Heat-response patterns of the heat shock transcription factor family in advanced development stages of wheat (*Triticum aestivum* L.) and thermotolerance-regulation by *TaHsfA2–10*

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**Abstract**

**Background:** Heat shock transcription factors (*Hsfs*) are present in majority of plants and play central roles in thermotolerance, transgenerational thermomemory, and many other stress responses. Our previous paper identified at least 82 *Hsf* members in a genome-wide study on wheat (*Triticum aestivum* L.). In this study, we analyzed the *Hsf* expression profiles in the advanced development stages of wheat, isolated the markedly heat-responsive gene *TaHsfA2–10* (GenBank accession number MK922287), and characterized this gene and its role in thermotolerance regulation in seedlings of *Arabidopsis thaliana* (L. Heynh.).

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Results: In the advanced development stages, wheat Hsf family transcription profiles exhibit different expression patterns and varying heat-responses in leaves and roots, and Hsfs are constitutively expressed to different degrees under the normal growth conditions. Overall, the majority of group A and B Hsfs are expressed in leaves while group C Hsfs are expressed at higher levels in roots. The expression of a few Hsf genes could not be detected. Heat shock (HS) caused upregulation about a quarter of genes in leaves and roots, while a number of genes were downregulated in response to HS. The highly heat-responsive gene TaHsfA2–10 was isolated through homologous cloning. qRT-PCR revealed that TaHsfA2–10 is expressed in a wide range of tissues and organs of different development stages of wheat under the normal growth conditions. Compared to non-stress treatment, TaHsfA2–10 was highly upregulated in response to HS, H2O2, and salicylic acid (SA), and was downregulated by abscisic acid (ABA) treatment in two-leaf-old seedlings. Transient transfection of tobacco epidermal cells revealed subcellular localization of TaHsfA2–10 in the nucleus under the normal growth conditions. Phenotypic observation indicated that TaHsfA2–10 could improve both basal thermotolerance and acquired thermotolerance of transgenic Arabidopsis thaliana seedlings and rescue the thermotolerance defect of the T-DNA insertion mutant athsfA2 during HS. Compared to wild type (WT) seedlings, the TaHsfA2–10-overexpressing lines displayed both higher chlorophyll contents and higher survival rates. Yeast one-hybrid assay results revealed that TaHsfA2–10 had transactivation activity. The expression levels of thermotolerance-related AtHsps in the TaHsfA2–10 transgenic Arabidopsis thaliana were higher than those in WT after HS.

Conclusions: Wheat Hsf family members exhibit diversification and specificity of transcription expression patterns in advanced development stages under the normal conditions and after HS. As a markedly responsive transcriptional factor to HS, SA and H2O2, TaHsfA2–10 involves in thermotolerance regulation of plants through binding to the HS responsive element in promoter domain of relative Hsps and upregulating the expression of Hsp genes.

Keywords: Heat shock transcription factor, Wheat, Expression pattern, Thermotolerance, Transcription activity, Binding activity.

Background
Owing to greenhouse gas emissions, the global mean surface temperature has increased about 0.65 °C from 1956 to 2005 [1]. The rising temperature has become one of the major climatic disasters restricting crop growth and development around the world [2]. Wheat (Triticum aestivum L.) is the main cereal crop in many countries of the world and the high and stable yield is the most important breeding target. However, wheat crops frequently suffer from cross-stresses of heat and dry wind, causing recent decreases in both quantity and quality [3]. It is therefore necessary to analyse molecular mechanisms of thermotolerance and develop wheat cultivars with high resistance to heat stress (HS).

Heat shock transcription factors (Hsfs) in plants play central roles in regulating plant thermotolerance. Hsfs can activate the expression of heat shock protein (Hsp) genes and thermotolerance-related genes by binding to HS responsive elements (HSEs) within promoters [4–7]. Since the cloning of yeast Hsf in the 1980s, many Hsfs have been recently identified at the genome-wide scale in a variety of species [8–12], including the first plant Hsf gene from tomato (Solanum lycopersicum L.) [13]. Plant Hsfs are divided into group A, B, C and are further divided into several subgroups based on different protein structures [4]. The number of Hsf gene family members varies greatly between species. So far, studies have identified 21 Hsfs in Arabidopsis thaliana, 16 Hsfs in tomato, and 82 Hsfs in wheat [7, 14].

Most previous studies on Hsfs have been limited to A1 and A2 Hsf subclasses within the model plants Arabidopsis thaliana and Solanum Lycopersicum (S. lycopersicum) [15–18]. The S. lycopersicum HsfA1 gene is constitutively expressed at low level and the protein coded by the gene localizes to both the nucleus and cytoplasm under the normal growth conditions. HsfA2 is localized in the cytoplasm due to a strong cytoplasmatic localization signal, while its nuclear entry relies on the binding of HsfA2 to HsfA1 to form a hetero-oligomer during HS [8, 17]. HsfA2 expression is strictly induced by HS and HsfA2 proteins can accumulate after continuous or repeated HS and during recovery from HS [8, 17]. Only one HsfA2 exists in both Arabidopsis thaliana and S. lycopersicum [13].

Arabidopsis thaliana HsfA2 is localized in both the nucleus and the cytoplasm and can activate downstream Hsp gene expression upon binding with and activation by AtHsfA1. When AtHsfA1 is deleted, AtHsfA2 can enter the nucleus and regulate the expression of a series of Hsps and chaperone genes [18]. AtHsfA1 mainly acts as a transcription factor while AtHsfA2 regulates acquired thermotolerance by activating the expression of genes related to reactive oxygen species and carbohydrate and lipid metabolism to maintain cell membrane...
stability in the later period of HS [19]. In addition, AtHsfA2 can partially perform certain functions of AtHsfA1 during exposure to different heat ranges and oxygen stress and can rescue AtHsfA1 mutant phenotypes [20–22]. Most recently, AtHsfA2 was found to regulate transgenerational thermomemory induced by HS in Arabidopsis thaliana by directly activating the H3K27me3 demethylase REF6 (Relative of early flowing 6) [23], suggesting that HsfA2 may participate in diverse thermotolerance regulation [15, 16, 20, 21, 24].

Studies to determine characteristics and functions of wheat Hsf genes have only recently begun. In 2008, seven TaHsfS were identified in wheat, one of which was dramatically upregulated by HS, suggesting that these TaHsfs help regulate thermotolerance [25]. In addition, TaHsfA4a is upregulated by cadmium stress and participates in cadmium tolerance [26]. Expression of the TaHsfA2d gene in Arabidopsis thaliana improves thermotolerance, salinity tolerance, and drought tolerance of seedlings, with the seedlings growing at moderately high temperatures displaying increased biomass and yield [27]. For seedlings of Arabidopsis thaliana expressing TaHsf3, both thermotolerance and cold resistance can potentially be improved [28]. In 2014, 56 Hsf members from families A, B, and C were identified in T. aestivum, many of which are constitutively expressed, and others in subgroups A2, B2, and A6 are significantly upregulated by HS [29]. TaHsfA6f directly regulates the expression of genes TaHsps, TaGAAP (Golgi anti-apoptotic protein, GAAP), and TaRof1 (a co-chaperone) and thus enhances seedling thermotolerance [30]. TaHsfs vary in expression levels and sensitivity to abiotic stresses including heat, salinity, drought, and cold [31]. TaHsfC2a is highly expressed in the filling stage of wheat and its overexpression upregulates the expression of genes related to drought, heat, and abscisic acid (ABA) responses, TaHsfC2a also provides proactive heat protection in developing wheat grains via an ABA-mediated regulatory pathway [32].

We previously reported that TaHsfB2d can regulate HS responses through a salicylic acid (SA) signalling pathway, which is dependent on H2O2 levels [33]. Both basal and acquired thermotolerances are improved in Arabidopsis thaliana overexpressing TaHsfA2e, with increased expression of multiple Hsp genes belonging to different Hsf group [34]. Hsp genes can improve the thermotolerances of transgenic Arabidopsis thaliana, though expression response to HS was different [34]. In another recent report, we identified 82 wheat Hsf genes in a genome-wide study. These TaHsf family members showed diverse expression patterns in both leaf and roots, and under osmotic stresses such as SA, H2O2, and ABA in two-leaf-old seedlings of wheat. Among the 82 wheat Hsf genes, 9 members of subclass A2 and 17 members of other subclass were newly identified [14]. However, little is known about the characteristics and functions of these genes nowadays.

The average temperature over land from 2006 to 2015 was 1.53 ºC higher than that from 1850 to 1900 and the warming temperature led to reduction of crop yield [35]. It is estimated that the yield of global wheat fall by 6% with 1 ºC increasing of global temperature [36]. So it is important to thoroughly investigate Hsf gene expression profiles in advanced development period of wheat and understand the thermotolerance-regulating functions of individual Hsf members during HS responses. This is especially relevant for subclass A2, which has previously been reported to be important for acquired thermotolerance during advanced development periods of wheat [20]. The aim of this study is to investigate the expression characterization of wheat Hsf family in the advanced development stages under HS and further elucidate thermotolerance regulatory function of individual wheat Hsf. The results may enable further understanding of biological functions and molecular mechanisms of Hsf family members and identify target genes for improving thermotolerance of wheat varieties.

Results

Expression patterns of wheat Hsf gene during HS in advanced development stages of T. aestivum

Flag leaves and roots of wheat under the normal growth conditions and after HS at 37 ºC were sampled at the anthesis stage and latter 10 d and 20 d, and used to analyse expression profiles of wheat Hsf genes via RNA-Seq (Fig. 1). Eighty wheat Hsf family genes were detected in both leaves and roots, except for TaHsfA2–11 and TaHsfA2–18. Transcription profiles of TaHsfs revealed complex expression patterns in leaves and roots. Under the normal conditions, no difference was detected in the expression profiles of most genes in leaves and roots of wheat in different stages. However, some genes were expressed at higher levels in leaves at the anthesis stage than the latter two development stages of wheat. These genes included the subclass A2 members TaHsfA2–7, TaHsfA2–8, TaHsfA2–9, TaHsfA2–13, the TaHsfB1 members, the B2 subclass members of TaHsfB2–6, TaHsfB2–7, TaHsfB2–8, and the C2 subclass members of TaHsfC2–2, TaHsfC2–3, and TaHsfC2–4. Expression levels of TaHsfA1–1, TaHsfA1–2, and TaHsfA1–3 increased in leaves in the two development stages after anthesis, and similar expression profiles of TaHsfB1–1, TaHsfB1–2, and TaHsfB1–3 were observed in wheat roots. Overall, the majority of class A and B Hsfs were expressed at higher levels in leaves while class C Hsfs were expressed at higher levels in roots.

Hsf expression in T. aestivum during advanced development stages exhibited multiple HS response patterns
Fig. 1 (See legend on next page.)
(Fig. 1). In both leaves and roots, Hsf expression levels were increased to different degrees under HS, especially those genes of subclasses A2, B1, and B2. Especially, TaHsfA2−10 and TaHsfA2−12 were increased most obviously under HS. In contrast, three TaHsfA1s were downregulated during HS in leaves and roots of wheat during three development stages. The expression levels of three A6 subclass members were remarkably upregulated by HS in leaves, but not in roots. In addition, the homeologous genes TaHsfC1−7, TaHsfC1−8, TaHsfC1−9, and both TaHsfC3−4 and HsfC3−10 were upregulated by HS in roots, but not in leaves. Additionally, the expression of those genes were undetectable during normal and HS conditions, including all subclass B4 members, six subclass C1 members, all subclass C3 members in wheat leaves, all subclass B4 members and three subclass C1 members in roots.

Amplification of TaHsfA2−10 cDNA and structural analysis of the encoded protein in T. aestivum

The cDNA sequence of TaHsfA2−10 was cloned using homoeologous cloning from young leaves of T. aestivum Cang 6005 after HS at 37 °C. The full-length sequence of TaHsfA2−10 is 1119bp long and encodes 372 amino acids. TaHsfA2−10, which is located on chromosome 5AL, is homoeologous to previously identified TaHsfA2−12 on chromosome 5DL [14]. The amino acid sequence of TaHsfA2−10 contained a DNA-binding domain (DBD), an oligomerization domain (OD), a nuclear localization signal (NLS), a nuclear export signal (NES), and an activator peptide motif (AHA). Protein similarity analysis indicated that TaHsfA2−10 is highly identical to AtHsfA2a-like from Aegilops tauschii, HvHsfA2a from Hordeum vulgare, BdHsfA2a from Brachypodium distachyon, and PhHsfA2a from Panicum hallii (Fig. 2).

TaHsfA2−10 expression in different tissues and organs of T. aestivum under abiotic stress

qRT-PCR analysis revealed that TaHsfA2−10 is constitutively expressed in many tissues and organs in different development stages of T. aestivum, with the highest expression levels in mature embryos, and expression levels in other tissues and organs were relative lower, suggesting that Hsf genes expression exist tissue-specific variations (Fig. 3a). TaHsfA2−10 expression levels in leaves were upregulated by HS, peaking at 90 min of the control levels while subjected to HS (Fig. 3b). TaHsfA2−10 levels also increased after application of exogenous SA (Fig. 3c) and H2O2 (Fig. 3d) with peak levels nearly 40 times and 25 times of their own controls at 120 min and 90 min after subjected to different stresses, respectively. In contrast, the expression of TaHsfA2−10 was downregulated by exogenous ABA (Fig. 3e).

Subcellular localization of TaHsfA2−10

The recombinant vector of TaHsfA2−10 with N-terminal of GFP fusion (pCAMBIA1300-TaHsfA2−10-GFP) and the recombinant vector of TaHsfA2−10 with C-terminal of GFP fusion (pCAMBIA1300-GFP-TaHsfA2−10) were constructed. The two constructs and the empty vector pCAMBIA1300-GFP were infiltrated into tobacco (Nicotiana tabacum L.) epidermal cells, respectively. Observation results showed that TaHsfA2−10 was nucleus localized under the normal growth conditions (Fig. 4).

Analysis of transactivation activity of TaHsfA2−10 in yeast

The transactivation activity of TaHsfA2−10 was evaluated in the yeast medium SD/Trp’/His’/Ade’/X-a-gal. As shown in Fig. 5, positive controls containing pGBK7−53 grew well while the negative control hardly grows. Yeast transformed with pGBK7−TaHsfA2−10 grew similarly as positive control (Fig. 5). This result suggested that TaHsfA2−10 possesses transactivation activity in yeast.

Evaluation of thermotolerance regulation by TaHsfA2−10 in transgenic Arabidopsis thaliana

Three transgenic Arabidopsis lines overexpressing TaHsfA2−10 of T3 generation were selected, with semi-RT-PCR confirming TaHsfA2−10 expression (Fig. 6a). Next, basal and acquired thermotolerance of these TaHsfA2−10-expressing Arabidopsis seedlings were evaluated with WT seedlings. No obvious phenotypic differences between three transgenic lines and WT plants were observed under the normal growth conditions (Fig. 6b, d); however, the growth vigour of all TaHsfA2−10-expressing plants was higher than that of WT controls after two types of HS regimes treatment. Out of the transgenic lines generated, line 11_26 exhibited the strongest basal (Fig. 6c) and acquired thermotolerance phenotypes (Fig. 6e). Chlorophyll levels and survival
rates decreased with increasing thermotolerance, but transgenic lines had significantly higher chlorophyll content (Fig. 6f) and survival rates (Fig. 6g) compared to WT under HS conditions. The seedlings of line 11_26 had the highest chlorophyll content (Fig. 6f) and survival rates (Fig. 6g) among the different genotypes.

**Rescued thermotolerance of the Arabidopsis thaliana mutant athsfA2 by TaHsfA2**

Three TaHsfA2–10/athsfA2 complimentary lines, M16_30, M18_14, M21_25, were created and used to investigate thermotolerance. Semi-RT-PCR analysis confirmed expression of TaHsfA2–10 in three T3 transgenic lines while WT and the mutant athsfA2 lacked TaHsfA2–10 expression (Fig. 7a). Phenotypic observation revealed that growth vigour of WT, athsfA2, and TaHsfA2–10/athsfA2 lines were similar under normal growth conditions (Fig. 7b). However, seedlings wilted to different degrees during the recovery period after HS treatment (Fig. 7c). The growth vigour of WT was better than that of the athsfA2 while complementation lines M16_30 and M21_25 showed similar growth vigour as WT. In addition, the M18_14 line showed the least amount of discolouration, suggesting that TaHsfA2–10 can rescue the thermotolerance defect of the mutant athsfA2. M18_14 also showed higher survival rates and chlorophyll levels compared to WT, athsfA2 mutant, and M16_30 and M21_25 lines (Fig. 7d, e) after HS treatment.

TaHsfA2–10-regulating Hsp gene expression is related to HS in Arabidopsis thaliana

The expression levels of Hsps, including AtHsa32, AtERDJ3A, AtHsp70T, AtHsp90.1, and AtHsp101, were
measured by qRT-PCR. Results showed that the expression levels of these five \textit{AtHsps} in the \textit{TaHsfA2--10} transgenic line 11_26 were slightly higher than that in WT plants under the normal conditions (Fig. 8a). Individual Hsp genes were upregulated to different degrees after HS, with peak expression levels appearing 1 h or 2 h after treatment. The expression levels of \textit{AtHsfA32} and \textit{AtHsp70T} were upregulated by 4–5 times during HS in the \textit{TaHsfA2--10} line compared to WT (Fig. 8b–f). After the production of acquired thermotolerance by HS, the expression levels of most Hsp genes gradually decreased in both WT and transgenic line 11_26, except for \textit{AtHsp90.1}, which showed higher expression level in line 11_26 than in WT plants 4 h after HS. However, during the recovery periods, the expression levels of \textit{AtHsp90.1} in the transgenic line were higher than those in WT plants. Overall, Hsp expression levels were higher after HS that induced basal thermotolerance than HS that induced acquired thermotolerance.

Five \textit{AtHsps} were then selected to study the direct binding of HSEs in promoters with \textit{TaHsfA2--10} under the normal conditions using the yeast one-hybrid assay.
Results revealed that TaHsfA2–10 can bind with HSEs in promoters of all tested AtHsps (Fig. 9); further indicating that TaHsfA2–10 can regulate Hsp genes expression by binding with their HSEs.

**Discussion**

Increasing global temperatures have caused diverse and profound effects on plant growth, development and reproduction [37, 38], and greatly threaten global crop yields. Plants have evolved sophisticated epigenetic machinery to respond quickly to heat [39]. Thermotolerance can be generated upon expression of Hsp genes induced by HS. In the advanced development stages of wheat, acquired thermotolerance is the predominant factor determining HS responses [40]. Reports from model plants revealed that members of the subclass HsfA2s play central roles in regulating acquired thermotolerance, in recovery from HS, and in transgenerational thermomemory [8, 23]. Therefore, in this study, we identified genes expressed in advanced development stages in *T. aestivum* and evaluated the thermotolerance-regulating roles of individual Hsf gene family members.

![Subcellular localization of TaHsfA2-10 in tobacco epidermal cells under the normal growth conditions.](image)

**Fig. 4** Subcellular localization of TaHsfA2–10 in tobacco epidermal cells under the normal growth conditions. 

- **a** Epidermal cells of tobacco expressing 35S:TaHsfA2–10-hGFP (both C and N terminal fusions) under white light.
- **b** Epidermal cells of tobacco expressing 35S:TaHsfA2–10-hGFP under green channel fluorescence (both C and N terminal fusions).
- **c** Epidermal cells of tobacco expressing 35S:TaHsfA2–10-hGFP under DAPI blue fluorescence (both C and N terminal fusions).
- **d** Merge of DAPI and GFP green channel fluorescence (both C and N terminal fusions).

![Yeast one-hybrid analysis of TaHsfA2-10 trans-activation.](image)

**Fig. 5** Yeast one-hybrid analysis of TaHsfA2–10 trans-activation. Positive control, Negative control and TaHsfA2–10 represent yeast cells transformed with pGBK7–53, pGADT7 and pGBK7-TaHsfA2–10 on the medium of SD/Trp- and SD/Trp-/His-/-Ade-- (dyed with X-α-gal), respectively.
Our RNA-Seq results reveal that *T. aestivum* *Hsf* genes exhibit complex expression profiles and heat-response patterns in the advanced stages of wheat development (Fig. 1). The majority of class A and B *Hsf* s were predominantly expressed in wheat leaves while class C *Hsf* s were more highly expressed in wheat roots. Under the normal conditions, no obvious gene expression differences among developmental stages were observed. However, *TaHsfA2–7, TaHsfB2–6, TaHsfC2–2*, and their two homoeologous genes were more highly expressed during the anthesis stage of leaves. The expression levels of three *TaHsfA1* members increased in leaves of the later developmental stages of wheat, and the same trends were observed for three *TaHsfB1* members in wheat roots. These results indicate that *TaHsf* s are differently expressed among tissue types. The study by Xue et al. [29] revealed that members A2b/c/e, A5b, A6c/d/e were predominantly expressed in the endosperm, subclass B1.
members were expressed at higher levels in reproductive organs than in young leaves and young roots, and three C1 and C2 members were highly expressed in embryos of wheat. Most of these genes expression were very low in both leaves and roots of our experiments. However, our results showed that subclass B4 members and three C1 members were nearly undetectable in roots, while Xue’s study indicated that B4 subclass members are expressed in roots and embryos of wheat. We speculate that these differences may be caused by differences in the specific wheat variety examined. Like subclass B4 members, 13 TaHafC3s showed very low expression level in leaves but higher in roots in three advanced development stages detected in our experiments under the normal conditions.

RNA-Seq results under HS revealed that the expression of three TaHsfA1s was downregulated during HS in both leaves and roots of wheat (Fig. 1), this perhaps...
caused by sampling time, because the HsfA1s always respond to heat earlier than HsfA2s, and function at early stage of HS [15]. The expression of three HsfA6s was upregulated by HS only in wheat leaves at anthesis and two following detective stages, showing tissue-special expression under HS. Wheat TaHsfA6f was expressed constitutively in green organs but was markedly up-regulated during HS. Wheat TaHsfA6f is a transcriptional activator that directly regulates TaHsp, TaGAAP, and TaRof1 genes in wheat and its gene regulatory network has a positive impact on thermotolerance [30]. Arabidopsis AtHsfA6b operates as a downstream regulator of the ABA-mediated stress response and is required for heat stress resistance, though it response to ABA but not heat [41]. No more reports have been known about HsfA6s. Additionally, in our experiment, the expression of the homologues TaHsfC1–7, TaHsfC3–4, and TaHsfC3–10 was upregulated only in wheat roots, and the expression levels of subclass B4 members, six members of subclass C1, and 13 subclass C3 members were almost undetectable in leaves during HS while the expression of subclass B4 and three C1 members were almost undetectable in roots in three detected stages of wheat. These results expand those obtained using two-leaf-old wheat seedlings reported by Duan and co-authors, in which the subclass HsfC3s mainly responded to ABA [14], suggesting that these genes perhaps mainly participate in ABA signal transduction. These results further support the existence of a proactive TaHsfC2-mediated protective mechanism involving an ABA-dependent pathway for regulating heat protection in developing grains of wheat [32]. Our results enrich the expression characterization of wheat Hsf by providing more underlying perception on the temporal and spatial expression of wheat Hsf family. Results of cis-element analysis showed that majority of TaHsfCs promoter contain ABA responsive motifs, only the promoter of TaHsfC3–1, TaHsfC3–2 and TaHsfC3–11 contain heat responsive motif (Additional file 1). In addition, TaHsfB1s and most TaHsfB2s were upregulated in both leaves and roots, suggesting they are involved in heat response of wheat. All TaHsfB1s and TaHsfB2s contain HSE in their promoter (Additional file 1), revealing
these genes can be upstreamly regulated by Hsf. Up to now, few genes are known about TaHsfBs function involved in thermotolerance regulation, previous studies showed they serve as coregulators or repressors of the HsfAs for lacking a defined activation domain [42]. Zhao et al. reported that TaHsfB2d can improve both basal and acquired thermotolerances of transgenic Arabidopsis thaliana [33], the Arabidopsis seedlings transformed with CaHsfB2 from Cicer arietinum display relatively high drought resistance and thermal tolerance [43]. Lots of work needs to be performed about characteristics and functions of class HsfBs.

Studies of model plants indicate that, HsfA2 members participate in responses to many osmotic stresses, including heat, salt, oxygen, drought, and both ABA- and SA-mediated signal transduction. Once activated by HsfA1, HsfA2 induces the expression of many Hsp genes as a key thermotolerance-regulating factor during HS [27]. Among the 82 Hsf genes identified in our previous study, most TaHsfA2s genes exhibit diverse response patterns to osmotic stresses [14]. In this study, as one of A2 members, TaHsfA2–10 was shown to be markedly expressed both in leaves and roots under HS at anthesis and later developing stages of wheat (Fig. 1) and in mature embryos (Fig. 3a), also is significantly upregulated by heat, SA, and H2O2 in two-leaf-old seedlings (Fig. 3b-d), indicating that TaHsfA2–10 perhaps involve in thermotolerance regulation in wheat different developing stages as a key factor. SA is reported to upregulate AtHsfA2 expression depending on presence of H2O2 [44], TaHsfB2d regulates HS responses through an SA-mediated signalling pathway in plants which depends on the presence of H2O2 [33]. TaHsfC2a appears to serve a proactive role in heat protection in developing wheat grains via an ABA-mediated regulatory pathway [32]. In both our results and Duan’s report [14], TaHsfA2–10 expression were downregulated by ABA in two-leaf-old seedlings and later development stages of wheat, speculating that TaHsfA2–10 perhaps participates in diverse thermotolerance regulation through an SA-mediated signalling pathway but not involving ABA-mediated signal transduction, though the promoter of TaHsfA2–10 contains both heat and ABA responsive cis-element (Additional file 1). However, whether this pathway depends on H2O2 need more researches.

There is only one HsfA2 gene in both tomato and Arabidopsis, tomato HsfA2 was localized in cytoplasm, the nuclear translocation of HsfA2 need to rely on the heterooligomer formed between HsfA2 and HsfA1 [17], while Arabidopsis HsfA2 was localized both nuclear and cytoplasm. Different from above, TaHsfA2–10 was confirmed to be localized in nuclear by two constructs of N and C terminal of GFP fusions. We speculate that perhaps nuclear localization enables Hsf to induce downstream genes expression more quickly to improve
thermotolerance. Though all contain functional domains such as DBD, NLS, NES, AHA, different localizations of same subclass Hsf exist in different species, suggesting diversity and complexity of Hsf characteristics and function.

Further phenotype observation provided convincing evidence for the above hypothesis (Figs. 6 and 7). By expressing TaHsfA2–10 in Arabidopsis, we found that TaHsfA2–10 both improves basal thermotolerance and acquired thermotolerance of the seedlings transgenic Arabidopsis thaliana. In addition, TaHsfA2–10 can rescue the thermotolerance defect of the mutant athsfa2 during HS. Growing vigour of the TaHsfA2–10/athsfa2 complimentary lines is better than WT, suggesting TaHsfA2–10 perhaps has stronger thermotolerance regulation ability than AtHsfA2. The survival rate and chlorophyll contents measurement results provide powerful evidences simultaneously. A previous study demonstrated that thermal tolerance, salinity tolerance, and drought tolerance of TaHsfA2d-expressing Arabidopsis seedlings were all improved and that seedlings growing at moderately high temperatures could accumulate relatively high amounts of biomass and yield when compared to WT counterparts [27]. Up to now, there is no any report about TaHsfA2–10. More diverse gene functions of TaHsfA2s need to be deeply investigated in future research.

As molecular chaperones, Hsps play central roles in protecting against stress damage and in assisting with the folding, intracellular distribution, and degradation of proteins [45–47]. Hsfs can specifically bind to HSEs in the promoter region of Hsp genes as key regulators of Hsp genes [4]. Functional HSEs bound by TaHsfA2b were previously identified in promoter regions of TaHsp17, TaHsp26.6, TaHsp70d, and TaHsp90.1-A1, implying that TaHsp17 and TaHsp90.1-A1 are likely direct targets of TaHsfA2b [29]. In this study, qRT-PCR of AtHsp90.1, AtHsp70T, AtHsp101, AtERDJ3A, and AtHsa32 showed that these Hsp genes were upregulated to different degrees within 4 h of HS, both in WT and transgenic lines (Fig. 8). AtHsp101 and AtHsa32 appear involved in long-term acquired thermotolerance in Arabidopsis [20, 48, 49], and our results suggest that they also participate in basal thermotolerance. In fact, TaHsfA2–10 can induce Hsp expression in transgenic Arabidopsis lines under normal growth conditions, although the resulting expression levels are relatively low (Fig. 8a). In transgenic Arabidopsis lines, AtHsfA2 activated the expression of Hsp genes like AtHsp101, AtHsa32, and AtHsp-Cl, but not AtHsp90, in the absence of HsfA1 member expression under non-stressed conditions [22]. TaHsfA2e and TaHsfA2f dramatically upregulate AtHsp70T expression with the improving of basal or acquired thermotolerance [32, 50], and ZmHsfB5 can activate AtHsp21 and AtHsp90 expression during HS [24], revealing different Hsfs involves in heat response by activating special Hsps expression. Yeast one-hybrid analysis further showed that these detected Hsp genes were the direct target genes of TaHsfA2–10 (Fig. 9). These results confirm the regulatory role of TaHsfA2–10 on Hsp gene expression during HS and suggest that different Hsf members of the same subclass only activate expression of certain Hsp genes in different thermotolerance regulation.

Conclusions

Our results expanded the expression characterization of wheat Hsf by acquiring new insights on the underlying mechanisms governing temporal and spatial expression of wheat Hsf family members. TaHsfA2–10 was one of a few markedly responsive genes to HS. TaHsfA2–10 showed transactivation activity in yeast and activated expression of a suite of thermotolerance-related Hsp genes in transgenic Arabidopsis thaliana plants. TaHsfA2–10 improved the basal thermotolerance and acquired thermotolerance of transgenic Arabidopsis seedlings and rescued the thermotolerance phenotype defect of the mutant athsfa2 during HS. These findings enrich understanding of the diversity and specificity of Hsf expression in wheat. The results may also spur further investigation of the biological functions and molecular mechanisms of Hsf family members and the identification of target genes for the genetic improvement of wheat thermotolerance.

Materials and methods

Plant materials, growth conditions, and stress treatments

The T. aestivum cultivar Cang 6005, used in this study, was provided by the Cangzhou Academy of Agriculture and Forestry Sciences, Hebei province (E116.83, N38.33). This wheat variety is a winter wheat with a total growth period of about 244 days. It has a reputation for heat and salt-tolerance and is mainly planted in the southeast region of Hebei province. Selected seeds were surface sterilized in 0.1% HgCl2 for 10 min, rinsed in distilled water repeatedly, and then germinated in a tray. When buds were about 1 cm in size, they were divided into two groups. One group about 30 buds were transplanted into one pot with mesh containing Hoagland nutrient solution, and the other group buds were vernalized at 4 °C for 40 d, then transferred into potted soil (soil:vermiculite, 3:1) in big pots with 8 plants per pot. The plants were cultivated in a greenhouse at 22 ± 2 °C/18 ± 2 °C (day/night) with a 16 h/8 h light/dark cycle and 50% humidity under approximately 150 μmol photons m−2 s−1 light intensity. For stress treatments, seedlings with two leaves were treated with HS, H2O2, SA, or ABA for different time following methods described in Zhao’s paper [33]. For HS treatment, 40 seedlings were put into a new pot containing Hoagland nutrient solution preheated at 37 °C in another chamber, then treated for 30, 60, 90, 120, 240 min. For H2O2 treatment, 40 seedlings were put into a new pot containing Hoagland nutrient solution with the final concentration of 10 mM
H₂O₂ for 30, 60, 90, 120, 240 min. For SA treatment, 40 seedlings were put into a new pot containing Hoagland nutrient solution with the final concentration of 10 mM SA for 30, 60, 90, 120, 240 min. For ABA treatment, 40 seedlings were put into a new pot containing Hoagland nutrient solution with the final concentration of 10 mM ABA for 2, 4, 6, 8, 12, 24 h. After stress treatments, the second expanded leaf was obtained from all experiments per treatment. Young root, young shoot and young leaf were sampled at wheat growth stage Feekes 6.0. Root, shoot, leaf, stamen, pistil, sepal and function leaf (flag leaf) were sampled at wheat growth stage Feekes 10.5.2. Immature embryos and mature embryos were obtained at wheat growth stage Feekes 11.1 and Feekes 11.4 respectively. All qRT-PCR results came from three biological experiments and each experiment included three technical replicates. During anthesis (Feekes 10.5.2), 10 daa and 20 daa, pots with 8 plants per pot were transferred to a new growth chamber at 37 °C. Flag leaves and roots of 50 plants were sampled after heat treatment for 60 min (leaves) and 90 min (root) and samples frozen immediately in liquid nitrogen for RNA-Seq analysis of Hsf family expression.

The T-DNA insertion mutant line SALK_008978 was provided by Dr. Yee-Yung Chiang (Agricultural Biotechnology Research Center, Academia Sinica, Taipei), which was named athsfa2 derived from the Arabidopsis Biological Resource Center (Ohio State University, USA). Seeds of WT (ecotype Columbia), athsfa2 and transgenic lines were surface sterilized and sown on MS medium which contained 1% (w/v) sucrose and 0.8% gelrite, then kept at 4 °C for 3 days. Plants were grown to the greenhouse at 22 °C/18 °C (day/night) with a 16 h/8 h light/dark cycle and 50% humidity under approximately 100 μmol photons m⁻² s⁻¹ light intensity.

**RNA extraction**

Total RNA of different tissues from wheat and Arabidopsis thaliana was extracted using the RNaose Reagent Systems kit (Shanghai Huashun Biotechnological Co., Ltd.) according to the manufacturer’s protocol, and genomic DNA contamination was removed by RNase-free DNase I. A NanoDrop 2000 (Thermo Fisher Scientific, Rockford, USA) was used to detect the RNA concentration and quality.

**RNA-Seq analysis of wheat family Hsfs**

Flag leaves and roots of anthesis (Feekes 10.5.2) and post-anthesis wheat were sampled for RNA-Seq analysis after stress treatment. RNA-Seq analysis was performed following methods described in [14]. Total RNA of each sample was extracted from 50 plants and genomic DNA was removed by RNase-free DNase I. An Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) was used to detect RNA integrity. For RNA sample preparation, about 2 μg RNA of each sample was used as input material. The sequencing libraries were prepared for Illumina by VAHTSTM mRNA-seq V2 Library Prep Kit. The paired-end sequencing of the library was carried out by the HiSeq Xten sequencers (Illumina, San Diego, CA, USA). The sequenced data quality was evaluated by FastQC (version 0.11.2). The clean reads were selected by Trimmmomatic (version 0.36). The clean reads to the wheat reference genome was mapped by HISAT2 (version 2.0) using default parameters. The gene expression abundance of the transcripts was calculated by String Tie (version 1.3.3b). DEGs (differentially expressed genes) were determined by DESeq2 (version 1.12.4). Each sample was detected by RNA-Seq analysis once. A heatmap was drawn to illustrate the relative expression profiles of wheat TaHsfs by TBtools version0.66831 [51].

**Cloning of TaHsfA2–10 cDNA and sequence analysis**

A total of 1 μg purified RNA was used to synthesize first-strand cDNA using the SuperScript IV First-Strand Synthesis System (Invitrogen). The primers used were: forward primer: 5′-CGGTTGGTGTCTTGGGA-3′; reverse primer: 5′-CCCTCATTTTCTCGTCA-3′. In addition, the high-fidelity enzyme Pyrobest (TaKaRa) was used for PCR amplification. The PCR system and the reaction procedures were performed according to methods described in [33]. The reaction mixture contained 1× reaction buffer, 2.5 mM dNTP mixture, 1 μL first-strand cDNA, 20 μM forward primer, 20 μM reverse primer and 2 U DNA polymerase in a total volume of 50 μL. The reaction procedure were: 1 min at 94 °C, 32 cycles of 10 s at 98 °C, 30 s at 56 °C, 1 min at 72 °C, and final extension 5 min at 72 °C.

**Expression analysis by quantitative real-time PCR**

For the expression analysis of TaHsfA2–10 in wheat, the specific primers for amplifying TaHsfA2–10 were designed based on the sequence of 5′-UTR (Forward primer: 5′-CACCTTCGGAATGCCCTG-3′, Reverse primer: 5′-GAAAATGTCGCCCTCCTGTA-3′). The internal reference gene was TaRP15 (F: 5′-GCACACGTGCTTTCGAGATAAG-3′; R: 5′-GCCCTCAAGCTCACAAGTAAACT-3′) [29]. The expression level in young roots was set to 1 for the tissue-specific expression analysis and the expression level at 0 h was set as 1 for the stress treatments of wheat. For the expression of AtHsps in Arabidopsis thaliana, the TaHsfA2–10 transgenic line 11_26 (T3 generation homozygote) was used. Rosette leaves of the 5-day-old Arabidopsis seedlings were sampled at 0 h, 1 h, 2 h, 4 h, and 8 h after heat treatment, as described in the thermotolerance assay section. Five Arabidopsis Hsp genes were selected for expression analysis. The internal reference gene was AtActin8 and the
expression level of WT at 0 h was set as 1. Primers used are listed in Additional file 4.

PCR reactions were 20 μL in total: 10 μL SYBR Premix Ex TaqII, 0.8 μL 10 μM forward primer, 0.8 μL 10 μM reverse primer, 1 μL 1st strand cDNA, and 7.4 μL ddH2O. PCR reactions were performed using a 7500 Real-time PCR System (Applied Biosystems, USA) and reaction procedures carried out according to methods described in [33]. PCR reactions were pre-denatured at 95 °C for 30 s, then performed 40 cycles of 5 s at 95 °C and 34 s at 60 °C. The data were analyzed using the 2–ΔΔCt method after the reaction. Each group of experiments included three biological replicates and each biological sample included three technical replicates. The data are represented by mean values ± standard error of three biological replicates for each experiment.

**Determination of TaHsfA2–10 subcellular localization using transient expression in tobacco epidermal cells**

For N-terminal fusions of TaHsfA2–10 with GFP, specific primers (Forward primer was 5′-GACGAGCTGTACAAAGGAGCTCATGGGACCCTTTTCAC-3′ and reverse primer was 5′-CGATCGGGGAATTCGAGCTTCCATGGTAGCTGCGG-3′). Underlined letters were restriction enzyme sites SacI respectively and bold letters belonged to coding sequence of TaHsfA2–10.) were designed to amplify the coding region of TaHsfA2–10 by PCR. The product of PCR was ligated into the vector pCAMBIA1300-GFP digestion with the restriction enzymes SacI (The plasmid map was Additional file 6B). For C-terminal fusions of TaHsfA2–10 with GFP, specific primers (Forward primer was 5′-GAGAAACGGGGGACTCTAGAATGGACCCCTTTCAC-3′ and reverse primer was 5′-CGATCGGGGAATTCGAGCTTCCATGGTAGCTGCGG-3′). Underlined letters were restriction enzyme sites SacI respectively and bold letters belonged to coding sequence of TaHsfA2–10.) were designed to amplify the coding region of TaHsfA2–10 by PCR. The product of PCR was ligated into the vector pCAMBIA1300-GFP digestion with the restriction enzymes SacI (The plasmid map was Additional file 6B).

**Transcription activation activity and one-hybrid assays in yeast**

Transcription activation activity assays were performed in yeast according to the manufacturer’s protocol (TaKaRa, Dalian, China). The coding regions of TaHsfA2–10 were cloned by PCR using primers (Forward primer was 5′-GAGGAGGACCTGCATATGAGACCCCTTTTCAC-3′ and reverse primer was 5′-GTTATGCGGCCGCTGCAGTCACATGGTAGCTGCGG-3′). Underlined letters were restriction enzyme sites NdeI and PstI respectively, bold letters belonged to coding sequence of TaHsfA2–10.) was constructed into the yeast expression vector pGBK7 digestion with NdeI and PstI (The plasmid map was Additional file 6D). The constructs driven by T7 promoter, the pGBK7–53 as positive control or the empty vector pGBK7 as negative control with pGADT7 respectively were transformed into AH109, the yeast cell. The yeast cells in exponential growth were diluted to OD600 of 0.1 and grown on the deficiency medium plates of SD/Trp-/His/Ade/X-α-gal. Then the plates were placed at 30 °C until the yeast cells grew well. Finally, the yeast cells were photographed after 3–5 days.

Yeast one-hybrid assays were performed to detect the binding activity between TaHsfA2–10 and promoters of AtHsps according to the methods described by Li et al. [24]. Briefly, the coding region of TaHsfA2–10 was obtained by PCR using primers (Forward primer was 5′-GCCATGGGAGCAGTCATCAGATGGACCCCTTTCAC-3′ and reverse primer was 5′-CGATCGGGGAATTCGAGCTTCCATGGTAGCTGCGG-3′). Underlined letters were restriction enzyme sites EcoRI and BamHI respectively, bold letters belonged to coding sequence of TaHsfA2–10.) was constructed into vector pGADT7 digestion with EcoRI and BamHI (The plasmid map was Additional file 6E). The promoter sequences of different AtHsps were cloned by PCR using primers (Additional file 5) and constructed into vector pHIS2.1 digestion with EcoRI and SaeI (The plasmid map was Additional file 6F). The pGADT7-TaHsfA2–10 driven by T7 promoter and different constructs of pHIS2.1-promoter driven by minimal HIS3 promoter were transformed into the yeast cell Y187. The SD/Trp-/Leu-/His-selective medium containing 3-AT (3-amino-1,2,4-triazole) were used in the assay. The yeast cells grew at 30 °C for 3–5 days before they were photographed.

**Generation of transgenic Arabidopsis thaliana lines**

WT and T-DNA insertion mutant athsfa2 (SALK_008978, the Arabidopsis Biological Resource Center, Ohio State University) plants of Arabidopsis thaliana.
(ecotype Columbia) were used for genetic transformation. Seeds were surface sterilized with 75% alcohol for 30 s then with 10% sodium hypochlorite for 10 min. Sterile seeds were sown on 0.5x Murashige and Skoog (MS) medium (containing 1% sucrose and 0.8% gelrite, San-EiGenFFl Inc., Osaka, Japan, 1x MS salts and vitamins, pH 5.8) in plastic Petri dishes. After incubation for 3 days at 4 °C in the dark to ensure synchronized germination, plants were grown in a growth chamber under normal conditions (22 °C/18 °C with 16 h light/8 h dark cycles and light intensity at 100 mmol photons m⁻² s⁻¹).

The coding region of TaHsfA2–10 was amplified by PCR using the primers (Forward primer: 5′-GAGAAC ACGGGGACTCTAGAAATGACCCCTTTCAAGG-3′, Reverse primer: 5′-CGATCGGGGAAATTGACG CTCTCAGTTAGCTGCGGG-3′). Underlined letters were restriction enzyme sites XbaI and SalI respectively, bold letters belonged to coding sequence of TaHsfA2–10). The products of PCR were purified and cloned into the binary vector pCAMBIA1300 after digesting the destination plasmid with XbaI and SalI and cloning into the binary vector pCAMBIA1300 after digesting the destination plasmid with XbaI and SalI (The plasmid map was Additional file 6A). The resulting constructs driven by 35S CaMV promoter were transformed into Agrobacterium tumefaciens strain GV3101. Constructs were then transformed into WT and the Arabidopsis thaliana mutant athsfa2 plants using the floral dip method under vacuum conditions as described by Clough et al. [52]. All transgenic plants were selected on MS plates containing 25 mg/mL hygromycin until T3 generation homozygous lines were screened.

RT–PCR analyses of transgenic lines
Samples of 100 ng of purified mRNA were used for synthesis of the first cDNA strand using Reverse-transcription RT Kit (Invitrogen, Carlsbad, CA, USA). All polymerase chain reactions were performed with Pyrobest DNA Polymerase (Takara Biotech Co. Ltd) in a total volume of 25 mL reaction mixture consisting of 10 x Pyrobest buffer, 2.5 mL; 2.5 mM dNTP, 2 mL; 1st strand cDNA, 2 mL; 20 mM forward primer, 0.25 mL; 20 mM reverse primer, 0.25 mL; Pyrobest DNA polymerase, 0.25 mL; ddH₂O, 17.75 mL (Forward primer, 5′-ACGCCTTT CCTGAACAAAG-3′, Reverse primer, 5′-ATCTGCTG CCTGCTTCTG-3′). The internal reference gene was Atactin8 (Forward primer: 5′-CTATGGTCTG TGACATAGG-3′; Reverse primer: 5′-AACCTCG TAGATAGGCA-3′). The reaction program was as follows: 98 °C for 10 s; 55 °C for 5 s; 72 °C for 2 min, 30 cycles. The products were ligated into the T-vector (pEasy-blunt simple cloning kit, TransGen Biotech, Beijing, China) for sequencing (Shanghai Biotech Co.).

Thermotolerance assays
For basal thermotolerance assays, WT, mutant athsfa2, and three independent T3 generation homozygous transgenic Arabidopsis lines were used. For basal thermotolerance, 5-day-old seedlings of WT and TaHsfA2–10 transgenic lines and on agar plates were subjected to heat shock for 50 min at 45 °C. For acquired thermotolerance assays, 5-day-old seedlings of WT and TaHsfA2–10 transgenic lines on agar plates were kept at 37 °C for 60 min, then recovered for 2 d at 22 °C and subjected to HS for 60 min at 46 °C. For rescued thermotolerance assays, 5-day-old WT, the mutant athsfa2, and TaHsfA2–10 complementary line seedlings on agar plates were subjected to HS for 70 min at 44 °C, and then allowed to continue growth for 8 days at 22 °C and photographs were taken. More than 50 plants of each line were used per plate and experiments repeated three times.

Measurements of chlorophyll content
Chlorophyll content was spectrophotometrically measured as previously described by Li et al. [53]. About 0.2 g fresh leaves of Arabidopsis thaliana were taken into a capped test tube containing 20 mL acetone and ethanol mixture (acetone: ethanol: ddH₂O, 4.5:4.5:1.0). The homogenate was filtered after the leaves were completely bleached. The content of Chlorophyll a and Chlorophyll b were calculated according to the value of A645 and A663 of the filtrate respectively.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-020-02555-5.

Additional file 1. Cis-elements in the promoter of TaHsf family.
Additional file 2. Raw data of Figs. 3, 4, 7 and Fig. 8.
Additional file 3. Original, unprocessed versions of the blots in Figs. 6 and 7.
Additional file 4. The primers for Arabidopsis Hsp genes related to thermotolerance in qRT-PCR.
Additional file 5. The primers of the promoters of AtHsps used in yeast one hybrid.
Additional file 6. The vector maps used in this paper.

Abbreviations
Hsf: Heat shock transcription factor; HS: Heat shock; SA: Salicylic acid; ABA: Abscisic acid; WT: Wild type; Hsp: Heat shock protein; HSE: HS responsive element; DBD: DNA-binding domain; OD: Oligomerization domain; NILS: Nuclear localization signal; NES: Nuclear export signal; AHA: Activator peptide motif

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Authors’ contributions
GL and XG designed the experiments and wrote the article. SY, HZ, and YZ1 carried out the majority of experiments. YZ2 performed vector construction and subcellular localization experiments. GW and YL revised the article. The authors read and approved the final manuscript.
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Availability of data and materials
The dataset supporting the conclusions of this article is available in the NCBI-SRA repository, [PRJNA604299 in https://www.ncbi.nlm.nih.gov/bioproject/PRJNA604299], the article and its additional files.

Ethics approval and consent to participate
No applicable.

Consent for publication
No applicable.

Competing interests
The authors declare that they have no competing interests.

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