzed after calculating the mean $\pm$ standard deviation (SD) of each experiment. A Student's *t*-test was used to assess the statistical differences, while $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered extremely statistically significant.

Abbreviations

ABA: Absciscic acid; BLAST: Basic local alignment search tool; CDS: Coding sequences; DRE: dehydration-responsive element; GSDB: Gene Structure Display Server; LTR: low temperature responsive element; MBS: MYB binding site involved in drought-inducibility; MEME: Multiple Expectation Maximization for Motif Elicitation; MYB: MYB recognition site; MYC: MYC recognition site; Pi: [isoelectricpoint](#); SMART: Simple Modular Architecture Research Tool.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this article and its supplementary information files. However, the sequence data in this study can also be accessed at http://genedenovoweb.ticp.net:81/Wheat_GDR1246/index.php?m=index&f=index. In addition, al

databases used in this study are open for public and the links are as follows:

Chinese Crop Germplasm Resources Information System: <http://www.cgris.net/zhongzhidinggou/index.php>

Ensembl Plants: https://plants.ensembl.org/Triticum_aestivum/Info/Index

ExPasy: http://web.expasy.org/compute_pi/

GSDS: <http://gsds.cbi.pku.edu.cn/>

JGI: <https://phytozome.jgi.doe.gov/pz/portal.html>

MEME: <http://meme-suite.org/>

plantCARE: <http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>

SMART: http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

Z.K. and H.M. conceived and initiated the research; H.M. designed the experiments; L.D., S.L., L.D. and X.C. performed the experiments. H.M. analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

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References

1. Paul MJ, Gonzalez-Uriarte A, Griffiths CA, Hassani-Pak K. The role of trehalose 6-phosphate in crop yield and resilience. *Plant Physiol.* 2018;177(1):12–23.
2. Fichtner F, Lunn JE. The role of trehalose 6-phosphate (Tre6P) in plant metabolism and development. *Annu Rev Plant Biol.* 2021;72:737–760.
3. Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, et al. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc Natl Acad Sci USA.* 2002;99(25):15898–15903.
4. Paul MJ, Primavesi LF, Jhurrea D, Zhang Y. Trehalose metabolism and signaling. *Annu Rev Plant Biol.* 2008;59(1):417–441.
5. Mu M, Lu XK, Wang JJ, Wang DL, Yin ZJ, Wang S, et al. Genome-wide identification and analysis of the stress-resistance function of the *TPS* (Trehalose-6-Phosphate Synthase) gene family in cotton. *BMC Genet.* 2016;17:54.
6. Yadav UP, Ivakov A, Feil R, Duan GY, Walther D, Giavalisco P, et al. The sucrose-trehalose 6-phosphate (Tre6P) nexus: specificity and mechanisms of sucrose signalling by Tre6P. *J Exp Bot.* 2014;65(4):1051–1068.
7. Astrid W. The function of trehalose biosynthesis in plants. *Phytochemistry.* 2002;60(5):437–440.
8. Zeid IM. Trehalose as osmoprotectant for maize under salinity-induced stress. *Research Journal Agriculture Biology Science.* 2009;5(5):613–622.
9. Ali Q, Ashraf M. Induction of drought tolerance in maize (*Zea mays* L.) due to exogenous application of trehalose: growth, photosynthesis, water relations and oxidative defence mechanism. *J Agron Crop Sci.* 2011;197(4):258–271.
10. Julieta RS, Ramón S, Jesús C, Gabriel I. Trehalose accumulation in *Azospirillum brasilense* improves drought tolerance and biomass in maize plants. *Fems Microbiology Lett.* 2010;296(1):52–59.

11. Satoh-Nagasawa N, Nagasawa N, Malcomber S, Sakai H, Jackson D. A trehalose metabolic enzyme controls inflorescence architecture in maize. *Nature*. 2006;441(7090):227–230.
12. Carillo P, Feil R, Gibon Y, Satoh-Nagasawa N. A fluorometric assay for trehalose in the picomole range. *Plant Methods*. 2013;9(1):21–21.
13. Nuccio ML, Wu J, Mowers R, Zhou HP, Meghji M, Primavesi LF, et al. Expression of trehalose-6-phosphate phosphatase in maize ears improves yield in well-watered and drought conditions. *Nat Biotechnol*. 2015;33(8):862–869.
14. Figueroa CM, Lunn JE. A tale of two sugars: trehalose 6-phosphate and sucrose. *Plant Physiol*. 2016;172(1):7–27.
15. Griffiths CA, Sagar R, Geng Y, Primavesi LF, Patel MK, Passarelli MK, et al. Chemical intervention in plant sugar signalling increases yield and resilience. *Nature*. 2016;540(7634):574–578.
16. Wahl V, Ponnu J, Schlereth A, Arrivault S, Langenecker T, Franke A, et al. Regulation of flowering by trehalose-6-phosphate signaling in *Arabidopsis thaliana*. *Science*. 2013;339(6120):704–707.
17. Hulsmans S, Rodriguez M, Coninck BD, Rolland F. The *SnRK1* energy sensor in plant biotic interactions. *Trends Plant Sci*. 2016;21(8):648–661.
18. Cabib E, Leloir LF. The biosynthesis of trehalose phosphate. *J Biol Chem*. 1958;231(1):259–259.
19. Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G. Insights on the evolution of trehalose biosynthesis. *BMC Evol Biol*. 2006;6(1):109.
20. Lunn JE. Gene families and evolution of trehalose metabolism in plants. *Funct Plant Biol*. 2007;34(6):550–563.
21. Dijk PV. The Cytophaga hutchinsonii ChTPSP: First characterized bifunctional TPS-TPP protein as putative ancestor of all eukaryotic trehalose biosynthesis proteins. *Mol Biol Evol*. 2010;27(2):359–369.
22. Yang HL, Liu YJ, Wang CL, Zeng QY, Natarajan K. molecular evolution of trehalose-6-phosphate synthase (*TPS*) gene family in populus, *Arabidopsis* and rice. *Plos One*. 2012;7(8):e42438.
23. Vandesteene L, Lopez-Galvis L, Vanneste K, Feil R, Maere S, Lammens W, et al. Expansive evolution of the trehalose-6-phosphate phosphatase gene family in *Arabidopsis*. *Plant Physiol*. 2012;160(2):884–896.
24. Krasensky J, Broyart C, Rabanal FA, Jonak C. The redox-sensitive chloroplast trehalose-6-phosphate phosphatase *AtTPPD* regulates salt stress tolerance. *Antioxid Redox Signal*. 2014;21(9):1289–1304.
25. Lin Q, Yang J, Wang Q, Zhu H, Wang K. Overexpression of the trehalose-6-phosphate phosphatase family gene *AtTPPF* improves the drought tolerance of *Arabidopsis thaliana*. *BMC Plant Biol*. 2019;19(1):381–381.
26. Lin Q, Wang S, Yihang D, Wang J, Wang K. *Arabidopsis thaliana* trehalose-6-phosphate phosphatase gene *TPPI* enhances drought tolerance by regulating stomatal apertures. *J Exp Bot*. 2020;71(14):4285–4297.
27. Pramanik M, Imai R. Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. *Plant Mol Biol*. 2005;58(6):751–762.
28. Shima, Matsui, Tahara, Imai. Biochemical characterization of rice trehalose-6-phosphate phosphatases supports distinctive functions of these plant enzymes. *FEBS J*. 2007;274(5):1192–1201.
29. Kretschmar T, Pelayo M, Trijatmiko KR, Gabun Ad A L, Alam R, Jimenez R, et al. A trehalose-6-phosphate phosphatase enhances anaerobic germination tolerance in rice. *Nat Plants*. 2015;1(9):15124.
30. Claeys H, Vi SL, Xu X, Satoh-Nagasawa N, Eveland AL, Goldshmidt A, et al. Control of meristem determinacy by trehalose 6-phosphate phosphatases is uncoupled from enzymatic activity. *Nat Plants*. 2019;5(4):352–357.
31. Appels R, Eversole K, Feuillet C, Keller B, Rogers J, Stein N, et al. Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science*. 2018;361(6403):eaar7191.
32. Holub EB. The arms race is ancient history in *Arabidopsis*, the wildflower. *Nat Rev Genet*. 2001;2(7):516–527.
33. Xu G, Guo C, Shan H, Kong H. Divergence of duplicate genes in exon-intron structure. *Pro Nat Acad Sci USA*. 2012;109(4):1187–1192.
34. Lescot, M. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res*. 2002;30(1):325–327.
35. Yamaguchi-Shinozaki K, Shinozaki K. Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci*. 2005;10(2):88–94.
36. Li F, Mei F, Zhang Y, Li S, Mao H. Genome-wide analysis of the *AREB/ABF* gene lineage in land plants and functional analysis of *TaABF3* in *Arabidopsis*. *BMC Plant Biol*. 2020;20(1):558.
37. Feldman M, Levy AA. Allopolyploidy - a shaping force in the evolution of wheat genomes. *Cytogenet Genome Res*. 2005;109(1–3):250–258.
38. Marcussen T, Sandve SR, Heier L, Spannagl M, Pfeifer M, Jakobsen KS, et al. Ancient hybridizations among the ancestral genomes of bread wheat. *Science*. 2014;345(6194):1250092.
39. Flagel LE, Wendel JF. Gene duplication and evolutionary novelty in plants. *New Phytol*. 2009;183(3):557–564.
40. Li Z, Zhang C, Guo Y, Niu W, Wang Y, Xu Y. Evolution and expression analysis reveal the potential role of the *HD-Zip* gene family in regulation of embryo abortion in grapes (*Vitis vinifera* L.). *BMC genomics*. 2017;18(1):744.
41. Ruan J, Zhou Y, Zhou M, Yan J, Khurshid M, Weng W, et al. Jasmonic acid signaling pathway in plants. *Int J Mol Sci*. 2019;20(10):2479.

42. A KJL, B TDS. Analysis of Relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402–408
43. Thompson J, Gibson T, Higgins D. Multiple sequence alignment using ClustalW and ClustalX. *Curr Protoc Bioinformatics*. 2002;Chap. 2, Unit 2.3.
44. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*. 2013;30(12):2725–2729.
45. Trapnell C RA, Goff L, Pertea G, Kim D, Kelley DR. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc*. 2012;7(3):562–578.
46. Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol*. 2013;31(1):46–53.
47. Yoo SD, Cho YH, Sheen J. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc*. 2007;2(7):1565–1572.
48. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J*. 1998;16(6):735–743.

Figures

Figure 1

Phylogenetic relationships of the TPP family in plants. The phylogenetic tree of TPP proteins from wheat, maize, rice, *Populus*, *Arabidopsis*, and *Brachypodium*. AMEGA 6.0 was used to build a neighbor-joining tree, which was visualized with the online tool Evolview. TPPs from one plant species

are marked with leaf labels. I-VII denotes the seven plant TPP protein subfamilies. The percentage bootstrap scores were calculated from 1000 replications.

Figure 2

Mapping and analysis of synteny of TaTPP genes B. distachyum and wheat chromosomes are represented as circles. The location of each BdTPP and TaTPP gene is denoted with a small black line on the circle. Colored curves indicate syntenic relationships between B. distachyum and wheat TPPs genes.

Figure 3

Gene structures and phylogenetic relationships of wheat TPP genes (A) Phylogenetic tree of 31 full-length wheat TPP proteins generated with MEGA 6.0 and the Neighbor-Joining (NJ) method with 1,000 bootstrap values. (B) Exon/intron structures of TPP genes in wheat. Exons and introns denoted by purple boxes and black lines, respectively.

Figure 4

Conserved motif analysis of TPP proteins in wheat A colored box denotes each motif, while black lines indicate non-conserve sequences. Conserved TPP domains consist of motifs 4-10.

Figure 5

Subcellular localization of TaTPP-GFP fusion proteins in wheat mesophyll protoplasts. The 35S::TaTPP6-GFP, 35S::TaTPP7-GFP, 35S::TaTPP9-GFP, and 35S::TaTPP11-GFP fusion vectors, and 35S::GFP control vectors were all independently transformed into wheat mesophyll protoplasts via PEG transfection. A laser scanning confocal microscope was used to observe the green fluorescence.

Figure 6

Promoter cis-element analysis of TaTPP genes 2-kb upstream promoter sequence for all TaTPP genes was obtained from wheat genome database, while PlantCARE was used to scan all the cis-acting regulatory elements. Numbers denote the sum of how different cis-acting elements respond to similar stimuli.

Figure 7

TaTPP gene expression profiles in ten different organs or tissues Heatmap drawn from Log10-transformed expression values. Blue or red indicates the lower or higher levels of expression of each transcript in each sample, respectively. R, root of wheat seedling at five-leaf stage; S, stem of wheat seedling at five-leaf stage; L, leaf of wheat seedling at five-leaf stage; FL, flag leaf at heading stage; YS5, young spike at early booting stage; YS15, spike at heading stage; GR5, grain of five days post-anthesis; GR10, grain of 10 days post-anthesis; GR15, grain of 15 days post-anthesis; GR20, grain of 20 days post-anthesis.

Figure 8

TaTPP gene expression profiles responding to abiotic stresses (A) TaTPP gene expression patterns after ABA treatment. (B) TaTPP gene expression pattern under low-temperature treatment. (C) TaTPP gene expression pattern under salt stress treatment.

Figure 9

TaTPP gene expression profile in seedling roots and leaves under drought stress conditions Red and blue columns represent level of TaTPP gene expression under 20% PEG treatment in plant leaves and roots obtained from wheat seedlings, respectively. X-axis indicates time points following drought treatment. Expression data from the control sample were normalized to 1, while error bars indicate standard error from three replicates.

Figure 10

Comparing plant morphology between WT plants and *TaTPP11* overexpression (A) *TaTPP11* expression profiles in various tissues. R, root of wheat seedling at five-leaf stage; L, leaf of wheat seedling at five-leaf stage; YS5, young spikes of 5 days after booting; YS15, spikes 15 days after booting; GR5, grain of five days post-anthesis; GR10, grain of 10 days post-anthesis; GR15, grain of 15 days post-anthesis; GR20, grain of 20 days post-anthesis; GR25, grain of 25 days post-anthesis. (B) RT-PCR identification of Arabidopsis lines overexpressing *TaTPP11*-7D. (C-D) Seed germination assay between 35S:TaTPP11-7D transgenic and WT plants. (E-F) Plant phenotype (E) and seed morphology (F) of wild-type and 35S:TaTPP11-7D transgenic Arabidopsis plants.

Figure 11

Drought stress tolerance of Arabidopsis plants overexpressing TaTPP11. (A) Phenotypes of 35S:TaTPP11-7D transgenic Arabidopsis and wild-type (WT) plants before and after drought stress. (B) Statistical analysis of survival rates following drought-stress treatment. Survival rates and standard errors were obtained using data acquired from four independent experiments. Each experiment used 12 plants for each line. (C) Trehalose contents in WT and 35S:TaTPP11-7D transgenic lines. (D-I) Proline, soluble sugar, and MDA contents in WT and 35S:TaTPP11-7D transgenic lines under well-watered (WW) and water-deficit (WD) conditions. Values indicates mean $\pm$ SD of three replicates. Asterisks represent significant differences from the WT per on student's t-test (* $P < 0.05$; ** $P < 0.01$).

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