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ORIGINAL ARTICLE

Characteristics and Regulating Role in Thermotolerance of the Heat Shock Transcription Factor *ZmHsf12* from *Zea mays* L.

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Abstract Plant heat shock transcription factors (Hsfs) are important regulators of heat shock signal transduction pathway. There are 30 members of the Hsf family in maize, only two of which, *ZmHsf06* and *ZmHsf12*, belong to subclass A1. Our previous work demonstrated that *ZmHsf06*-overexpressing Arabidopsis lines showed improved tolerance to heat and drought stresses. In this study, we isolated *ZmHsf12* from young leaves of maize (*Zea mays* L.) using homologous cloning methods. The CDS (coding sequence) of *ZmHsf12* is 1,494 bp and encodes a putative protein consisting of 497 amino acids which possesses domains such as DBD (DNA-binding domain), OD (oligomerization domain), NLS (nuclear localization signal), NES (nuclear export signal), and an AHA (activator) motif. The ZmHsf12-GFP fusion protein is localized to the cell nucleus. *ZmHsf12* was expressed in many maize organs, and its expression was up-regulated by heat shock. Furthermore, we characterized the function of *ZmHsf12* in yeast and Arabidopsis. Yeast cells overexpressing *ZmHsf12* showed enhanced heat tolerance. *ZmHsf12*-overexpressing Arabidopsis seedlings displayed significant increases in both basal and acquired thermotolerance. Compared to WT seedlings, the *ZmHsf12*-overexpressing lines displayed both increased chlorophyll contents and higher survival rates. Also, the expression of *AtHsps* was increased higher in the *ZmHsf12*-overexpressing Arabidopsis lines after heat stress. The results of our study strongly suggested that *ZmHsf12* may take part in plant response to heat stress.

Keywords: Heat stress, Maize, Thermotolerance, *Zea mays* L., *ZmHsf12*

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Introduction

Crop plants are often exposed to environmental stresses during agricultural production in warm-climate regions, and are frequently subjected to changes in climate, particularly rising temperatures (Sewelam et al. 2014; Driedonks et al. 2016), which can have a significantly adverse impacts on crop productivity and grain quality. Kang and Eltahir (2018) predicted that the North China Plain will be threatened by deadly heatwaves as a result of climate change and the vast expansion of irrigation after fifty years. In order to survive, plants have developed a series of physiological and biochemical mechanisms to respond to various stresses and adapt to environmental changes. It has become clear that plants can acquire thermotolerance by regulating a large of target genes, such as heat shock protein (Hsp) genes that encode heat shock proteins (Hsps) during acclimation to permissive high temperatures (Schöffl et al. 1998). In this process, heat shock transcription factors (Hsfs) act as key regulatory factors of the Hsps and control the mRNA levels of the target genes by binding to heat shock elements (HSEs) in the promoter regions of *Hsps* (Nover et al. 2001; Wahid et al. 2007). Some *Hsps* such as *Hsp40*, *Hsp70*, *Hsp90*, *Hsp101* and particularly small *Hsps*, were induced during the heat shock conditions (Queitsch et al. 2000; Hahn et al. 2011; Hu et al. 2012; Wu et al. 2013; Zhong et al. 2013; Huang et al. 2018). The production and accumulation of Hsps in cells are necessary to protect plants from the effects of heat stress (Kotak et al. 2007). Hsfs are the most important regulatory proteins during the heat shock (HS) response in plants (Nover et al. 2001; Kotak et al. 2007; Wang et al. 2018).

Hsfs were found in all eukaryotic organisms. *Drosophila melanogaster,* yeast and *Caenorhabditis elegans* have only one *Hsf* gene respectively, and vertebrates have four *Hsfs* in their genomes (Pirkkala et al. 2001). But in plants, Hsfs are

encoded by complex gene family that can contain many members, for example, there are 21 members in Arabidopsis (Guo et al. 2008), 25 members in rice (Scharf et al. 2012), 30 members in maize (Scharf et al. 2012), 56 members in wheat (Xue et al. 2014) and 26 members in tomato (Yang et al. 2016). Plant Hsfs belong to three classes (A, B and C) based on the peculiarities of oligomerization domain (OD) and the flexible linkers between DNA-binding domain (DBD) and OD. Each class is comprised of seveval subclasses. AHA motifs are found in the C-terminal regions of all class A. and many AHA motifs serve to positively regulate the HS response in plants (Busch et al. 2005; Nishizawa et al. 2006; Li et al. 2014; Xue et al. 2014). *HsfA1* from tomato was the first plant *Hsf* gene to be isolated and it was shown that constitutively expressed HsfA1 proteins are in an inactive monomer state and interact with Hsp90/Hsp70 in the absence of heat stress (Hahn et al. 2011). In model species such as Arabidopsis and tomato, *HsfA1s* are the early HS response genes that act as transcriptional activators for the late response *Hsfs* such as *HsfA2* (Charng et al. 2007; Liu et al. 2011). The tomato HsfA1a is a master regulatory factor that triggers the HS response and any other Hsfs in tomato can replace it (Mishra et al. 2002). In both acquired thermotolerance (AT) and basal thermotolerance (BT), the expression levels of *Hsfs* and *Hsps* induced by HS were found to be up-regulated in transgenic tomatoes expressing *HsfA1a* and were strongly down-regulated in the co-suppression lines (Mishra et al. 2002; Scharf et al. 2012). No obvious morphological and developmental changes were observed at the control temperature (Mishra et al. 2002). Four Hsfs, HsfA1a, HsfA1b, HsfA1d and HsfA1e, were present in Arabidopsis. HsfA1a, HsfA1b and HsfA1d function as the main positive regulators in Arabidopsis response to HS (Yoshida et al. 2011; Scharf et al. 2012; Liu and Charng 2013). Single knockout mutants of *HsfA1a*, *HsfA1b*, *HsfA1d* or *HsfA1e* showed no effects on the expression of *Hsps* and there were no changes in influence on morphology or phenotype, but plants of all four *HsfA1* knockout lines showed a complete loss of thermotolerance and displayed diverse phenotypes and developmental retardation (Yoshida et al. 2011; Nishizawa-Yokoi et al. 2011; Liu et al. 2011; Scharf et al. 2012).

In maize, it was initially shown that the mRNA levels of three Hsfs were different during different stage of pollen development (Gagliardi et al. 1995), and one *Hsf* (*ZmHsfa*, GenBank No. S61458) was expressed constitutively while the others (*ZmHsfb*, GenBank No. NP_001307887 and *ZmHsfc*, GenBank No. S61459) were induced by heat stress. Based on the genomic sequences and phylogenetic analyses, 25 Hsfs were initially identified in maize (Lin et al. 2011) and 30 Hsfs were reported in 2012 (Scharf et al. 2012). However, little is known about the characteristics of gene expression and the biological functions of individual members of the Hsf family under abiotic stress conditions. Only four maize Hsf genes, *ZmHsf04*, *ZmHsf05*, *ZmHsf06* and *ZmHsf25*, have been isolated and characterized (Li et al. 2015; Zhao et al. 2017; Jiang et al. 2018; Li et al. 2019). The proteins encoded by these genes belong to HsfA2, HsfA1 and HsfB subclasses, respectively. There are two HsfA1 subclass members in maize, ZmHsf06 and ZmHsf12 (Lin et al. 2011). It was previously reported that maize *ZmHsf06* was expressed in many green organs, and was up-regulated by HS and other stresses (Li et al. 2014). Arabidopsis seedlings over-expressing *ZmHsf06* showed increased tolerance to heat and drought stresses (Li et al. 2015).

ZmHsf12 is another member of the maize HsfA1 subclass, and its expression patterns and functions in thermotolerance are unclear. In the present study, we cloned the CDS of *ZmHsf12* and quantitatively analyzed gene expression in different organs and under heat stress. The subcellular localization of ZmHsf12 was determined by fusing it with GFP. To understand the biological function of ZmHsf12, we introduced the gene into yeast and also generated transgenic Arabidopsis plants. We then performed thermotolerance assays on the recombinant yeast strains and the transgenic plants, and the expression of heat protection genes induced by *ZmHsf12* was analyzed further.

Results

Cloning of the *ZmHsf12* and Analysis of the Putative Protein Sequence

Sequence analysis showed that the CDS of *ZmHsf12* (GenBank No. MK733910) was 1,494 bp in length and is predicted to encode a protein of 497 amino acid residues. The protein possesses a DBD which is a typical structure of Hsfs family, an adjacent OD, a NLS, a NES and an AHA motif (Fig. 1). The amino acids alignment results of the HsfA1 members in plant showed that ZmHsf12 protein shared 79%, 82%, 71%, 68% and 58% identities with ZmHsf06 (GRMZM2G115456_P01, the other HsfA1 member from maize), SbHsfA1 (XP_002466067, HsfA1 from *Sorghum bicolor*), SiHsfA1 (XP_004980999, HsfA1 from *Setaria italica*), OsHsfA1 (XP_015630421, HsfA1 from *Oryza sativa*) and HvHsfA1 (BAJ86136, HsfA1 from *Hordeum vulgare*), respectively (Fig. 2). The alignment results showed that DBD, OD, NLS, AHA and NES domains of ZmHsf12 were highly conserved with those of other plant HsfA1. The amino acid sequence analysis in HEATSTER (http://applbio.biologie.uni-frankfurt.de/hsf/heatster/) suggested that ZmHsf12 contained eight A1 signature sequences which are marked by bold solid black lines (Fig. 2). These results clearly showed that ZmHsf12 is a member of HsfA1 subclass.

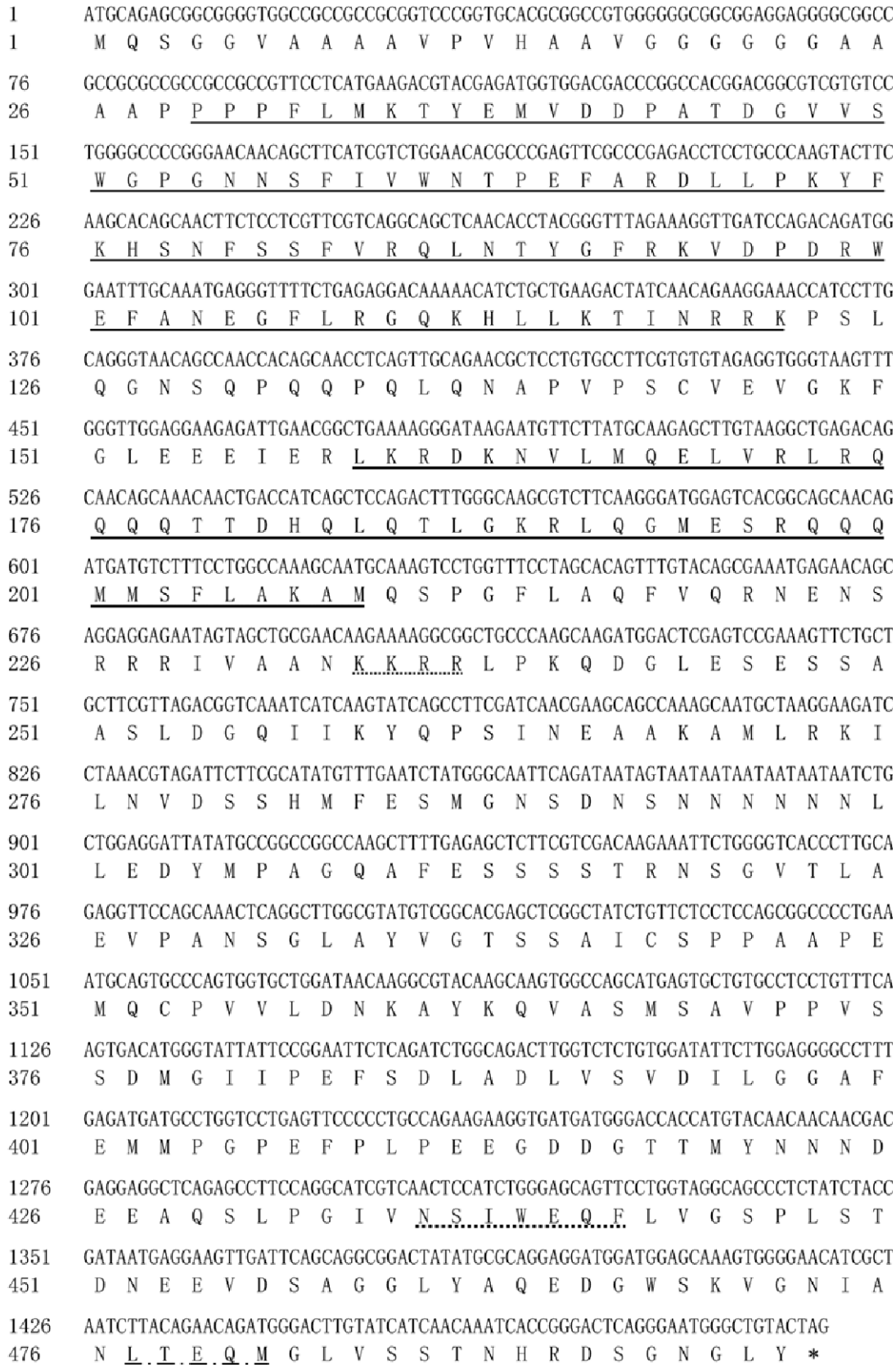

Fig. 1. Nucleotide and the predicted amino acid sequences of *ZmHsf12*. The amino acids underlined in black indicate a typical DBD and the adjacent OD domain is underlined in bold. NLS (dotted underline), AHA-motif (bold dotted underline) and NES (dashed and dotted underline) are indicated.

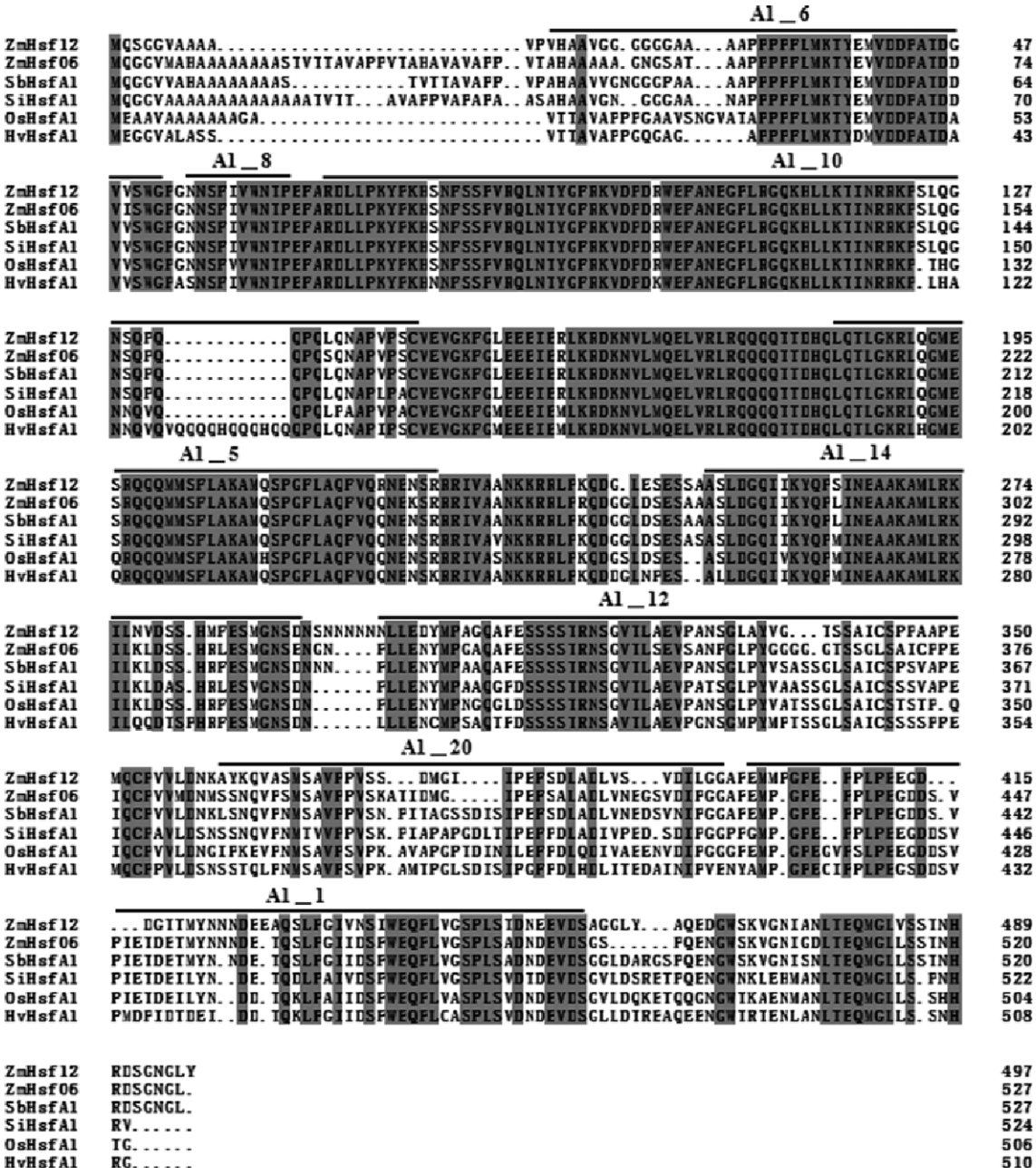

Fig. 2. Sequence alignment of ZmHsfA1 with HsfA1 subclass members from other plants. The accession number of in maizeDGB (https://www.maizegdb.org) for ZmHsf06 is GRMZM2G115456_P01. The accession numbers for the other proteins in NCBI (https://www.ncbi.nlm.nih.gov) are as follows: SbHsfA1, XP_002466067; SiHsfA1, XP_004980999; OsHsfA1, XP_015630421; HvHsfA1, BAJ86136. HsfA1 signature sequences are indicated by bold solid black lines above the sequence alignment, and highly conserved amino acids are highlighted in gray.

Expression Profile of *ZmHsf12*

Using qRT-PCR assays, we quantified the expression of *ZmHsf12* in maize organs at normal temperature and in response to HS. The results demonstrated that *ZmHsf12* was expressed in many organs such as roots, shoots and leaves of maize seedlings and functional leaf, ear, pollen, immature embryos of anthesis under normal conditions. The expression of *ZmHsf12* was found to be highest in young leaves and lowest in ears and immature embryos (Fig. 3A). Expression analysis was performed in order to examine the role of *ZmHsf12* in the maize response to HS. The relative expression of *ZmHsf12* was up-regulated by heat stress at 42°C in both leaves and roots, with the highest levels of 1.5-fold in leaves and 6-fold in roots occurring at 30 min after the start of HS treatment (Fig. 3B). With prolonged heat treatment, the up-regulation

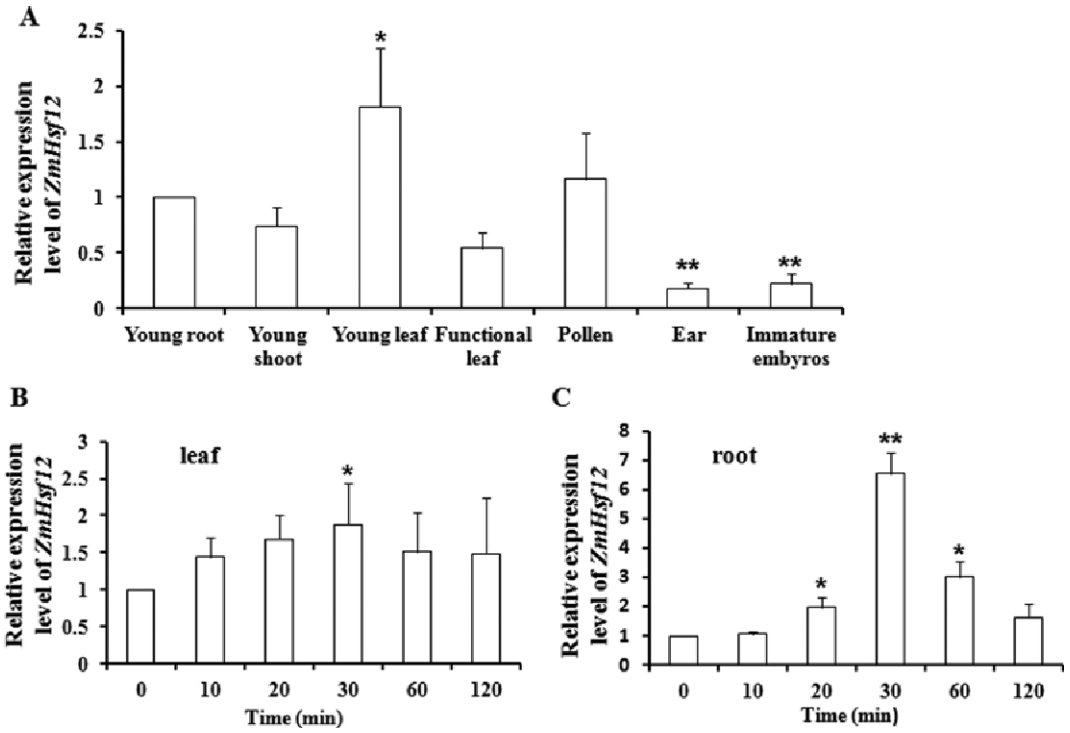

Fig. 3. Expression patterns of *ZmHsf12* in maize. (A) Tissue-specific expression patterns of *ZmHsf12*. The expression level in young roots was set as 1. Analysis of *ZmHsf12* expression changes in response to heat shock at 42°C in leaves (B) and roots (C). The expression of the untreated samples was set as 1. Reference gene *β-actin* from maize was used as an internal control to normalize the signals of different samples. Error bars refer to the standard deviations of three biological replicates. The asterisks * and ** indicate significant differences compared with controls at $P<0.05$ and $P<0.01$ level (*t* test), respectively.

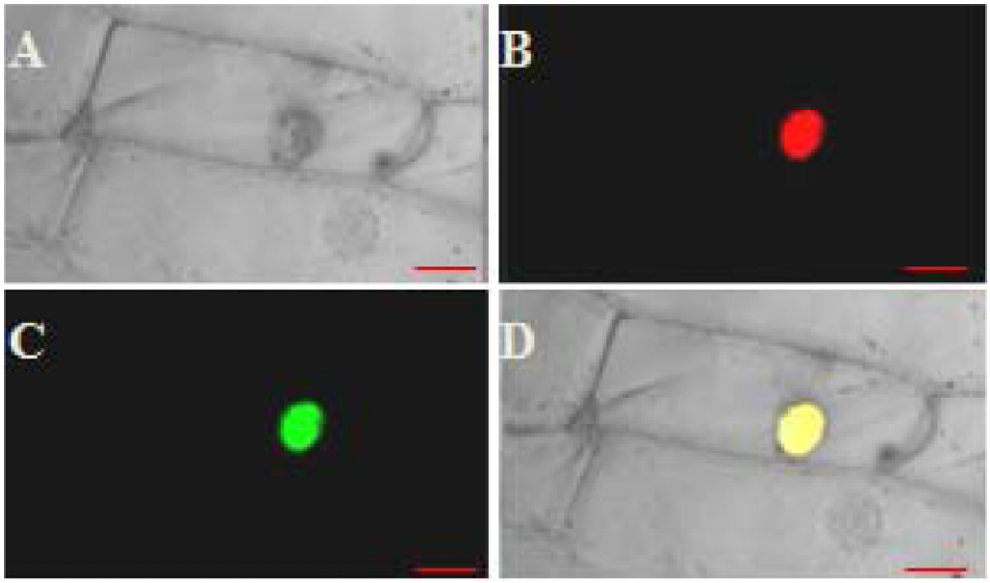

Fig. 4. Subcellular localization of the ZmHsf12-GFP fusion protein in onion epidermal cells. (A) Cells expressing ZmHsf12-GFP under white light; (B) Cells expressing ZmHsf12-GFP under DAPI red fluorescence; (C) Cells expressing ZmHsf12-hGFP under green channel fluorescence; (D) Cells merged of A, B and C. The scale bar represents 50 μm.

of transcription declined, but it was still a little higher than in the controls at 120 min after treatment. These results suggested that *ZmHsf12* is expressed constitutively in the tested organs and is up-regulated during HS treatment.

The ZmHsf12-GFP Fusion Protein is Localized to the Plant Cell Nucleus

To determine the subcellular localization of ZmHsf12, we

constructed the pCAMBIA1300-35S::ZmHsf12-GFP fusion expression vector. The constructs were bombarded into the cells of onion epidermis. The onion epidermis were incubated at 22°C overnight. After stained with staining solution DAPI of nuclei-special dye, the epidermic cells was checked by a fluorescence microscope. It showed that the fluorescence signal derived from the ZmHsf12-GFP protein was only detected in the cell nuclei, and matched perfectly with the DAPI fluorescence. These results suggested that ZmHsf12 localized to the nucleus (Fig. 4) and that it functions there.

Overexpression of *ZmHsf12* Enhanced Thermotolerance in Yeast

Tomato Hsf proteins can bind to yeast HSE and functionally replace ScHsf1 (Boscheinen et al. 1997). The biological function of *ZmHsf12* was determined in transgenic yeast. Under normal conditions, the growth rate of yeast cells harboring pYES2-*ZmHsf12* was similar to the pYES2 control (Fig. 5A). When exposed to HS, the growth rate of pYES2-*ZmHsf12* expressing cells was higher than that of pYES2 transformed cells, especially after 12 h of HS treatment (Fig. 5B). Yeast cells were further observed on solid media containing Gal. There were no obvious differences in colony formation between cells harboring pYES2 and pYES2-*ZmHsf12* grown on the same Gal plate without heat stress, but the yeast cells overexpressing *ZmHsf12* showed much higher tolerance to HS than that did the controls which failed to grow at higher cell dilutions, because the thermotolerance was decreased after exposed to HS for 15 min (Fig. 5C). The results showed that expression of maize *ZmHsf12* improved heat tolerance in yeast.

Overexpression of *ZmHsf12* Improved Thermotolerance in Transgenic Arabidopsis

To verify the thermotolerance resulting from the expression of *ZmHsf12* in Arabidopsis, we obtained *ZmHsf12*-overexpressing lines using a pCAMBIA1300::35S::ZmHsf12 construct. Three T3 generation homozygous progeny (lines 4_11, 6_14 and 10_10) displayed different *ZmHsf12* expression levels as determined by semi RT-PCR (Fig. 6E) were used in the functional study. Seedlings of the three *ZmHsf12* expressing lines and WT were used to assay thermotolerance in Arabidopsis. For basal and acquired thermotolerance, 5-day-old seedlings of all the genotypes were subjected to two

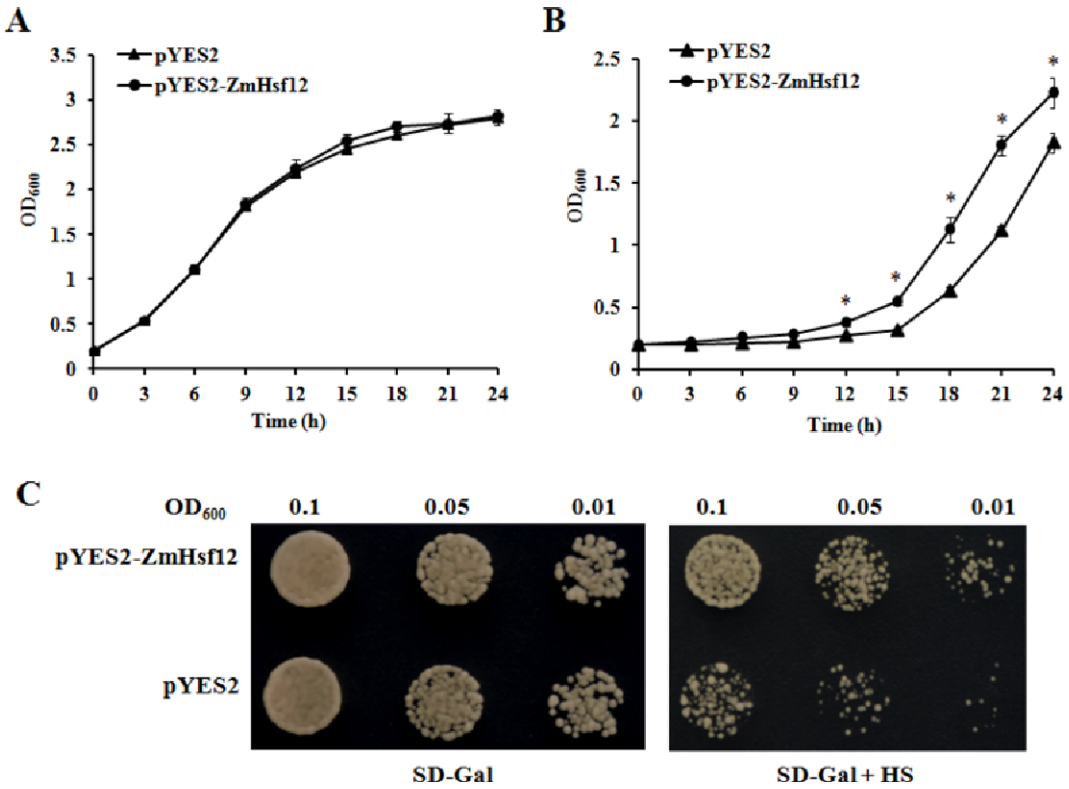

Fig. 5. The growth patterns of yeast cells transformed with pYES-ZmHsf12 and empty vector pYES2 with or without HS at 50°C for 15 min. The growth rates of yeast cells expressing the pYES-ZmHsf12 (A) and empty vector (B) in liquid SD-Gal inducing medium for different times after without or with HS treatment at 50°C for 15 min. (C) Growth of yeast cells transformed with different vectors on solid SD-Gal inducing medium with or without HS at 50°C for 15 min. After incubation for 3 days at 30°C, plates were photographed. Error bars refer to the standard deviations of three biological replicates. The asterisk * indicates significant differences compared with the control at $P < 0.05$ level (*t* test).

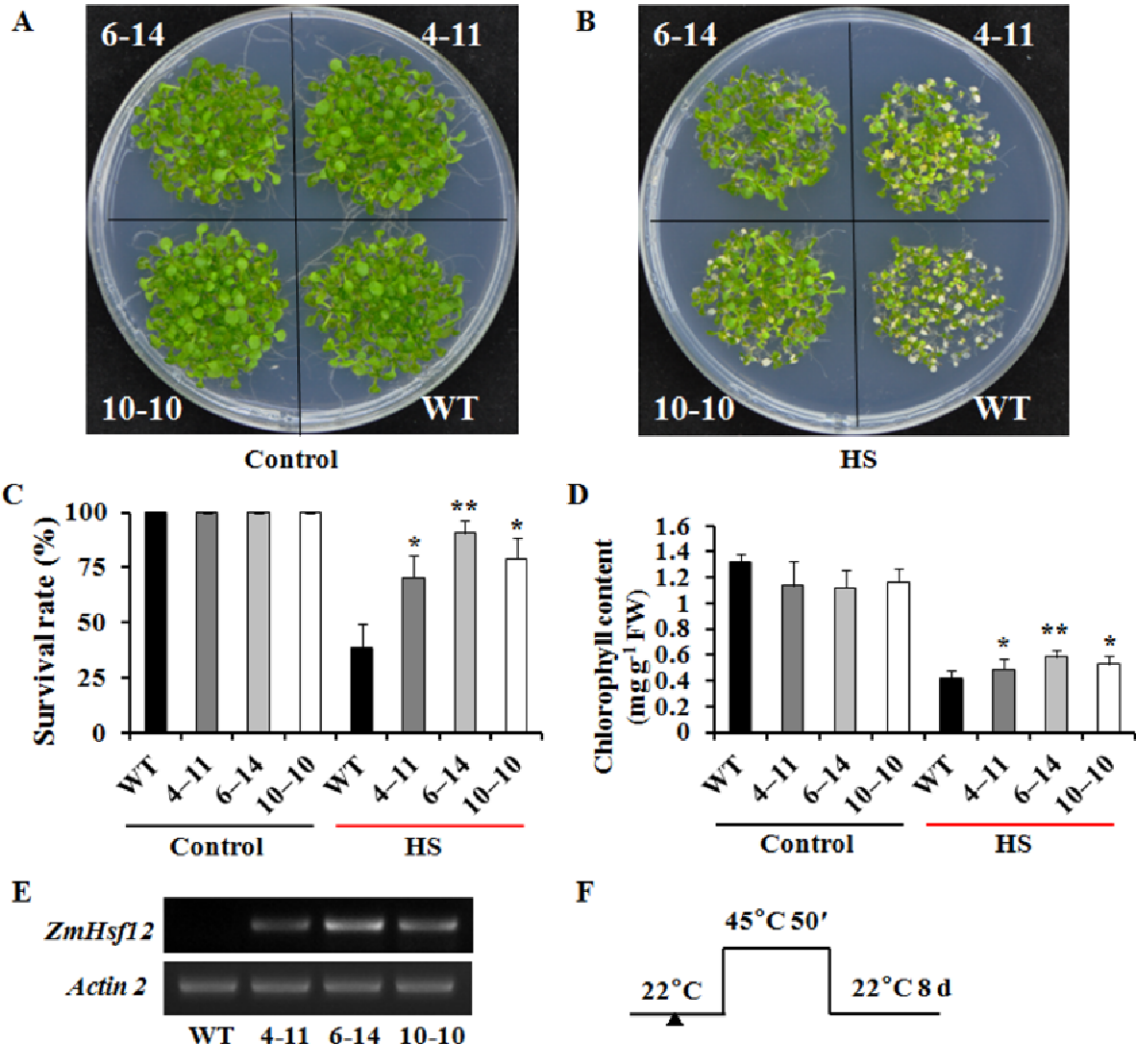

Fig. 6. Overexpressing *ZmHsf12* improved the basal thermotolerance in Arabidopsis seedlings. (A) 13-day-old seedlings of WT and three overexpressing lines (4-11, 6-14 and 10-10) grown on the same plate without heat stress were used as a control. (B) 5-day-old seedlings of all genotypes under HS at 45°C for 50 min and recovered at normal conditions for 8 days. (C) Survival rate of WT and three *ZmHsf12*-overexpressing lines with or without heat stress. More than 30 seedlings of each line were used in the experiment. Data are means (± SD) of three independent experiments. (D) Chlorophyll contents of seedlings with 0.1 g fresh tissue under normal and HS conditions in each experiment. Data are means (± SD) of three independent experiments. (E) Semi RT-PCR analysis of the *ZmHsf12* mRNA levels in seedlings of all genotypes. The *Actin2* expression levels were used as a gene expression control. (F) Schematic representation of the HS regimes. The asterisks * and ** indicate significant differences compared with WT at $P < 0.05$ and $P < 0.01$ level (*t* test), respectively.

different heat stress regimes (Fig. 6F; Fig. 7E), then allowed to recover and grow for another 8 days at 22°C. Under non-heat stress conditions, there were no obvious phenotypic and developmental changes between WT and the transgenic Arabidopsis seedlings (Fig. 6A; Fig. 7A). When exposed to 45°C for 50 min, the basal thermotolerance of the *ZmHsf12*-overexpressing lines was increased significantly compared to the WT seedlings after recovery at normal conditions for 8 days (Fig. 6B). When pretreated at 37°C for 1 h, allowed to recover under normal conditions for 2 days, and then exposed to 46°C for 60 min, seedlings of the *ZmHsf12*-overexpressing lines displayed increased acquired thermotolerance compared to WT seedlings after recovery at 22°C for 8 days (Fig. 7B). After exposure to heat stress, the three lines showed higher survival rates (Fig. 6C; Fig. 7C) and chlorophyll contents (Fig. 6D; Fig. 7D) than did WT seedlings. These results indicated that *ZmHsf12* may participate in both basal and acquired thermotolerance in Arabidopsis.

Expression of *AtHsps* was Affected by *ZmHsf12* Overexpression in Arabidopsis

For qRT-PCR analysis the expression profiles of *AtHsps* under HS conditions, one high-level expression line 6-14 and

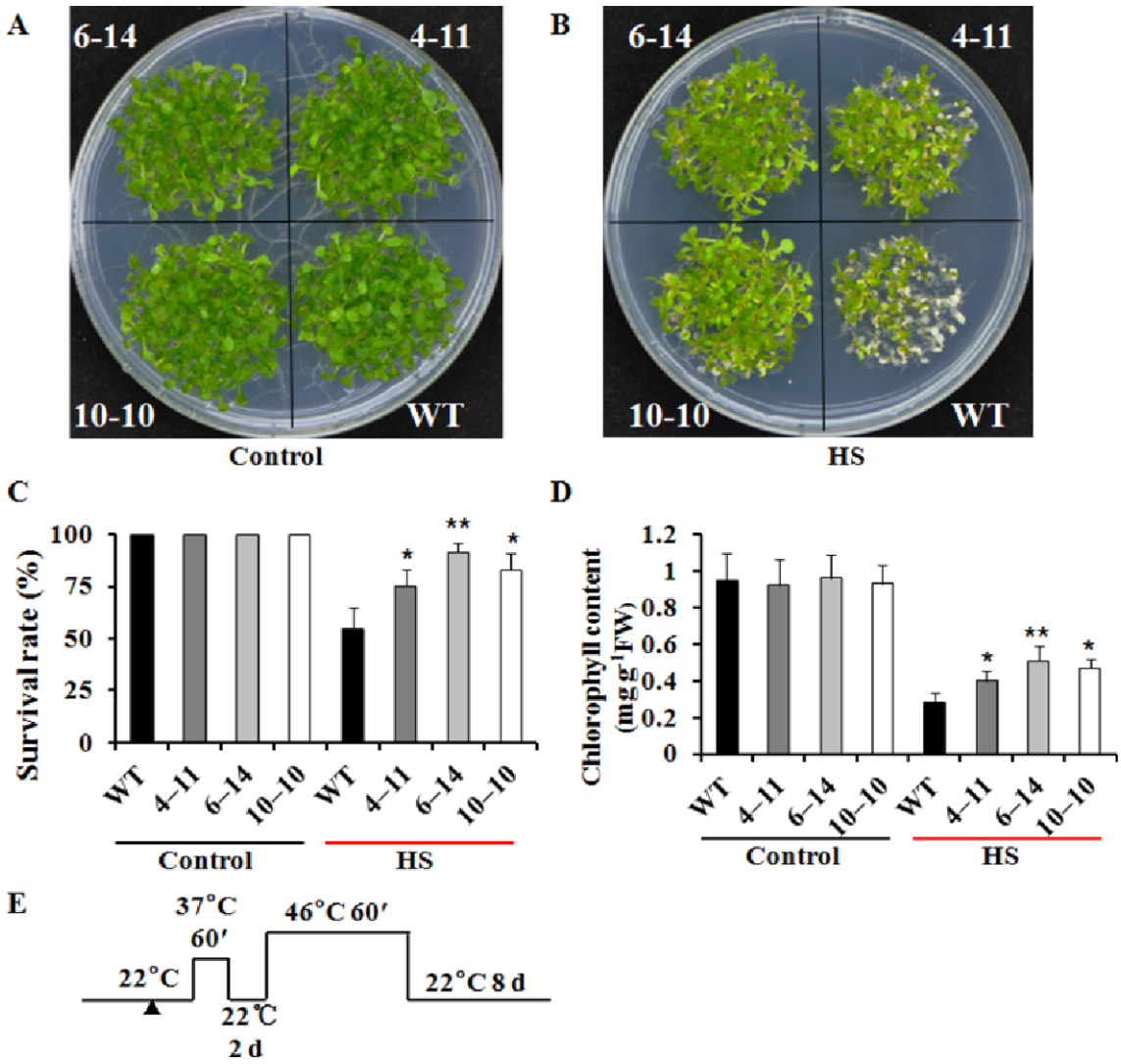

Fig. 7. The acquired thermotolerances were improved in Arabidopsis seedlings overexpressing *ZmHsf12*. (A) 15-day-old seedlings of WT and the three overexpressing lines (4-11, 6-14 and 10-10) grown on the same plate without heat stress were used as a control. (B) Seedlings of all genotypes grown at normal conditions for 5 days pretreated at 37°C for 1 h were recovered at normal conditions for 2 days before exposed to 46°C for 60 min, and then recovered for another 8 days. (C) Survival rate of WT and three overexpressing lines with or without heat stress. Each experiment contained over 30 seedlings of each line. Data represent the means (± SD) of three biological experiments. (D) Chlorophyll contents of seedlings with 0.1 g fresh tissue under normal and HS conditions in each experiment. Data represent the means (± SD) of three independent biological experiments. (F) Schematic representation of the HS regimes. * and ** indicate significant differences compared with WT at $P < 0.05$ and $P < 0.01$ level (*t* test), respectively.

WT were selected to test whether the expression of *ZmHsf12* affects the expression of *AtHsps* in Arabidopsis. The seedlings were sampled after 50 min of HS treatment at 45°C. The results showed that the expression of *Hsp* genes (*AtHsp18.2*, *AtHsp21*, *AtHsa32*, *AtHsp70b*, *AtHsp90.1*, and *AtHsp101*) in seedlings of the *ZmHsf12*-overexpressing line 6-14 were 1.4- to 2-fold higher than in WT without HS and 3- to 35-fold after heat treatment (Fig. 8). Expression of *AtHsp18.2*, *AtHsp21*, *AtHsa32*, *AtHsp70b*, *AtHsp90.1*, and *AtHsp101* genes in the *ZmHsf12*-overexpressing line 6-14 plants treated with HS was more than that in WT plants. The induction of these genes expression was more obvious in the *ZmHsf12*-overexpressing line 6-14 than that in WT. These results demonstrated that changes in the expression *AtHsps* may result in the themotolerance differences observed between transgenic and WT Arabidopsis seedlings.

Discussion

Based on the structural characteristics of the proteins and phylogenetic comparisons, plant Hsfs can be divided into three classes (A, B, and C). The members of subclass HsfA1 play key roles in the early response to heat shock based on

studies in model plants, and they act as transcriptional activators of down-stream *Hsps* expression to increase thermotolerance (Scharf et al. 1998; Mishra et al. 2002; Liu et al. 2013; Gong et al. 2014). Four members of the HsfA1 subclass are present in the genomes of the model plant Arabidopsis and tomato respectively. HsfA1 in tomato is a master regulator in plant response to heat shock. However, HsfA1s are functionally redundant for responding to heat shock in Arabidopsis (Lohmann et al. 2004; Liu et al., 2013). The genome of maize contained two members of subclass HsfA1, *ZmHsf06* and *ZmHsf12*. Alignment of the ZmHsf06 and ZmHsf12 protein sequences showed that the two proteins share 79% amino acid identities (Fig. 2), and they have the structure and domains typical of heat shock transcription factors. It has been speculated that the two proteins might have originated from the same ancestor. The ZmHsf12 protein contained a NLS and the subcellular localization assay showed that the ZmHsf12-GFP fusion protein is localized to the nucleus (Fig. 4). The alignment results showed that ZmHsf12 contains all of the functional domains including DBD, OD, NLS, AHA and NES, that are highly conserved in other plant HsfA1 proteins. Sharf et al. (2012) proposed the current Hsf classification scheme and further developed a web server (HEATSTER) for identification and classification of Hsfs in plants (Berz et al. 2019). Based on this method, we found that ZmHsf12 includes eight HsfA1 signature sequences (Fig. 2). Therefore, ZmHsf12 is a member of subclass HsfA1.

Except for the *HsfA1s* in wheat leaves (Xue et al. 2014), expression of the *HsfA1* gene family members in plants were induced by HS (Liu et al. 2013; Li et al. 2014; Gong et al. 2014). *ZmHsf12* was constitutively expressed in all detected organs of maize and was induced by HS in roots and leaves (Fig. 3). But the increase in expression of *ZmHsf12* in roots was higher than it was in leaves at 30 min after HS treatment. These results indicated that *ZmHsf12* may participate in plant response to heat stress.

In recent years, the quality and yield have decreased dramatically in many crops because of the rising temperature (Tito et al. 2018). At present, the functional mechanisms that control the plant response to HS are not entirely clear. During the process of triggering the heat shock response, we know that Hsfs play vital roles in plants (Schöffl et al. 1998; Nover et al. 2001; Mishra et al. 2002;Guo et al. 2016). The members of the tomato HsfA1 subclass are master regulators in the HS response. In Arabidopsis, *HsfA1a* and *HsfA1b* are constitutively expressed and act as the early response *Hsfs* under heat stress (Liu et al. 2011). HsfA1a and HsfA1b also act as transcriptional activators to induce the expression of late response genes (Liu et al. 2011). The other two members of the HsfA1 subclass in Arabidopsis, HsfA1d and HsfA1e, can serve as regulators involved in the regulation of HsfA2 transcription in the signal network of Hsf regulation (Nishizawa-Yokoi et al. 2011). Under non-heat stress conditions, HsfA1 proteins expressed constitutively are in inactive monomers that interact with repressors such as Hsp90/Hsp70 (Hahn et al. 2011). Research in the model plant species tomato and Arabidopsis showed that HsfA1s are vital regulators when plants are exposed to heat stress (Schöffl et al. 1998; Mishra et al. 2002; Nishizawa-Yokoi et al. 2011).

In maize, *ZmHsf06* was identified as a member of the HsfA1 subclass. *ZmHsf06*-overexpressing Arabidopsis showed enhanced thermotolerance after heat stress treatment (Li et al. 2014, 2015). We isolated *ZmHsf12*, the other member of the maize HsfA1 subclass in this study. When exposed to HS, the growth rate of yeast cells harboring pYES2-ZmHsf12 in liquid Gal medium (Fig. 5B) and yeast cells overexpressing *ZmHsf12* on solid Gal medium (Fig. 5C) showed much more tolerance to HS than did the controls. The Arabidopsis seedlings overexpressing *ZmHsf12* showed significantly higher basal thermotolerance (Fig. 6B) and acquired thermotolerance (Fig. 7B) compared to WT seedlings. The survival rates and chlorophyll contents of the *ZmHsf12*-overexpressing lines were higher than in WT seedlings for both basal thermotolerance (Fig. 6C, D) and acquired thermotolerance (Fig. 7C, D). Thus, the two HsfA1 subclass proteins, *ZmHsf12* and *ZmHsf06*, may perform the same function in the maize HS response. *ZmHsf06* overexpression enhanced drought-stress tolerance in transgenic Arabidopsis, but *ZmHsf12* did not provide the same tolerance (data not shown). Further study may reveal the functional diversification of the two proteins.

When subjected to heat stress, plants are stimulated to produce Hsps for the acquisition of thermotolerance (Verling 1991; Queitsch et al. 2000; Liu et al. 2006; Hu et al. 2012; Wu et al. 2013). Hsfs regulate the expression of target genes by their binding affinity for HSEs in the promoters of *Hsps* (Wahid et al. 2007). Our results suggested that the ectopic expression of *ZmHsf12* in Arabidopsis affected the expression of *AtHsps*. All of the tested genes, *AtHsp18.2*, *AtHsp21*, *AtHsa32*, *AtHsp70b*, *AtHsp90.1* and *AtHsp101*, showed higher expression levels in the *ZmHsf12*-expressing line 6-14 than in WT after HS at 45°C (Fig. 8). These results indicated that *ZmHsf12* play a role in plant thermotolerance by regulating the expression of *Hsps*.

In Arabidopsis, AtHsfA1a can be phosphorylated by the active AtCBK3 kinase, leading to the accumulation of Hsp proteins, thereby increasing plant thermotolerance under HS conditions (Liu et al. 2008). In maize, further research will be required to identify the protein that regulates *ZmHsf12* expression. It is important to study the upstream signals in heat shock signal transduction pathway in maize in the future.

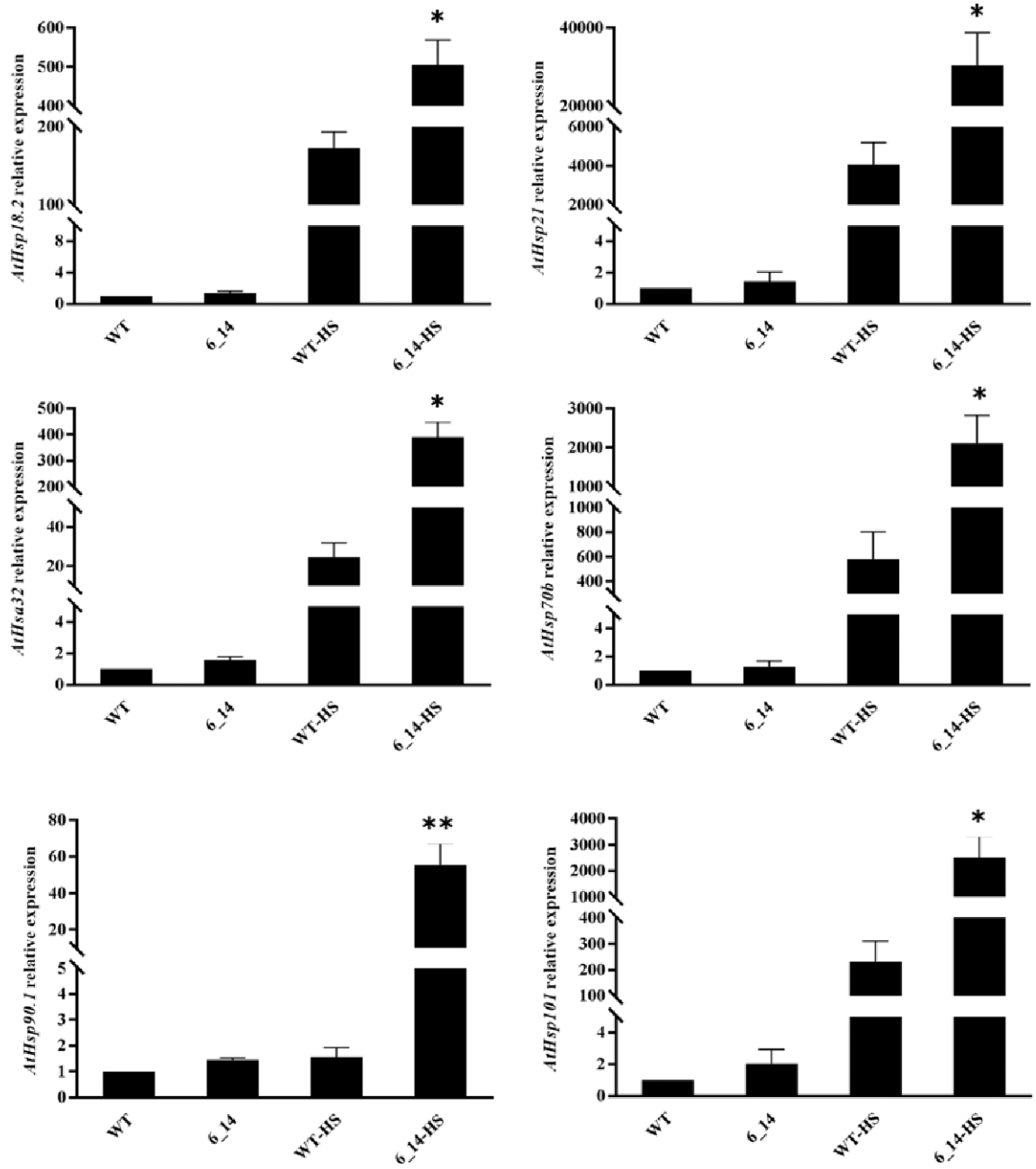

Fig. 8. Effect of *ZmHsf12* on *Hsps* expression during HS in Arabidopsis. The expression levels of *AtHsps* in seedlings of WT and *ZmHsf12*-overexpressing line 6-14 with and without HS at 45°C for 50 min. The expression of untreated seedlings of WT was set as 1. Reference gene *Atactin8* (At1g49240) was used as an internal control to normalize the loading of different samples. Error bars refer to the standard deviations of three biological replicates. * indicates significant differences compared with WT-HS at $P < 0.05$ level (*t* test). The accession number of the genes: *AtHsp18.2*, At5g59720; *AtHsp21*, At4g27670; *AtHsa32*, At4g21320; *AtHsp70b*, At1g16030; *AtHsp90.1*, At5g52640; *AtHsp101*, At1g74310.

Materials and Methods

Plant Growth Conditions

Mature seeds of maize (inbred line H21) were surface-sterilized with 0.1% $HgCl_2$, rinsed thoroughly with sterile water and allowed to germinate in an incubator at 28°C. The seedlings were then transplanted into individual pots and watered with Hoagland nutrient solution under 16/8 h day/night (100 μmol m^{-2} s) at 28/20°C and 60% RH. After the second leaf had fully expanded, the second leaves, shoots and roots were collected an immediately frozen in liquid nitrogen for use in the expression analysis experiments. For stress tests, uniformly-grown seedlings were selected and subjected to different treatments. The functional leaves, ears, pollen and immature embryos were harvested for expression analysis in different growth stages. For heat stress tests, uniform maize seedlings with two fully expanded leaves were transferred into Hoagland nutrient solution that had been

preheated in an incubator at 42°C and the shoots were exposed to 42°C air temperature for appropriate time. The second leaves were sampled after the HS treatments and stored in liquid nitrogen for use in the gene expression experiments. The controls were seedlings grown at 28°C under normal conditions.

Arabidopsis thaliana (ecotype Columbia) was used for genetic transformation with the maize *ZmHsf12* gene. Surface sterilization and seedling culture conditions were as described by Li et al. (2015). After 1 week, the seedlings were transplanted into potted soil and grown. The plants were watered once a week with Hoagland solution.

Preparation of cDNA and Cloning the CDS Sequence of *ZmHsf12*

Total RNA was isolated using TRIpure Reagent (Aidlab, Beijing, China). DNase I (TaKaRa) was to treat each RNA sample to remove contaimating genomic DNA. Purified mRNA was used for the first strand synthesis of cDNA with the PrimeScript RT Reagent Kit (Takara, Shiga, Japan). The RNA quantity was determined with a NanoDrop 2000 spectrophomoter (Thermo, Wilmington, DE, USA). The full-length coding sequence of *ZmHsf12* was amplified by RT-PCR using forward primer (5'-ATGCAGGGCGGCGGGGTGGC-3') and reverse primer (5'-CTAGTACAGCCCATTCCCTGA-3') with PrimeSTAR HS DNA polymerase (TaKaRa). The PCR reaction mixture and amplification program were as described by Li et al. (2015). The amplification products were sequenced after they were cloned into T-vector (Transgen, Beijing, China).

Quantitative Real-time PCR

Quantitative real-time PCR (qRT-PCR) assays were performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (TaKaRa). The maize *β-actin* gene was used as an endogenous control gene to normalize gene expression, whereas the *AtActin8* gene was used as the control in Arabidopsis. Three biological replicates at least were carried out and relative gene expression was analyzed using the method previously described by Li et al. (2015). Primer sequences used in this experiment are listed in Table S1.

Semi RT-PCR Analysis

Using semi RT-PCR, we measured the relative mRNA abundance for *ZmHsf12* in two-week-old transgenic Arabidopsis seedlings. For semi RT-PCR, the first strand of cDNA was synthetized by reverse transcription from 1 μg of total RNA. The *ZmHsf12* fragment were obtained using gene-specific primers (5'-GCTTCATCGTCTGGAACAC-3' and 5'-TTCAATCTCTTCCTCCAACC-3'). *AtActin2* (At3g18780), the loading control, was amplified with the specific primers (5'-CAATCGTGT-GTGACAATGG-3' and 5'-AACCCTCGTAGATTGGCA-3'). The PCR products were examined by electrophoresis on a 1% agarose gel and images were captured with the Gel Doc System Universal Hood II (Bio-Rad, Hercules, CA, USA).

Subcellular Localization of ZmHsf12 in Onion Epidermis Cells

The *ZmHsf12* CDS amplified by PCR without the stop codon using the following primers (forward: 5'-GAGAACACGGGGGACTCTAG-AATGCAGGGCGGCGGG-3'; reverse: 5'-GCCCTTGCTCACCATG-GATCCGTACAGCCCATTCCC-3'), the amplified fragment was inserted into the pCAMBIA1300-GFP vector which was restricted in advance with the restriction enzymes *Xba* I and *BamH* I according to the ClonExpress II one step cloning kit (Vazyme, Jiangsu Sheng, China) instruction.

Gold particle embedding and bombardment were performed as described by Li et al. (2014). The bombarded onion epidermis was placed on MS agar plates, sealed and cultured in the dark at 22°C overnight. The epidermis was then stained with DAPI (10 μg ml^{-1}) for 5 min and rinsed with physiological saline three times, each for 5 min. The fluorescence was then examined in the stained cells using a Zeiss LSM510 META confocal laser microscope.

Thermotolerance Assay of Yeast Cells Overexpressing *ZmHsf12*

The yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA) contains GAL1 promoter. Expression of genes cloned downstream of the GAL1 promoter can be induced by galactose (but not by glucose) in the medium in *Saccharomyces cerevisiae*, which is often used to select ura3-genotype host strain transformants using the URA3 gene. The PCR primers (forward, 5'-GGGAATATTAAGCTTGGTACCA-TG-GAGGGCGGCGGGGTG-3'; reverse, 5'-TGATGGATATCTGC-AGAATTCCTAGTACAGCCCATTCCC-3') were designed to amplify the CDS of *ZmHsf12*. Using the ClonExpress II one step cloning kit (Vazyme), the PCR products were integrated into the pYES2 vector digested with *Kpn* I and *EcoR* I (NEB, Ipswich, MA, USA).

The pYES2-*ZmHsf12* construct and the pYES2 control vector were transformed into competent cells of yeast strain *INVSc*1 using LiAc method (Gietz et al. 1992). SC-Glu-Ura^- medium was used to select the transformants. Pick single colony into 50 μl ddH_2O, mixed, froze and thaw in liquid nitrogen for 3 times. Using the PCR method, positive yeast clones were obtained. For the thermotolerance test on solid SD-Gal inducing medium, positive clones of the two strains were picked into liquid SC-Glu-Ura^- medium and the cultures were shaken overnight (250 rpm) until the OD_{600} was 0.6–0.7. The cultures were diluted to and OD_{600} of 0.2 with SC-Glu-Ura^- liquid medium and shaken for 2-3 h to allow the cells to enter the exponential phase of growth (OD_{600} of 0.4-0.8), after which they were collected by centrifugation, washed twice with sterile water, and then serially diluted to OD_{600} of 0.1, 0.05, and 0.01. There were two groups in the experiment: one group was subjected to HS in a water bath at 50°C for 15 min, and the other group was the control (no HS). Small aliquots (8 μl) of each culture dilution were plated on SC-Gal-Ura^- medium, the plates were incubated at 30°C and colonies were observed and photographed after 3 days. For the thermotolerance test in liquid SD-Gal inducing medium, exponential phase cells grown in liquid SC-Glu-Ura^- medium were diluted to an OD_{600} of 0.2 with the same liquid medium, and divided into two groups as before. One group was the control, and the other group was subjected to HS in a 50°C water bath for 15 min. The two groups were then shaken and the OD_{600} was measured every three hours.

Plasmid Constructs and Plant Transformation

The CDS of *ZmHsf12* was amplified using the gene-specific PCR primers (5'-GAGAACACGGGGGACTCTAGAATGCAGGGCG-GCGGG-3' and 5'-CGATCGGGGAAATTCGAGCTCCTAGTACA-GCCCATTCCC-3'). The amplified fragment was integrated into the pCAMBIA1300 vector digested with *Xba I* and *Sac I* (NEB). *Agrobacterium tumefaciens* strain GV3101 was then transformed with the pCAMBIA1300-35S::*ZmHsf12* construct. Arabidopsis transformation was performed using the floral dip method described by Clough and Bent (1998). MS culture medium containing 25 μg l^{-1} hygromycin was used to screen transgenic Arabidopsis lines,. In this experiment, the homozygous T3 generation of the transgenic lines was used.

Thermotolerance Analysis in Arabidopsis

Seedlings of WT Arabidopsis and transgenic lines were sown together on the same 0.5 × MS plate. In the basal thermotolerance assay, seedlings grown for 5 days under normal conditions were exposed to 45°C for 50 min in a growth chamber and then allowed to recover at 22°C. In the acquired thermotolerance assay, 5-day-old seedlings were exposed to 37°C for 1 h in a chamber, returned to

normal growth conditions for 2 days, exposed to 46°C for 60 min again, and then allowed to recover at 22°C. The growth changes in the seedlings of the different genotypes were photographed after recovery for 8 days and the viable seedlings were counted. The seedlings that had grown new leaves were considered to be survivors. Referring to the method described by Li et al. (2015), the chlorophyll content of the different genotypes was mesured.

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Author's Contributions

GL and XG designed the experiments and wrote the article. YZ and GL carried out most of the experiments. HZ, YZ and LZ performed the vector construction and the experiment of subcellular localization. ZL and HZ revised the article. All authors read and approved the final manuscript.

Supporting Information

Table S1. qPCR primers of *Zea mays* L. and *Arabidopsis thaliana*.

References

Boscheinen O, Lyck R, Queitsch C, Treuter E, Zimarino V, Scharf KD (1997) Heat shock transcription factors from tomato can functionally replace Hsf1 in the yeast *Saccharomyces cerevisiae*. Mol Gen Genet 255:322–331

Busch W, Wunderlich M, Schöffl F (2005) Identification of novel heat shock factor-dependent genes and biochemical pathways in *Arabidopsis thaliana*. Plant J 41:1–14

Charng YY, Liu HC, Liu NY, Chi WT, Wang CN, Chang SH, Wang TT (2007) A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. Plant Physiol 143:251–262

Charng YY, Liu HC, Liu NY, Hsu FC, Ko, SS (2006) Arabidopsis *Hsa32*, a novel heat shock protein, is essential for acquired thermotolerance during long recovery after acclimation. Plant Physiol 140:1297–1305

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16: 735-743

Driedonks N, Rieu I, Vrizen WH (2016) Breeding for plant heat tolerance at vegetative and reproductive stages. Plant Reprod 29: 67–79

Gagliardi D, Breton C, Chaboud A, Vergne P, Dumas C (1995) Expression of heat shock factor and heat shock protein 70 genes during maize pollen development. Plant Molecular Biology 29: 841–856

Gietz D, Jean AS, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20:1425

Guo M, Liu HJ, Ma X, Luo DX, Gong ZH, Lu MH (2016) The plant heat stress transcription factors (HSFs): Structure, regulation and function in response to aboitic stresses. Front Plant Sci 7:1–14

Guo J, Wu J, Ji Q, Wang C, Luo L, Yuan Y, Wang Y, Wang J (2008) Genome-wide analysis of heat shock transcription factor families in rice and Arabidopsis. J Genet Genomics 35:105–118

Hahn A, Bublak D, Schleiff E, Scharf KD (2011) Crosstalk between Hsp90 and Hsp70 chaperones and heat stress transcription factors in tomato. Plant Cell 23:741–755

Hu C, Lin SY, Chi WT, Charng YY (2012) Recent gene duplication and subfunctionalization produced a mitochondrial GrpE, the nucleotide exchange factor of the Hsp70 complex, specialized in thermotolerance to chronic heat stress in Arabidopsis. Plant physiol 158:747–758

Huang LJ, Cheng GX, Khan A, Wei AM, Yu QH, Yang SB, Luo DX, Gong ZH (2018) *CaHSP16.4*, a small heat shock protein gene in pepper, is involved in heat and drought tolerance. Protaplasma online (https://doi.org/10.1007/s00709-018-1280-7)

Jiang Y, Zheng Q, Chen L, Liang Y, Wu J (2018) Ectopic overexpression of maize heat shock transcription factor gene *ZmHsf04* confers increased thermo and salt stress-tolerance in transgenic Arabidopsis. Acta Physiol Plant 40:9

Kang S, Eltahir EAB (2018) North China Plain threatened by deadly heatwaves due to climate change and irrigation. Nat Commun 9: 2894

Kotak S, Larkindale J, Lee U, von Koskull-Doring P, Vierling E, Scharf KD (2007) Complexity of the heat stress response in plants. Curr Opin Plant Biol 10:310–316

Li GL, Zhang HN, Shao H, Wang GY, Zhang YY, Zhang YJ, Zhao LN, Guo XL, Sheteiwy MS (2019) *ZmHsf05*, a new heat shock transcription factor from *Zea mays* L. improves thermotolerance in *Arabidopsis thaliana* and rescues thermotolerance defects of the *athsfa2* mutant. Plant Sci 283:375–384

Li HC, Li GL, Guo XL (2014) Cloning, expression characteristics and subcellular-location of heat shock transcription factor *ZmHsf06* in *Zea mays*. J Agric Biotechnol 23:41–51

Li HC, Zhang HN, Li GL, Liu ZH, Zhang YM, Zhang HM, Guo XL (2015) Expression of maize heat shock transcription factor gene ZmHsf06 enhances the thermotolerance and drought-stress tolerance of transgenic Arabidopsis. Funct Plant Biol 42:1080–1090.

Lin YX, Jiang HY, Chu ZX, Tang XL, Zhu SW, Cheng BJ (2011) Genome-wide identification, classification and analysis of heat shock transcription factor family in maize. BMC Genomics 12: 76–89

Liu HT, Gao F, Li GL, Han JL, Liu DL, Sun DY, Zhou RG (2008) The calmodulin-binding protein kinase 3 is part of heat-shock signal transduction in *Arabidopsis thaliana*. Plant J 55:760–773

Liu HT, Liao HT, Charng YY (2011) The role of class A1 heat shock factors (HSFA1s) in response to heat and other stresses in Arabidopsis. Plant Cell Environ 34:738–751

Liu HC, Charng YY (2013) Common and distinct functions of Arabidopsis class A1 and A2 heat shock factors in diverse abiotic stress responses and development. Plant Physiol 163:276–290

Lohmann C, Eggers-Schumacher G, Wunderlich M, Schöffl F (2004) Two different heat shock transcription factors regulate immediate early expression of stress genes in Arabidopsis, Mol Genet Genomics 271:11–21

Mishra SK, Tripp J, Winkelhaus S, Tschiersch B, Theres K (2002) In the complex family of heat stress transcription factors, HsfA1 has a unique role as master regulator of thermotolerance in tomato. Genes Dev 16:1555–1567

Nishizawa A, Yabuta Y, Yoshida E, Maruta T, Yoshimura K (2006) Arabidopsis heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. Plant J 48:

535–547
Nishizawa-Yokoi A, Nosaka R, Hayashi H, Tainaka H, Maruta T, Tamoi M, Ikeda M, Ohme-Takagi M, Yoshimura K, Yabuta Y, Shigeoka S (2011) HsfA1d and HsfA1e involved in the transcriptional regulation of HsfA2 function as key regulators for the Hsf signaling network in response to environmental Stress. Plant Cell Physiol 52:933–945
Nover L, Bharti K, Döring P, Mishra SK, Ganguli A, Scharf KD (2001) Arabidopsis and the heat stress transcription factor world: how many heat stress transcription factors do we need? Cell Stress Chaperones 6:177–189
Pirkkala L, Nykänen P, Sistonen L (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J. 15:1118–1131.
Queitsch C, Hong SW, Vierling E, Lindquist S (2000) Heat shock protein 101 plays a crucial role in thermotolerance in Arabidopsis. Plant Cell 12:479–492
Sewelam N, Oshima Y, Mitsuda N, Ohma-Takagi M (2014) A step toward understanding plant responses to multiple environmental stresses: a genome-wide study. Plant Cell Environ 37:2024–2035
Schöffl F, Prändl R, Reindl A (1998) Regulation of heat-shock response. Plant Physiol 117:1135–1141
Scharf KD, Berberich T, Ebersberger I, Nover L (2012) The plant heat stress transcription factor (Hsf) family: structure, function and evolution. Biochim Biophys Acta 1819:104–119
Scharf KD, Heider H, Höhfeld I, Lyck R, Schmidt E, Nover L (1998) The tomato HSF system: HSFA2 needs interaction with HSFA1 for efficient nuclear import and may be localized in cytoplasmic heat stress granules. Mol Cell Biol 18:2240–2251
Tito R, Vasconcelos HL, Feeley KJ (2018) Global climate change increases risk of crop yield losses and food insecurity in the tropical Andes. Glob Chang Biol 24:e592-e602
Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42:579–620
Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. Environ Exp Bot 61:199–223
Wang X, Shi X, Chen S, Ma C, Xu S (2018) Evolutionary origin, gradual accumulation and functional divergence of heat shock factor gene family with plant evolution. Front Plant Sci 9:71
Wu TY, Juan YT, Hsu YH, Wu SH, Liao HT, Fung RWM, Charng Y (2013) Interplay between heat shock proteins HSP101 and HSA32 prolongs heat acclimation memory posttranscriptionally in Arabidopsis. Plant physiol 161:2075–2084
Xue GP, Sadat S, Drenth J, Mcintyre CL (2014) The heat shock factor family from *Triticum aestivum* in response to heat and other major abiotic stresses and their role in regulation of heat shock protein genes. J Exp Bot 65:539–557
Yang X, Zhu W, Zhang H, Liu N, Tian S (2016) Heat shock factors in tomatoes: genome-wide identification, phylogenetic analysis and expression profiling under development and heat stress. Peer J 4:e1961
Yoshida T, Ohama N, Nakajima J, Kidokaro S, Mizoi J, Nakashima K, Maruyama K, Kim JM, Seki M, Todaka D, Osakabe Y, Sakuma Y, Schöffl F, Shinozaki K, Yamaguchi-Shinozaki K (2011) Arabidopsis HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. Mol Genet Genomics 286:321–332
Zhao LN, Duan SN, Zhang HN, Guo XL, Li GL (2017) Cloning, characteristics and regulating role in thermotolerance of heat shock transcription factor (*ZmHsf25*) in *Zea mays* L. Acta Agronomica Sinica 43:1021–1029
Zhong L, Zhou W, Wang H, Ding S, Lu Q, Wen X, Peng L, Zhang L, Lu C (2013) Chloroplast small heat shock protein HSP21 interacts with plastid nucleoid protein pTAC5 and is essential for chloroplast development in Arabidopsis under heat stress. Plant cell 25:2925–2943