Genome-wide investigation of heat shock transcription factor family in wheat (*Triticum aestivum* L.)

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Abstract

Background: Heat shock transcription factors (HSFs) play crucial roles in resisting heat stress and regulating plant development. Investigating the HSF family is essential for understanding the fertility conversion mechanism in thermo-sensitive male sterile wheat. Previous studies have investigated the HSF family in wheat but it is necessary to conduct more in-depth and systematic analyses based on the newly published reference genome. **Results:** In the present study, 61 wheat Hsf (TaHsf) genes were identified using two main strategies and renamed based on their physical locations on chromosomes. According to the gene structure and phylogenetic analyses, the 61 TaHsf genes were classified into three categories and eleven subclasses. The genes were unequally distributed on 21 chromosomes, including two pairs of tandem duplication genes and 52 TaHsf segmental duplication genes. According to the cis-elements identified, most of the TaHsfs can be activated by Ca⁺⁺ and MYB, and they respond to drought, light, copper, and other stresses as well as heat shock. RNA-seq analysis indicated that the A2 class TaHsf genes exhibited persistently upregulated expression levels in the leaves/shoots, roots (except in the vegetative growth and reproductive growth stages), spikes, and grains in wheat under normal conditions. The A and B class TaHsf genes were positively regulated during the resistance to heat, whereas the C class genes were involved in drought regulation in wheat. Only the A and B class TaHsf genes were upregulated under fertile conditions in thermo-sensitive male sterile wheat. **Conclusion:** In this study, 61 wheat Hsf genes were identified based on the complete wheat reference genome. This comprehensive analysis provides novel insights into the TaHsf genes, including their diverse functions and involvement in metabolic pathways.

Background

The heat shock response is common in plants where a series of stress responses are generated under external stresses, especially during heat stress [1]. Under heat stress, heat shock elements (HSEs) present in the promoter regions upstream of the heat shock protein (HSP) genes are recognized by the activated heat shock transcription factors (HSFs), and they induce the transcription of *Hsp* genes as molecular chaperones to help refold, assemble, distribute, and degrade related proteins, as well as repairing damaged proteins and maintaining cell survival [2-4]. In particular, regulation of the activity of HSFs is the key mechanism responsible for the transcription and expression of *Hsp* genes.

A typical HSF has four components comprising the N-terminal DNA-binding domain (DBD), oligomeric domain (OD), nuclear localization signal (NLS), and nuclear export signal (NES). Some HSFs also have a C-terminal activation domain (CTAD) [5]. DBDs are located at the N-terminus of HSFs and they are highly conserved regions with three helical structures (H1, H2, and H3) and four inverted parallel β -sheets (β 1, β 2, β 3, and β 4) [6]. The helix-turn-helix (H2-T-H3) structure at the hydrophobic centre of the DBD allows the precise localization and recognition of HSE sequences (5-AGAAnnTTCT-3) [7]. The OD region is also located at the N-terminus of HSFs and it is linked to the DBD region by a basic amino acid sequence measuring 15 to 80 amino acids (aa) in length. The OD region comprises two hydrophobic heptad repeat regions: A and B (HR-A/B). HR-A contains 5–6 sets of hydrophobic heptapeptide repeats, whereas HR-B

has two overlapping heptapeptide repeats, which form a helical coiled-coil structure in space [8]. According to the characteristics of the HR-A/B region, plant HSFs can be classified into class A, class B, and class C. Class A and class C HSFs have 21 and 7 aa inserts between regions HR-A and HR-B, respectively, whereas class B HSFs are relatively simple with no amino acid insertions [9]. The synergy between the NLS and NES maintains the cell balance, thereby allowing plant HSFs to be freely distributed in the cytoplasm and nucleus [10]. The CTAD is characterized by aromatic and large hydrophobic amino acids, and acidic amino acid (AHA) motifs specifically activate the HSFs in class A. By contrast, the B and C class HSFs have no activation functions due to the lack of an AHA domain [5].

The first HSF gene in plants was cloned in tomato (*Solanum lycopersicum* L.) [11] and they have now been characterized in *Arabidopsis*, rice [12], maize (*Zea mays* L.) [13], soybean (*Glycine max* L.) [14], chickpea [15], and sorghum [16], as well as in vegetables including Chinese cabbage [17] and pepper [18], and fruits such as apple [19] and pear [20]. In addition to those *Hsfs* who involved in the regulation of heat-resistance mechanisms were researched in *Arabidopsis* [21, 22], many *Hsfs* have been found to be participate in other response in various plants in recent years. The overexpression of *AtHsfA2* improves the heat tolerance of *Arabidopsis* but it also enhances the salt tolerance [23], hypoxia resistance [24], and resistance to oxidative stress [25]. Under high temperature stress, the overexpression of *LeHsfA2* increases the fruit set rate in tomato and enhances fruit development, probably because *LeHsfA2* activates the protective mechanism for tomato pollen [26]. Shim et al. found that the overexpression of *HsfA4a* in rice increased the tolerance of cadmium [27]. The sunflower *HaHsfA9* is involved in the development of the embryo and seed maturation. The overexpression of *HaHsfA9* alone or *HaHsfA9* together with *HaDreb2* can also promote the synthesis and accumulation of HSPs in tobacco seeds and increase the seed life [28]. The class B and C HSFs lack transcriptional activities but many studies in recent years have shown that class B and class C *Hsfs* are essential for the regulation of plant growth and development, metabolism, and stress responses. For example, the heat-induced tomato *LeHsfB1* is a coactivator of a class A *Hsfs* (e.g., *HsfA1a*) and it has an auxiliary activation function by enhancing the expression of target genes [29]. Little studies have shown that class C *Hsfs* can be induced by a variety of stresses [17].

Higher temperatures from wheat emergence until the tillering stage can decrease the number of tillers, while exposure to high temperatures from the jointing to flowering stages leads to a decreased numbers of panicles per ear and spikelets per panicle, and a lower grain weight [30]. Wheat is a major food crop so improving its heat resistance and ensuring food production is important for human society. Members of the HSF family in wheat have been identified based on the old reference genome [31], but the lack of sufficient information hinders in-depth studies of the stress resistance mechanisms related to *TaHsfs*. Indeed, only three members of the wheat HSF family have been functionally analyzed, i.e., *TaHsfA4a*, *TaHsfB2*, and *TaHsfA6e* [27, 32, 33]. Therefore, a more comprehensive and systematic analysis is required for *TaHsfs* based on the newly published wheat reference genome to provide a secure basis for subsequent research.

The fertility of thermo-sensitive male sterile wheat is controlled by the temperature and it has been applied in wheat hybridization to increase the wheat yield. Understanding the fertility conversion mechanism is important for accelerating the rate of production improvements. In our previous studies, we showed that the expression levels of *Hsfs* and *Hsps* differ significantly under different fertility conditions (different temperatures). In order to further explore their roles in the fertility conversion mechanism and the HSF family members that might be important for their roles, we used the newly published reference genome to identify the HSF family members in wheat. We analyzed their evolutionary relationships, gene structure, and expression patterns, and verified their preferential expression in thermo-sensitive male sterile wheat.

Materials And Methods

Genome-wide identification of the *TaHsf* family members in *Triticum aestivum* L.

The latest wheat reference genome and the hidden HMM profile of the HSF family in the wheat genome database IWGSC (IWGSC RefSeq annotation v1.1, <https://wheat-urgi.versailles.inra.fr/Seq-Repository/Annotations>) [55] and Pfam database (<http://pfam.xfam.org/family/PF00447>) were downloaded [56]. The HSF HMM profile was searched in the wheat genome with the HMMER search program and reliable results were screened based on an E-value less than or equal to $1e-20$. The results obtained were used to construct a wheat-specific HSF HMM profile, before searching the wheat reference genome again with an E-value threshold of $1e-3$. In order to avoid missing other HSF members, the HSF protein sequences for rice and *Arabidopsis* were downloaded from phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) and used as queries to BLAST the wheat reference genome, where the E-value threshold was $1e-10$. The results obtained were identified as wheat candidate genes and verified using CD-search (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) [57], SMART (<http://smart.embl.de/>), and pfam (<http://pfam.xfam.org/>) [58]. Coiled-coil structures were detected using the MARCOIL program (<https://bcf.isb-sib.ch/webmarcoil/webmarcoilC1.html>) [59]. Protein sequences that lacked the DBD domain or coiled-coil structure were removed. According to the positions of the genes on the chromosomes, the non-redundant *Hsfs* were designated as *TaHsf-01* to *TaHsf-61*. All high confidence level HSF protein sequences were submitted to Expy (https://www.expasy.org/structural_bioinformatics) to calculate the number of amino acids, molecular weight, and theoretical isoelectric point. NetNES (<http://www.cbs.dtu.dk/services/NetNES/>) and cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi#opennewwindow) were used to search for the NESs and NLSs in the *TaHsfs* [60, 61]. The gene annotation information for the *TaHsf* family members was submitted to the online site MapGene2 Chrom web V2 (http://mg2c.iask.in/mg2c_v2.0/) to map their chromosomal locations.

Multiple sequence alignment, phylogenetic analysis, and classification of *TaHsfs*

The full-length amino acid sequences of the HSFs in rice and *Arabidopsis* were used for evolutionary analysis with the full-length amino acid sequences of the *TaHsfs*. First, all of the sequences were

imported into MAGE7 for sequence comparison using the default parameters and the phylogenetic tree was then constructed based on the conserved regions in the alignment results with the maximum likelihood method, where the settings comprised the Poisson distribution mode and bootstrap tests were conducted with 1000 replicates. The classification of HSFs in wheat was based on the topology of the phylogenetic tree and the classification of HSFs in the other two plants.

Analysis of gene duplication and synteny relationships of *TaHsfs*

Segmental duplications and tandem duplications were determined for the *TaHsfs* using McscanX software [62]. Sequences on the same chromosome with a shared similarity greater than 75% and fragment lengths in the alignment that exceeded 75% of the length of the longer sequence were confirmed as tandem duplications. The KA/KS values were calculated for the tandem duplications using KAKS_calculator 2.0 [63]. A segmental duplication map of the genome was prepared using Circos software [64]. In addition to rice and *Arabidopsis thaliana*, the genome-wide CDS sequences and genome annotation information for Chinese cabbage

(http://brassicadb.org/brad/datasets/pub/Genomes/Brassica_rapa/V1.0/Scaffold1.0/), sorghum (<http://www.plantgdb.org/BdGDB/>), maize (http://interface.maizegdb.org/gbrowse/maize_v4/), and *Brachypodium* (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_BdistachyonBd21_3_er) were obtained, and the synteny relationships between the HSF family in wheat and these species were determined using Mcscanx.

Structural characterization, conserved motifs, cis-elements, and construction of the interaction network for *TaHsfs*

ClustalW was used to align the conserved DBD and HR-A/B domains in the *TaHsfs*. The gene structures of the *TaHsfs* were visualized using the online drawing tool Gene Structure Display Server 2.0 (GSDS, <http://gsds.cbi.pku.edu.cn/>) [65]. The MEME software installed in the Linux system obtained 15 conserved motifs in the *TaHsfs* with lengths of 6–50 aa. The gene structure and motifs in the *TaHsfs* were determined using TB tools [66]. Sequences located 1500 bp upstream of the *TaHsfs* were obtained and the cis-acting elements were predicted with PLACE (plant cis-acting regulatory element, <https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?sid=&lang=en&pj=640&action=page&page=newplace>) [67], and the results were displayed using GSDS. STRING (https://version-10-5.string-db.org/cgi/input.pl?sessionId=bHwqHDUnAsoF&input_page_show_search=on) and PlantRegMap (<http://plantregmap.cbi.pku.edu.cn/network.php>) were employed to obtain the regulation networks between *TaHsfs* and their interacting genes, which were visualized with Cytoscape (<https://cytoscape.org/>) [68-70].

RNA-seq data analysis

Part of the wheat transcriptome data was obtained from the Wheat Expression Browser powered by expVIP (<http://www.wheat-expression.com/>) to study the expression patterns of the *TaHsfs*. The RNA-seq data were derived from nine different growth periods and tissues in Chinese Spring under normal

conditions (grain in reproductive stage, leaves and shoots in seedling stage, roots in seedling stage, leaves and shoots in vegetative stage, spike in reproductive stage, spike in vegetative stage, leaves and shoots in reproductive stage, roots in vegetative stage, and roots in reproductive stage) [71], as well as under six types of stress and a normal control in the heat and drought tolerant wheat variety TAM107 (combined drought and heat stress for 6 h, combined drought and heat stress for 1 h, heat stress for 6 h, heat stress for 1 h, drought stress for 6 h, drought stress for 1 h, and no stress control) [72]. In addition, in order to study the mechanism responsible for regulating *TaHsfs* in thermosensitive male sterile wheat, transcriptome data were analyzed from a type of temperature-sensitive male sterile wheat KTM3315A which was selected by our research group in 2001. KTM3315A exhibits male sterility under low temperature conditions (< 18C) and fertility conversion under high temperature conditions (> 20C) [49]. Ten pots of KTM3315A were placed in two artificial climate incubators before the fertility conversion period (binucleate stage) and they were treated at two different temperatures until the flowering stage. RNA was extracted from the anthers of sterile and fertile plants during the uninucleate, binucleate, and trinucleate stages (AS1, AS2, AS3, AF1, AF2, and AF3), and six samples were used for RNA-seq [49]. The heat maps were drawn using the heatmap application in omicshare (<http://www.omicshare.com/tools/index.php/Home/help/index/html/combinemore>).

Results

Identification and basic characteristics of *TaHsf* genes

The newly constructed wheat-specific hidden Markov model (HMM) file was used to search the whole wheat protein sequences and 94 candidate *Hsf* genes were obtained, which were consistent with the wheat candidate *Hsf* genes obtained by alignments with *Arabidopsis* and rice *Hsf* genes. After identifying the DBD domain and coiled-coil structure, 61 non-redundant genes were finally identified in the wheat HSF family (Additional file 1: Table S1, Additional file 2: Table S2). These 61 *TaHsfs* were shown to be unequally distributed on 21 chromosomes (Additional file 3: Figure S1). The 5A chromosome contained the most *TaHsf* genes (6) and one to five *TaHsfs* were unevenly distributed on other chromosomes. We renamed these genes as *TaHsf*-01 to -61 according to their respective chromosome distribution. The gene name, transcript ID, gene position, exon number, aa length, molecular weight, and theoretical isoelectric point are listed for each of the *TaHsfs* in Table 1. The UniParct ID and old versions (IWGSC2.2) of the gene IDs are also included for clarity. The protein lengths of the *TaHsfs* ranged from 227 (*TaHsf*-22) to 569 (*TaHsf*-44), the molecular weights varied from 24.69 (*TaHsf*-22) to 59.76 (*TaHsf*-44), and the predicted isoelectric points ranged from 4.85 (*TaHsf*-12) to 9.52 (*TaHsf*-43).

Phylogenetic analysis of *TaHsfs*

The full-length amino acid sequences of 25 *Hsf* genes in rice, 22 *Hsf* genes in *Arabidopsis*, and 61 *Hsf* genes in wheat were obtained to co-construct a phylogenetic tree to classify the *TaHsfs* (Figure 1). Based on the clustering results, 61 *TaHsfs* were classified into three major categories: A, B, and C. The *TaHsfs* in class A belonged to six subclasses of A1–A6, the class B *TaHsfs* were divided into three subclasses of

B1, B2, and B4; and the class C *TaHsfs* were divided into two subclasses of C1 and C2. Compared with rice and *Arabidopsis Hsf* members, the *TaHsf* family lacked members belonging to A7, A8, A9, and B3. In every subclass, at least one *OsHsf* was found to be highly homologous with the *Hsf* genes in wheat, e.g., *OsHsf-13* (A1) was highly homologous with *TaHsf-46*, *TaHsf-32*, and *TaHsf-51* in A1; *OsHsf-07* (A3) shared high homology with *TaHsf-10*, *TaHsf-14*, and *TaHsf-07* in A3; *OsHsf-04* (A4b) in A4 was highly homologous with *TaHsf-28*, *TaHsf-18*, and *TaHsf-13*; and *OsHsf-06* (A5) and *TaHsf-52*, *TaHsf-53*, *TaHsf-54*, in A5 were clustered together due to their high homology. *OsHsf-17* (A6a) in A6, *OsHsf-23* (B1) in B1, *OsHsf-14* (B2a) and *OsHsf-24* (B2c) in B2, *OsHsf-10* (B4d) in B4, *OsHsf-02* (C1b) and *OsHsf-03* (C1a) in C1, and *OsHsf-05* (C2a) and *OsHsf-16* (C2b) in C2 were also highly homologous with some *Hsfs* in wheat. However, only *TaHsf-01* in A4 and *OsHsf-15* (A4d), and *TaHsf-13* in B4 and *OsHsf-19* (B4d) were orthologous genes. No orthologous genes were detected in the HSF family members in *Arabidopsis* and wheat, which indicates that the genetic relationship between wheat and rice is closer than that between wheat and *Arabidopsis*. Nineteen pairs of paralogous genes were found among the *TaHsfs*. A2 had the most paralogous genes with five pairs, as well as three pairs in C1 and B2, two pairs in C2, and one pair in each of the other subclasses. In addition to the initial the roles of heat stress response, those paralogous genes conferred new functions for wheat HSF family, which is very important for the functional differentiation of this gene family.

Syntenic analysis for *TaHsfs*

The synteny between species is strongly related to their evolution and kinship. In order to compare the kinship between wheat and other species based on HSFs, we performed synteny analysis between wheat with *Arabidopsis thaliana*, Chinese cabbage, rice, maize, sorghum, and *Brachypodium*. The results showed that *TaHsfs* were distantly related to the *Hsfs* in *Arabidopsis thaliana* and *Brassica rapa*, where only two and three genes were syntenic genes, respectively (Figure 2A). By contrast, the number of syntenic genes between wheat and rice (25), sorghum (39), corn (30), and *Brachypodium distachyon* (34), which all belong to the Gramineae, were higher (Figure 2B, Figure 2C). *Arabidopsis* and *Brassica rapa* are both cruciferous, so their 21 *Hsf* genes had synteny relationships (Figure 2D). Thus, the *Hsf* genes in plants from the same family were more closely related, thereby suggesting that the evolution of *Hsfs* is consistent with the evolutionary direction of the whole genome. To further investigate the evolutionary relationships in the *TaHsf* family, we performed synteny analysis within the *TaHsf* genome. Only two pairs of tandem duplications comprising *TaHsf-20/ TaHsf-19* and *TaHsf-30/ TaHsf-29* occurred in the *TaHsf* family on the 3A and 3D chromosomes, respectively (Additional file 3: Figure S1), and their KA/KS values were 0.367731 and 0.388683. Therefore, these two pairs of genes were subject to purifying selection. In addition, 52 *TaHsfs* participated in segmental duplication (Additional file 4: Table S3) and one to five *TaHsfs* were involved in each chromosome (Figure 3). The results showed that the probability of segmental duplication was greater for *TaHsfs* in the same chromosome than that between chromosomes. For instance, the segmental duplication on chromosome 2 only occurred between 2A, 2B, and 2D. Thus, the *Hsf* gene family in wheat mainly formed via segmental duplication.

Gene structure and motif composition in *TaHsfs*

Multiple sequence alignments were performed to further analyze the degree of conservation in the DBD and OD domains in the *TaHsfs*. The results showed that the DBD amino acid lengths in the *TaHsfs* were 76–94 aa, and thus they were found to be highly conserved (Figure 4A). In particular, *TaHsf-28* (76 aa) had a 17 aa deletion compared with most of the *TaHsfs*. The secondary structure of the DBD in the *TaHsfs* was predicted to comprise three alpha helices and four beta folds. According to the OD structure, the classification of the gene family was consistent with the results obtained by phylogenetic analysis, with 21 and seven aa insertions between the HR-A and HR-B in the class A and class C *TaHsfs*, respectively, and no insertion between the HR-A and HR-B in the class B *TaHsfs* (Figure 4B). The close phylogenetic relationships between the *TaHsfs* indicated the high evolutionary conservation of the ODs, and the sequences of the ODs in some pairs of paralogous genes were even identical. Figure 5 shows that the number of introns was very small in the *TaHsf* family members, ranging from 0 to 4, and the lengths of the introns varied greatly. Motif prediction identified 15 conserved motifs with high confidence ranging between 11–50 aa in the *TaHsf* family, where motif 1 was the longest (50 aa) and motif 6 was the shortest (Additional file 5: Table S4). None of the *TaHsf* contained all 15 motifs. Three *TaHsfs* in subclass A1 contained the most motifs (10) and *TaHsf-41* contained the least motifs (three). Motifs 1, 2, 5, and 6 corresponded to the DBD domain, motif 3 and 4 to the OD domain, motif 7 to the NLS, and motif 10 to the AHA motif.

Predicted cis-elements in *TaHsf* promoters and interaction network analysis

In order to facilitate subsequent mining of the functions and mechanisms of *TaHsfs*, we predicted the cis-elements in their upstream promoter regions (Figure 6). We screened six cis-elements comprising three related to stress responses (DRECRCOREAT (dehydration-responsive element), CURECORECR (copper-response element), and SORLIP1A (over-Represented in Light-Induced Promoters)) and the other three were CGCGBOXAT (Ca⁺⁺/calmodulin binds), MYBPLANT (plant MYB binding site), and POLLEN1LELAT52 (one of two co-dependent regulatory elements responsible for pollen specific activation). The results showed that except *TaHsf-14*, there was at least one cis-element binding site in each *TaHsf*. The combination of *TaHsf* with DRECRCOREAT, CURECORECR, and SORLIP1A also showed that *TaHsfs* function in the resistance to heat stress, but some *TaHsfs* also respond to drought, copper, and light stresses. The Ca⁺⁺/calmodulin pathway is the primary signaling pathway and almost every *TaHsf* gene was shown to contain more than one binding site. In our previous study, we found that *TaHsf* interacts with MYB, and this result was confirmed by the MYBPLANT binding sites in *TaHsf-15* (B2), *TaHsf-16* (C1), *TaHsf-28* (A4), and *TaHsf-35* (A2). Cis-element analysis allowed us to obtain the upstream factors that regulate their activity, but we determined their interactions using PlantReg Map (Figure 7A). The predicted results indicated interactions between 10 *TaHsfs*, including self-regulation between *TaHsf-02* and *TaHsf-44*, and both these and nine other genes had regulatory or regulated effects. In addition, we predicted their interacting genes using STRING and found that three main types of genes had interaction relationships with most of the *TaHsfs*, i.e., the target genes of *TaHsfs* comprised *Hsp70s* and *Hsp90s*, as well as the chaperone protein htpG (high-temperature protein G) (Figure 7B).

Expression profiles of *TaHsfs*

In order to fully understand the expression patterns of the *TaHsfs* during each plant growth period and their organization, we analyzed RNA-seq data from the roots, stems, and leaves in the seedling, vegetative growth, and reproductive growth stages, as well spikes from the vegetative growth and reproductive growth stages, and the mature grain (Wheat Expression Browser, <http://www.wheat-expression.com/>, developmental time-course of Chinese Spring). The results showed that in the roots during the seedling stage, only *TaHsfs* in subclasses A2 (*TaHsf-39* and *TaHsf-49*), C1 (*TaHsf-16*), and C2 (*TaHsf-25*, *TaHsf-36*, and *TaHsf-34*) were upregulated. In the roots during the vegetative growth and reproductive growth stages, the *TaHsfs* with upregulated expression belonged to seven subclasses comprising A1 (*TaHsf-46*, *TaHsf-32*, and *TaHsf-51*), A4 (*TaHsf-23*, *TaHsf-18*, *TaHsf-28*, and *TaHsf-01*), A5 (*TaHsf-52*, *TaHsf-53*, and *TaHsf-54*), B1 (*TaHsf-37*, *TaHsf-43*, and *TaHsf-47*), B2 (*TaHsf-38* and *TaHsf-44*), C1 (*TaHsf-16*), and C2 (*TaHsf-25*, *TaHsf-36*, *TaHsf-34*, and *TaHsf-42*). In the stems and leaves during the seedling stage, the upregulated genes only belonged to subclasses A2 (*TaHsf-05*) and A3 (*TaHsf-56*). In the stems and leaves during the vegetative growth period, the upregulated genes belonged to subclasses A2 (*TaHsf-03* and *TaHsf-04*) and C1 (*TaHsf-20*, *TaHsf-24*, and *TaHsf-29*). In the stems and leaves during the reproductive growth period, the upregulated genes belonged to subclasses A2 (*TaHsf-40*, *TaHsf-31*, *TaHsf-33*, *TaHsf-35*, *TaHsf-02*, *TaHsf-03*, *TaHsf-04*, *TaHsf-39*, and *TaHsf-49*) and B2 (*TaHsf-55*, *TaHsf-57*, *TaHsf-59*, *TaHsf-15*, *TaHsf-08*, *TaHsf-11*, *TaHsf-38*, *TaHsf-44*, and *TaHsf-48*). In the spikes during the vegetative growth phase, the upregulated genes mainly belonged to subclasses B4 (*TaHsf-06*, *TaHsf-13*), A2 (*TaHsf-45*), A6 (*TaHsf-07*, *TaHsf-10*, *TaHsf-14*), and A5 (*TaHsf-54*). In the spikes during the reproductive growth phase, the upregulated *TaHsfs* mainly belonged to C1 (*TaHsf-17*, *TaHsf-19*, *TaHsf-22*, *TaHsf-27*, *TaHsf-29*, and *TaHsf-21*) and A2 (*TaHsf-50*, *TaHsf-45*, *TaHsf-40*, *TaHsf-05*, *TaHsf-09*, and *TaHsf-12*). Among the mature grains, the upregulated *TaHsfs* mainly belonged to subclasses A2 (*TaHsf-05*, *TaHsf-09*, *TaHsf-12*, *TaHsf-40*, *TaHsf-50*), A3 (*TaHsf-56*, *TaHsf-58*), C2 (*TaHsf-61*, *TaHsf-55*, *TaHsf-25*, *TaHsf-60*), and A6 (*TaHsf-14*, *TaHsf-07*, *TaHsf-10*) (Figure 8A, Additional file 6: Table S5).

The analysis of cis-elements demonstrated that *TaHsfs* also regulate drought stress, so we investigated the expression patterns of *TaHsfs* under heat stress and drought stress (Wheat Expression Browser, <http://www.wheat-expression.com/>, drought and heat stress time-course in seedlings). The RNA-seq data showed that (Figure 8B, Additional file 7: Table S6) compared with the no stress control, the *TaHsfs* did not exhibit differences in their expression levels in response to drought stress for 1 h, but the *TaHsfs* in C1 (*TaHsf-16*, *TaHsf-21*, *TaHsf-17*, *TaHsf-22*, *TaHsf-26*, and *TaHsf-27*) and C2 (*TaHsf-60*, *TaHsf-36*, *TaHsf-61*, *TaHsf-25*, and *TaHsf-42*) were significantly upregulated under drought stress for 6 h. Under heat stress for 1 h and the combination of heat stress and drought stress for 1 h, the expression levels of the *TaHsfs* were similar, where the main upregulated *TaHsfs* belonged to A2 (*TaHsf-02*, *TaHsf-03*, *TaHsf-04*, *TaHsf-31*, *TaHsf-39*, *TaHsf-49*, *TaHsf-33*, *TaHsf-35*, *TaHsf-50*, *TaHsf-45*, and *TaHsf-40*), A3 (*TaHsf-56* and *TaHsf-58*), A4 (*TaHsf-23*, *TaHsf-18*, and *TaHsf-28*), A5 (*TaHsf-52*), B2 (*TaHsf-08*, *TaHsf-15*, *TaHsf-11*, *TaHsf-57*, *TaHsf-55*, and *TaHsf-59*), and B1 (*TaHsf-37*, *TaHsf-43*, and *TaHsf-47*). *TaHsfs* from A6 (*TaHsf-50*, *TaHsf-45*, and *TaHsf-40*) and A4 (*TaHsf-23*, *TaHsf-28*, *TaHsf-18*) were upregulated under heat stress for 6 h and the combination of heat stress and drought stress for 6 h, but *TaHsfs* from B2 (*TaHsf-48*, *TaHsf-38*, and

TaHsf-44) were upregulated in the former conditions whereas *TaHsfs* from A6 (*TaHsf-10*, *TaHsf-07*, and *TaHsf-14*) were upregulated under the latter conditions.

In addition, in order to study the responses of *TaHsfs* in thermo-sensitive male sterile wheat to different fertility conditions, we analyzed the RNA-seq data for 40 *TaHsfs* under two fertility conditions in three different periods. The results showed that compared with the sterile conditions, *TaHsfs* from A1 (*TaHsf-51*, *TaHsf-32*, and *TaHsf-46*), A2 (*TaHsf-50*, *TaHsf-02*, *TaHsf-04*, *TaHsf-05*, *TaHsf-09*, *TaHsf-35*, *TaHsf-12*, and *TaHsf-33*), B1 (*TaHsf-43* and *TaHsf-47*), and B2 (*TaHsf-11* and *TaHsf-55*) were upregulated in the uninucleate anthers under fertile conditions. *TaHsfs* from A2 (*TaHsf-12*, *TaHsf-33*, *TaHsf-45*, and *TaHsf-50*), A4 (*TaHsf-18* and *TaHsf-28*), A5 (*TaHsf-54*), A6 (*TaHsf-10*), B1 (*TaHsf-43*), and B4 (*TaHsf-06*) were significantly upregulated in binucleate anthers, and *TaHsfs* from A2 (*TaHsf-35*, *TaHsf-12*, and *TaHsf-33*), A4 (*TaHsf-07*), A5 (*TaHsf-53*), and B2 (*TaHsf-55*) in the trinucleate anthers under fertile conditions. However, no *TaHsfs* in subclass C were upregulated under fertile conditions during these three periods (Figure 8C, Additional file 8: Table S7).

The results indicated that the responses of *TaHsfs* from different subclasses varied in different developmental stages, tissues, stresses, and ecological conditions, thereby demonstrating that the different subclasses of *TaHsf* have diverse functions and they may participate in different regulatory links and pathways.

Discussion

Wheat is an important food crop throughout the world and it is of great economic importance to increase its stress resistance, yield, and quality. The release of the wheat genome sequence had provided important information for identifying excellent agronomic traits, stress resistance genes, and heat resistance genes in wheat at the genome level, thereby facilitating the cultivation of high quality and stress-resistant (heat) wheat varieties. In plants, HSFs are involved in various molecular processes and they have essential roles in the responses to abiotic stresses. In this study, we identified the members of the *Hsf* gene family in wheat as well as investigating their evolutionary relationships, gene structure, expression levels under different conditions, and their expression in thermo-sensitive male sterile wheat under different fertility conditions. Our findings facilitate the subsequent study of the stress resistance mechanism in wheat and they provide a reference for understanding the molecular temperature sensing and regulation mechanisms in plants with temperature-sensitive biological characteristics.

Previous studies identified 21, 25, 25, 25, and 22 *Hsf* genes in *Arabidopsis*, rice, maize, sorghum, tomato, and chickpea, respectively [12-15, 34]. These findings suggest that the number of the plant HSF family members is about 25 and that the number of genes in this family is highly conserved. However, in the present study, we searched for wheat *Hsfs* using various methods and finally identified 61 wheat HSFs with DBD structures. The number of HSF family members appears to be unrelated to the genome size, e.g., 35 *Hsfs* were found in Chinese cabbage (485 Mb) and 137 *Hsfs* in pear (527 Mb) [20, 35]. In the present study, in addition to employing two approaches for identifying *TaHsfs*, we also used the wheat

HSF sequence in the plantTFDB database and NCBI as the query sequence for aligning all of the wheat protein sequences. Verification using Pfam, SMART, Conserved Domain Database (CDD), MARCOIL, and other databases showed that the final results were exactly the same as those obtained using the two approaches employed in the present study. Thus, the method employed for constructing wheat-specific HSF HMM files with HSF HMM files (PF00447) in the present study was highly effective and reasonable, and the combination of multiple approaches avoided the failure to identify *TaHsfs*. However, after checking the wheat HSFs provided in plantTFDB, we found that several transcripts designated as HSFs by PlantTFDB had a DBD but they lacked the necessary coiled-coil structure for a HSF. These discrepancies may be explained as follows. First, the tools used for identification may have been different or the same tool could have been used with different parameter settings, e.g., changing the E-value threshold will affect the HSF screening results. Second, a different version of the genome could have been employed with different genome annotations, and thus the screening results obtained may have been different.

A gene family is a group of genes derived from the same ancestor and produced by a gene or by two or more copies of the gene. Orthologs are proteins that have evolved from a vertical lineage (species formation) of different species and they usually retain the same function as the original protein. A paralog is a protein derived from gene duplication in a certain species and it may develop new related functions [36]. In our study, the phylogenetic tree was constructed using the maximum likelihood method for similar regions in the wheat, rice, and *Arabidopsis thaliana* genomes. We detected two pairs of orthologous genes between *TaHsfs* and *OsHsfs* but 19 paralogous pairs, which indicate that the *Hsfs* gene in wheat was constructed before the differentiation of wheat and rice, and it expanded during the evolution of the respective species. The new functions of the wheat HSF family members mainly developed after differentiation. This phenomenon has also been verified widely in studies of other gene families in plants [37-39]. In contrast to the HSF clusters in rice and *Arabidopsis*, the wheat HSF family lacks the A7, A8, A9, and B3 subclasses, thereby indicating that gene deletion occurred in the wheat HSF family during its evolution. Two important events may occur during the evolution of genes: gene duplication and gene synteny. Synteny refers to the fact that two or more markers on a chromosome or chromosome segment from a species are located on homologous chromosome fragments in another species, but the relative order of these markers may vary, even within the same species. In this study, we analyzed the synteny relationships between wheat and *Arabidopsis thaliana*, Chinese cabbage, maize, rice, sorghum, and *Brachypodium*. The results showed that the syntenic relationships of *Hsfs* were stronger in species when the relationship with wheat was closer. Segmental duplication and tandem duplication are the two main causes of the expansion of gene families in plants [39]. After the occurrence of gene duplication, one of the genes exerts its original function and the other copy can accumulate mutations without a loss of function, thereby allowing the generation of new genes and regulatory networks, which can evolve new functions and network regulation mechanisms to allow plants to adapt better to the environment [40]. We found two pairs of tandem duplications and 59 pairs of segmental duplications in the HSF family in wheat, and thus segmental duplication has played a major role in the expansion of the HSF family in wheat. Similar to gramineous plants, the genes in *Arabidopsis* and maize

are more prone to genome-wide replication and segmental duplication, and these are the main modes of gene expansion in wheat.

The evolution of gene families is often accompanied by an increase or loss of exons. The structural differences between introns and exons are often important, and they have played key roles in the evolution of gene families [41]. We analyzed the number and distribution of exons and introns in each member of the *TaHsfs* family. We found that the 61 *TaHsfs* contained 1–3 exons and 0–4 introns. The lengths and positions of the *TaHsf* gene introns in the same subclass were relatively well conserved, but the introns varied greatly among the different *TaHsf* subclasses. The presence of introns allows a variety of splicing modes and the same DNA sequence can produce different protein products via alternative splicing of the gene after transcription [42]. This is one of the explanations for the functional diversity of the wheat HSF family during evolution and it is also a cause of the different temperature responses of *TaHsfs* [43]. The sizes of HSF sequences have differed during the evolution of eukaryotes but their basic structure is highly conserved. In our study, motifs 1, 2, 5, and 6 corresponded to the DBD domain located at the N-terminus of each *TaHsf* and their structures were found to be highly conserved, thereby making them highly compatible with HSEs to ensure the accuracy localization of the HSEs and the precise regulation of downstream gene expression [7, 44]. Motifs 3 and 4 corresponded to the OD domain and plant HSFs can be divided into three categories based on the unique features of the OD: class A, class B, and class C [5, 45]. This is one of the reasons why we excluded candidate *TaHsfs* without a coiled-coil structure but a DBD structure.

The heat shock response is an extremely complex regulatory process that involves multiple signals and genes. Plants have been shown to respond to high temperature stress mainly via the HSF-HSP pathway, calcium-calmodulin (Ca^{++} -CaM) pathway, reactive oxygen species, and hormonal pathways [46]. The plant plasma membrane receives the heat stress signal and the fluidity of the cell phospholipid bilayer increases, thereby allowing a large amount of Ca^{++} to flow into the cell. Next, the effect of the inflow of Ca^{++} is amplified by a signaling cascade to transmit the heat stress signal to the nucleus, which leads to the expression and accumulation of HSPs, osmoregulatory proteins, and antioxidant proteins, thereby alleviating the damage caused by high temperature and making the plant heat resistant [47, 48]. MYB is one of the largest families of plant transcription factors involved in the regulation of cell differentiation during the cell cycle as well as responding to hormones and the environment. In our previous studies, we showed that MYBs were significantly upregulated under high temperature conditions and they interacted with *TaHsfs* [49]. Therefore, the cis-elements of the *TaHsfs* promoter region were predicted. Except for *TaHsf-12*, *TaHsf-14*, and *TaHsf-24*, we predicted at least one CGCGbox as a Ca-binding site for each gene. Moreover, each class of *TaHsfs* was shown to have binding sites for MYB.

In the present study, we analyzed the expression patterns of *TaHsf* genes in nine different developmental stages and various tissues in order to classify their tissue-specific expression. The results showed that the *TaHsf* expression patterns were similar in the roots where they were mostly upregulated during the vegetative growth and reproductive growth stages. *TaHsf* subclasses A1, A4, A5, B1, B2, C1, and C2 all exhibited upregulated expression in the roots during these stages, but the expression levels of the

subclass A2 genes were not significantly upregulated, whereas these genes were upregulated in various tissues during other periods. In rice, the expression levels of A class *Hsfs* also vary in different specific tissues under normal conditions [23, 50]. The functions of genes that participate in regulation can be predicted according to the preferential expression levels of genes under different environmental conditions in the same tissue. The overexpression of *Hsfs* can increase the tolerance of heat in plants by increasing the expression of *Hsps* and other related genes. Our analysis showed that class A and B *TaHsfs* were significantly upregulated under heat stress whereas class C *TaHsfs* were not, as also found in maize and pepper [13, 18]. Under drought stress, the expression levels of class A and B *TaHsfs* were not significantly increased, but the C class *TaHsfs* were significantly upregulated, and thus it appears that the A and B class *TaHsfs* are involved in heat shock regulation whereas the C class *TaHsfs* are involved in drought regulation. Previous studies have shown that B1, B2, and B4 subclass *TaHsfs* are often induced by the heat shock reaction, and they may act as synergistic factors that allow A class *TaHsfs* to resist heat more effectively [29, 51, 52]. However, B3 subclass *TaHsfs* are downregulated as suppressors under heat shock conditions [18]. No A3 subclass *TaHsfs* were identified in wheat, so we could only check the coordinated expression of B1, B2, and B4 subclass *TaHsfs* and class A *TaHsfs* in response to heat. In our previous studies, we suggested that some *Hsfs* may be involved in pollen development in plants, which is related to fertility. In the present study, we showed that *TaHsfs* were specifically expressed under different fertility conditions, and that class A and B *TaHsfs* were upregulated under fertile conditions, and they were the same as the genes involved in the heat stress response. We originally considered that heat would induce the expression of these *TaHsf* genes and that their upregulated expression would lead to the upregulation of other fertility-related genes, thereby ensuring fertility. However, we found that some genes had pollen-specific expression-related binding sites based on our predictions of the cis-elements, and previous studies have shown that the AT2G26150 and AT4G13980 genes are critical during pollen reproduction [53, 54]. Our analysis of the *TaHsfs* related to wheat fertility provides a basis for future investigations of the important roles of *TaHsfs* in the fertility regulation mechanism.

Conclusions

In this study, we comprehensively analyzed the HSF family in wheat based on the latest release of the complete wheat reference genome. We identified 61 *TaHsfs* and they were unevenly distributed on 21 chromosomes. According to phylogenetic comparisons with *Arabidopsis* and rice, and structural analysis of the gene products, the wheat HSFs were divided into three classes (A, B, and C) and 11 subclasses (A1–A6, B1, B2, B4, C1, and C2). The synteny relationships showed that the HSF family in wheat is closely related to that in *Brachypodium*, and the expansion of the wheat HSF family mainly occurred by segmental duplication. The 61 wheat *Hsfs* contain 0–4 introns and 1–3 exons, and the gene structures are highly conserved in the same subclass. Cis-element prediction and RNA-seq analysis showed that the different members of the *TaHsf* family have diverse roles. In addition to resisting heat stress, *TaHsfs* also participate in the regulation of other stresses such as drought. Analysis of the expression levels in temperature-sensitive male sterile wheat confirmed that some *TaHsfs* are associated with pollen development and fertility conversion in wheat. Thus, we identified new members of the wheat HSF family,

thereby facilitating further research into the functions of HSFs and their roles in the temperature response mechanism in temperature-sensitive wheat.

Abbreviations

HSF: Heat shock transcription factor

Hsf: HSF gene

TaHsf: Wheat Hsf

HSE: Heat shock element

HSP: Heat shock protein

Hsp: HSP gene

DBD: DNA binding domain

OD: Oligomeric domain

NLS: Nuclear localization signal

NES: Nuclear export signal

CTAD: C-terminal activation domain

H2-T-H3: Helix-turn-helix

HR-A/B: Hydrophobic heptad repeat regions A and B

AHA: Acidic amino acid

HMM: Hidden Markov model

aa: Amino acid

DRECR COREAT: Dehydration-responsive element

CURE CORECR: Copper-response element

SORLIP1A: Over-Represented in Light-Induced Promoters

CGCGBOXAT: Ca⁺⁺/calmodulin binding region

MYBPLANT: Plant MYB binding site

POLLEN1LELAT52: One of two co-dependent regulatory elements responsible for pollen specific activation

htpG: High-temperature protein G

CDD: Conserved domain database

HSF-HSP: Heat stress transcription factor-heat shock protein

Ca⁺⁺-CaM: Calcium-calmodulin

Declarations

Competing interests

The authors declare that they have no competing financial interests.

Authors' contributions

Xiyue Song and Lingli Zhang designed the research. Jiali Ye and Xueting Yang performed the experiments. Jiali Ye, Sha Li, Qi Liu, and Wei Li analyzed the data. Jiali Ye wrote the manuscript.

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Tables

Due to technical limitations, Table(s) 1 and 2 are only available as a download in the supplemental files section.

Figures

Figure 1

Phylogenetic tree obtained for the HSF family in wheat, rice, and Arabidopsis.

2A

2B

2C

2D

Figure 2

Synteny relationships of Hsfs in wheat and six representative species. Gray lines in the background indicate the synteny blocks for wheat and other plant genomes, and the lines in other colors highlight the synteny of Hsf gene pairs.

Figure 3

Genomic locations of Hsfs and duplicated gene pairs in the wheat genome. Gray lines in the background indicate the syntenic blocks within the whole wheat genome, and red lines denote the segmental duplication Hsf gene pairs.

4A

4B

Figure 4

Analysis of the conservation of DBD domains and OD regions in the 61 Hsf genes in wheat. a. Multiple sequence alignment of the DBD domains in 61 TaHsfs. The scheme at the top depicts the protein secondary structure predicted based on the conserved DBD domain. b. Multiple sequence alignment of the HR-A/B regions in 61 TaHsfs. The scheme at the top shows the locations and boundaries of the HR-A core, insert, and HR-B regions within the HR-A/B regions.

Figure 5

Phylogenetic tree, gene structure, and architecture of conserved protein motifs in TaHsfs. Details of the clusters in the phylogenetic tree are shown in different colors. The left panel represents the intron–exon structure of the TaHsfs, and the right panel shows the motif compositions of the TaHsfs. Different motifs are indicated by different colors and they are numbered from 1–15.

Figure 6

Distribution of cis-element binding sites in the promoter regions of Hsf family members in wheat.

7A

7B

Figure 7

Interaction network between TaHsfs and related genes. a. Interactions between TaHsfs where the arrows denote regulation. b. Interactions between TaHsfs and other genes. The red circles represent TaHsfs and the hexagons in other colors represent the genes that interact with them. Lines connecting two genes show that an interaction exists between them.

Figure 8

Expression profiles for TaHsfs in different condition. a. Heat map of TaHsfs in nine different tissues and growth stages under normal conditions. b. Heat map of TaHsfs under five different abiotic stress conditions and in a normal control. c. Heat map of TaHsfs in the thermosensitive male sterile wheat KTM3315A at two different temperatures. Heatmap obtained using the count values from RNA-seq analysis.

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