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Functional characterization of a new maize heat shock transcription factor gene *ZmHsf01* playing important roles in thermotolerance

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Background. The yield of maize crop is influenced seriously by heat waves. Plant heat shock transcription factors (Hsfs) play a key regulatory role in heat shock signal transduction pathway. **Method.** In this study, a new heat shock transcription factor gene, *ZmHsf01* (accession number: MK888854) , was cloned from maize young leaves using homologous cloning method. The transcriptional level of *ZmHsf01* were detected by qRT-PCR in different tissues or under heat shock, abscisic acid (ABA) and hydrogen peroxide (H_2O_2) treatment. The transgenic yeast and Arabidopsis were used to study the gene function of *ZmHsf01*. **Result.** The coding sequence (CDS) of *ZmHsf01* was 1176 bp and encoded a protein that consisted of 391 amino acids. The homologous analysis result showed that ZmHsf01 and SbHsfA2d had the highest protein sequence identity. Subcellular localization experiments demonstrated that ZmHsf01 is localized to the nucleus. *ZmHsf01* was expressed in many maize tissues and was up-regulated by heat stress. *ZmHsf01* was up-regulated in roots and down-regulated in leaves by ABA and H_2O_2treatments. In yeast, *ZmHsf01*-overexpressing cells showed increased thermotolerance. In *Arabidopsis* seedlings, *ZmHsf01* complemented the thermotolerance defects of *athsfa2* mutant and *ZmHsf01*-overexpressing lines presented enhanced basal and acquired thermotolerance. Compared to wild type (WT) seedlings, *ZmHsf01*-overexpressing lines showed increased chlorophyll content after heat stress. The expression level of heat shock protein genes was up-regulated higher in *ZmHsf01*-overexpressing *Arabidopsis* seedlings than that in WT. These results suggested that ZmHsf01 plays a vital role in plant response to heat stress.

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Abbreviations

Hsfs, heat shock transcription factors; HSE, heat shock element; HS, heat shock; HSPs, heat shock proteins; RH, relative humidity; CDS, coding sequence; DBD, DNA-binding domain; OD, oligomerization domain; NLS, nuclear localization signal; NES, nuclear export signal; AHA motifs, C-terminal activator motifs; ABA, abscisic acid; H_2O_2, hydrogen peroxide; GFP, green fluorescence protein; WT, wild type; BT, basal thermotolerance; AT, acquired thermotolerance.

Abstract

Background. The yield of maize crop is influenced seriously by heat waves. Plant heat shock transcription factors (Hsfs) play a key regulatory role in heat shock signal transduction pathway.

Method. In this study, a new heat shock transcription factor gene, *ZmHsf01*(accession number: MK888854), was cloned from maize young leaves using homologous cloning method. The transcriptional level of *ZmHsf01* were detected by qRT-PCR in different tissues or under heat shock, abscisic acid (ABA) and hydrogen peroxide (H_2O_2) treatment. The transgenic yeast and Arabidopsis were used to study the gene function of *ZmHsf01*.

Result. The coding sequence (CDS) of *ZmHsf01* was 1176 bp and encoded a protein that consisted of 391 amino acids. The homologous analysis result showed that ZmHsf01 and SbHsfA2d had the highest protein sequence identity. Subcellular localization experiments demonstrated that ZmHsf01 is localized to the nucleus. *ZmHsf01* was expressed in many maize tissues and was up-regulated by heat stress. *ZmHsf01* was up-regulated in roots and down-regulated in leaves by ABA and H_2O_2treatments. In yeast, *ZmHsf01*-overexpressing cells showed increased thermotolerance. In *Arabidopsis* seedlings, *ZmHsf01* complemented the thermotolerance defects of *athsfa2* mutant and *ZmHsf01*-overexpressing lines presented enhanced basal and acquired thermotolerance. Compared to wild type (WT) seedlings, *ZmHsf01*-overexpressing lines showed increased chlorophyll content after heat stress. The expression level of heat shock protein genes was up-regulated higher in *ZmHsf01*-overexpressing *Arabidopsis* seedlings than that in WT. These results suggested that ZmHsf01 plays a vital role in plant response to heat stress.

Key words: *ZmHsf01*; Thermotolerance; Heat shock transcription factors; Maize

Introduction

High temperature is one of the abiotic stress factors, which greatly impact on crop yields and quality. In the North China Plain which has a typical temperature and monsoonal climate, agricultural production is often subjected to the frequency and intensity of heat waves(*Li et al.,2018; Suchul & Eltahir, 2018*). Both high temperature and drought stress, two reasons for production and quality decline, have strong effects on the maize growth period, especially at the early stage (*Caims et al., 2012*). Certain adaption exists in crops under high temperature, so controllable and appropriate heat acclimation could induce expression of related genes and synthesis of heat shock proteins (Hsps) and protective enzymes (*Nover et al., 1996*). These variations may help plants to obtain thermotolerance and to adapt to gravest high temperature. Studies have shown that the heat shock transcription factors (Hsfs) have a regulatory effect in the thermotolerance-formation process. Hsfs could bind the heat shock elements (HSEs) on the promoter region of *Hsps* or other related genes, and activate the downstream genes to generate heat shock response (*Nover et al., 2001*). Thus Hsfs have been proved to be an important regulator in the transcriptional activation, especially in the heat shock (HS) signal transduction pathway upon heat stress and other adversity stresses (*Aranda et al., 1999*).

Several *Hsfs* have been cloned in different species since yeast *Hsf* was isolated in the 1980s (*Baniwal et al., 2004; Bharti et al., 2004; Czarnecka-Verner et al., 2004; Kotak et al., 2004; Zhu et al., 2006; Guo et al., 2008; Yang et al., 2016*). As a common gene in plants, the first *Hsf* was cloned from tomato in 1990 (*Yokotani et al., 2008*). Based on the different structures, the *Hsfs* multi-gene family are divided into three classes (A, B and C), and each class contains different subclasses. The gene numbers of the *Hsfs* family varies greatly depending on organisms. There is only one *Hsf* gene in yeast and fruit fly, and vertebrates have at least four *Hsfs* (*Nakai, 2016*). However, plants possess more *Hsfs* than other organisms. So far, 21 *Hsfs* in *Arabidopsis* and 24 *Hsfs* in tomato have been identified, and at least 56 *Hsfs* in wheat were predicted (*Scharf et al., 2012; Xue et al., 2014*).

The functional analysis on Hsfs in plant is relatively less, and previous studies were limited to the A1 and A2 subclasses of *Arabidopsis* and tomato. From previous studies, the tomato *HsfA1a* is constitutively expressed and the deduced protein is localized in the nucleus and cytoplasm at normal growth conditions (*Scharf et al., 1998*). The *HsfA1a* has a gene regulatory role which induces the *Hsps* expression through activates the synthesis of *HsfA2* and *HsfB1* for heat shock resistance in tomato (*Scharf et al., 1998; Liu et al., 2013*). *HsfA2* with strong stability is strictly up-regulated by heat induction, and considerable accumulation of *HsfA2* appears at the later period and recovery

stage of heat stress in tomato (*Heerklotz et al., 2001*). Due to strong cytoplasmic localization signal, the nuclear localization of *HsfA2* has to rely on the fact that the *HsfA2* and *HsfA1* are bound together to form a heterooligomer (*Heerklotz et al., 2001*). In *Arabidopsis*, *AtHsfA2* is dramatically induced by heat shock. Not only basal and acquired thermotolerance, but also the resistance to salt and osmotic stresses of *AtHsfA2*-overexpressing *Arabidopsis* seedlings can be enhanced (*Ogawa et al., 2007*). Consistently, the basal and acquired thermotolerance and antioxidant capacity are reduced in *athsfa2* mutants (*Li et al., 2005*).

AtHsfA2 plays an important regulatory role in response to multiple abiotic stresses including heat stress (*Busch et al., 2005; Schramm et al., 2006; Nishizawa et al., 2006; Charng et al., 2007*). AtHsfA2 is a key regulator in plant response to environmental stresses (*Charng et al., 2007*). AtHsfA2 can directly bind to HSEs in the promoter region of target genes or interact with AtHsfA1s to form heterogenic complex to regulate the expression of downstream genes especially *Hsps* (*Liu & Charng, 2013; Nishizawa-Yokoi et al., 2011*). AtHsfA1s function as key regulators involving in the regulation of *AtHsfA2* expression (*Liu & Charng, 2013*). AtHsfA1s and AtHsfA2 have distinct but overlapping functions in response to abiotic stresses (*Liu & Charng, 2013; Nishizawa-Yokoi et al., 2011*).

In maize, four Hsfs including *ZmHsf01*, *ZmHsf04, ZmHsf05* and *ZmHsf17* were identified as HsfA2 subclass (*Lin et al., 2011*). Among subclass A2, *ZmHsf04* and *ZmHsf05* were cloned. Ectopic overexpression *ZmHsf04* and *ZmHsf05* improves the thermotolerance in transgenic *Arabidopsis* seedlings (*Li et al., 2019; Jiang et al., 2018*). In our study, *ZmHsf01*, another member of *ZmHsfA2*, was cloned and analyzed. Subcellular localization of ZmHsf01-GFP was observed. The functions of *ZmHsf01* in plant response to heat stress were detected and discussed.

Materials and Methods

Plant materials and culture conditions

Maize (*Zea mays* L.) inbred line H21 was used in the study. Healthy seeds were surface-sterilized with 0.1% $HgCl_2$ for 10 min and rinsed repeatedly with double distilled water. The seeds were germinated in the dark for 12 h and planted into a control-environment greenhouse at 28°C with the conditions of 16/8 h of day/night (100 μmol m^{-2} s^{-1}) and 60% RH. The two-leaf-old seedlings were used to stress experiments. Mature leaves, pollens and ears were separated in blooming period. Immature embryos were separated in two weeks after pollination. All the tissues and organs were used for gene expression analysis after frozen in liquid nitrogen.

Stress treatment

Uniform two-leaf-old maize seedlings were selected and subjected to the following treatments according to the methods described by Li et al. with some modification (*Li et al., 2019*). For heat stress, Hoagland nutrient solution was pre-treated at 42°C before the seedlings were immersed. The leaves and roots were sampled at the treatment time of 0, 10, 20, 30, 40, 50, 60 and 120 min, respectively. For ABA treatment, the seedlings were treated with the final concentration of 200 μM ABA. The treatment time was 0, 2, 4, 6, 12, 24 and 36 h. For H_2O_2 treatment, the seedlings were treated with the final concentration of 10 mM H_2O_2. The treatment times were 0, 15, 30, 60, 90, 120 and 240 min. For *Arabidopsis* treatment, 5-day-old seedlings of the transgenic line 36_7 and WT were selected to be used in this experiment. All the leaves of WT and overexpressing line were harvested at 2 h after different heat treatments as the basal and acquired thermotolerance assays. Three biological experiments were carried out. The samples were collected and frozen quickly in liquid nitrogen.

Gene cloning and sequencing

Total RNA was extracted from leaves using the RNArose Reagent Systems Kit (SBS, Beijing, China). The genomic DNA was digested by *DNase* Ⅰ (TaKaRa, Dalian, China) for 30 min at 37°C. One μg total RNA was used for the first standard synthesis of cDNA using Reverse Transcription Kit (Invitrogen, USA). The quantity of RNA samples was checked on NanoDrop 2000 (Thermo Scientific, USA).

A pair of primers (forward primer 5′-CGTGGCGAGATGGACCTGATGC-3′, reverse primer 5′-TTAACGCGATCATCTCTACTTC-3′) was designed to amplify the open reading frame of *ZmHsf01*. We submitted the full coding sequences (CDS) of ZmHsf01 to GenBank and received accession number MK888854. High-fidelity enzyme Pyrobest (TaKaRa, Dalian, China) was used for PCR amplification. The PCR reaction system consists of 1 × Pyrobest buffer; 0.2 mM dNTP mixture; 200 ng 1st strand cDNA; 0.2 μM forward primer; 0.2 μM reverse primer; 1.25 units Pyrobest DNA polymerase. The reaction procedure is: 98°C 10 s, 55°C 15 s, 72°C 2 min, 30 cycles. The PCR products were ligated into T-vector (TransGen Biotech, Beijing, China) and the genes on T-vectors were sequenced by Shanghai Biotech Company.

qRT-PCR analyses

The PCR reaction mixtures contain 1 × SYBR Premix Ex *Taq*II (Takara, Dalian, China), 0.4 μM forward primer, 0.4 μM reverse primer and 1 μg cDNA in a final volume of 20 μL. A 7500 Real-Time PCR system (Applied Biosystems, USA) was used in this experiment. The reaction procedure is as follows: pre-denaturation at 95°C for

10 min, and 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 1min. After the reaction, the data were analyzed using the $2^{-\Delta\Delta Ct}$ method. Three biological replicates were performed in every group of experiments. Analysis of the data was performed with Microsoft Excel 2010. For statistical analysis, each dataset was repeated at least three times. The expression level of young root was set as 1 for the expression analysis of tissues and organs, and that at 0 min was set as 1 for the expression analysis of different stress treatments. A maize gene of *β-Actin* was used as an endogenous control in maize, whereas the *AtActin8* (At1g49240) was used in *Arabidopsis*. The primers of *ZmHsf01* and other primers of some relevant genes were designed and listed in **Table S1.**

Subcellular localization of ZmHsf01 in tobacco epidermal cells

Using a ClonExpress II kit (Vazyme, Nanjing, China), we constructed a recombinant vector pCAMBIA1300-ZmHsf01-GFP driven by CaMV 35S promoter, which contained the ZmHsf01 CDS amplified by gene-specific primers (forward primer: 5′-GAGAACACGGGGGACTCTAGAATGGACCTGATGCTG-3′; reverse primer: 5′-GCCCTTGCTCACCATGGATCCCTTCGCCGTGGTGTT-3′) and GFP gene. The constructs were transformed into Agrobacterium tumefaciens EHA105 cells. ZmHsf01-GFP was expressed in the tobacco leaves epidermal cells by EHA105. The tobacco seedlings were raised in a glasshouse (12/12 h of day/night, 150 µmol m-2 s-1, 50% relative humidity, the temperature 19-23°C) for 72 h , then the leaves were harvested and were stained with DAPI (10 µg mL^{-1}) for 5 min. After rinsed with physiological saline three times, the tobacco epidermal cells were observed under the laser-scanning confocal microscope LSM 710 (Zeiss Microsystems, German).

Semi qRT-PCR assay

The transcription abundance of *ZmHsf01* in transgenic *Arabidopsis* was tested by semi qRT-PCR method. RNA extraction and the synthesis of cDNA were carried out according to the protocols mentioned in the previous report. Based on the coding region sequence of *ZmHsf01*, a pair of primers (forward primer, 5′-GTGACGGTAAAGGAGGAGTGGCCT-3′; reverse primer, 5′-GCCATAGGTGTTCAGCTGGCGGAC-3′) were synthesized. *AtActin2* (At3g18780) (forward primers 5′- CAATCGTGTGTGACAATGG-3′ and reverse 5′-AACCCTCGTAGATTGGCA-3′) was used as loading control.

Construction, transformation of yeast expression vectors and thermotolerance assay

The vector pYES2 (Invitrogen, USA) was used for detecting the target protein expression in *Saccharomyces cerevisiae*. Using the clonExpress Ⅱ recombination system (Vazyme, Nanjing, China), the PCR products of *ZmHsf01* CDS amplified by a pair of specific primers (5′-GGGAATATTAAGCTTGGTACCATGGACCTGATGCTGCCG-3′ and 5′- TGATGGATATCTGCAGAATTCCTACTTCGCCGTGGTGTT-3′) were inserted into pYES2 vector. The recombinants were transformed to the yeast INVSc1 competent cells described by Gietz et al. (*1992*), then the cells were diluted and plated on SC/Glu/Ura$^-$ agar screening plate for growth at 30° C. After 2~3 days, the positive clones were selected and verified by colony PCR.

For the thermotolerance assay, the positive clones were cultured with liquid SC/Glu/Ura$^-$ medium in a shaking incubator (250 rpm min^{-1}). When the OD_{600} of cells reached 0.6–0.7, the cells were diluted to OD_{600} 0.2 with SC/Glu/Ura$^-$ liquid medium, and cultured with shaking for 2-3 h. Cells were collected at OD_{600} 0.4-0.8, eluted two times with sterile water, and then serially diluted to OD_{600} of 0.1, 0.05 and 0.01. The two samples were separated into two groups. One group was subjected to HS treatment in 50°C water bath for 15 min, while no intervention was made in control. 8 μL treated yeast cells were plated on SC/Gal/Ura$^-$ agar and grown at 30°C. Yeast colony formation was examined and photographed after 2~3 days.

Plasmid construction and transformation in Arabidopsis

Special-primers (Forward: 5'- GAGAACACGGGGGACTCTAGAATGGACCTGATGCTG-3'; Reverse: 5'-CGATCGGGGAAATTCGAGCTCCTACTTCGCCGTGGTGTT -3') were designed and used to amplify the coding sequence of *ZmHsf01* by RT-PCR. Using a ClonExpress II cloning kit, we inserted the PCR products into the pCAMBIA1300 vector digested in advance with *Xba I* and *Sac I* which was driven by CaMV 35S promoter. After the construct infected *Agrobacterium* GV3101, wild *Arabidopsis* and deletion mutants were transformed with the new constructs by vacuum dipping method. The MS medium containing 25 μg ml^{-1} hygromycin were used to screen the progeny plants, until the homozygous seeds were harvested. The transgenic lines of T3 homozygous were used for identification of thermotolerance.

Thermotolerance assays in *Arabidopsis*

The sterilized seeds of WT, *athsfa2* and transgenic lines of *Arabidopsis thaliana* (Ecotype, Columbia) were planted in 1/2 MS solid plates containing 0.8% agar. The plates were placed in an incubator at 22°C (day) and 18°C (night) with the conditions of 16/8 h of day/night (100 μmol m^{-2} s^{-1}). The basal thermotolerance was evaluated by the

following protocol: 5-day-old seedlings of WT and three overexpressed lines were subjected to 45°C heat shock for 50 min, and then recovered at the normal growth conditions for 8 days. The acquired thermotolerance was evaluated by the treatment: 5-day-old seedlings of WT and three overexpressed lines were subjected to 37°C for 60 min, then recovered at normal conditions for 2 days, re-treatment under heat shock at 46°C for 60 min and recovered at the normal conditions for 8 days. The thermotolerance of complementary lines was assayed: 5-day-old seedlings of WT, *athsfa2* mutant and transgenic lines were treated at 44°C for 70 min, and recovered at normal conditions for 8 days. All the phenotypes were observed and photographed, and then the chlorophyll content of leaves were measured (*Li et al., 2015*). At least 30 seedlings were analyzed for each line. All the experiments were repeated 3 times.

Transcriptional activation analysis in yeast

According to the instruction of Y2H system (TaKaRa, Dalian, China), transcription activation activity assay s of ZmHsf01 were performed. Using the CloneExpress II cloning Kit, we make the recombinants with the pGBKT7 vector and the full CDS of *ZmHsf01* amplified by the specific primers (5′-ATGGCCATGGAGGC CGAATTCATGGACCTGATGCTG-3′ and 5′- CCGCTGCAGGTCGACGGATCCCTACTTCGCCGTGGT-3′). LiAc and PEG3350 were used in yeast transformation assay. The pGBKT7 vector, the pGBKT7-53 and pGADT7-T vectors and the pGBKT7-ZmHsf01 fusion vector were transformed into the yeast cell AH109, r espectively. Different concentration of transformed yeast cells were cultured in SD/Trp^- and SD/Trp^-/His^- /Ade^-/X-α-gal successively.

Results

Cloning of the ZmHsf01and protein subcellular localization

Using RT-PCR method, we cloned the CDS of *ZmHsf01* from H21 seedlings. Sequence analysis showed that the CDS of *ZmHsf01* was 1,176 bp length and encodes a deduced protein with 391 amino acid residues. The CDS and amino acid sequence exhibited in the Figure 11and containing The conserved DBD, OD, NLS, NES and AHA domains were marked with red lines (Fig. 1). Amino acid sequence alignment results showed that ZmHsf01 shared 86%, 70% and 69% identities with SbHsfA2d from *sorghum bicolor* (XP_002468465), DoHsfA2d from *Dichanthelium oligosanthes* (OEL38242) and SiHsfA2d from *Setaria italica* (XP_004985605), respectively (Fig. 1). These results indicated that *ZmHsf01* is a heat shock transcription factor gene belonged to A2 subclass.

The ZmHsf01-GFP fusion protein was observed for the subcellular localization of ZmHsf01. The CDS of *ZmHsf01* was connected to the N-terminal of green fluorescent protein (GFP) gene driven by a CaMV 35S promoter. The tobacco epidermal cells were used to express ZmHsf01-GFP fusion protein by *Agrobacterium*-mediated transformation. After cultured for 3 days, the tobacco epidermal cells were stained with DAPI which is a nuclei-special dye. The laser confocal microscopy examination showed that ZmHsf01-GFP fusion protein can only be detected in the nuclei, and co-localized with DAPI florescence (Fig. 2). These results suggested that ZmHsf01 localizes in the nucleus.

Expression analysis of ZmHsf01

Under the normal growth conditions, *ZmHsf01* was expressed in all detected maize organs such as young roots, young shoots, young leaves, mature leaves, pollens, ears and immature embryos (Fig. 3A). The expression level of *ZmHsf01* was highest in young leaves and lowest in ears. *ZmHsf01* was expressed more than twenty times in young leaves than that in roots.

The expression of *ZmHsf01* was up-regulated significantly in both roots and leaves after 42°C heat shock, and the expression level reached the peaks at heat shock 30 mins and then gradually decreased (Fig. 3B, C). After ABA treatment, the expression of *ZmHsf01* in roots was up-regulated and reached the peaks value at 24 h, but the expression of *ZmHsf01* in leaf appeared a down-regulation tendency (Fig. 4A, B). The expression level of *ZmHsf01* increased in roots and decreased in leaves by H_2O_2 treatment (Fig. 4C, D). These results showed that *ZmHsf01* was up-regulated by heat, ABA and H_2O_2 stresses.

Expressing ZmHsf01 yeast cells improved the thermotolerance

To further analyze the function of ZmHsf01, the pYES2-ZmHsf01 yeast expression vector was used to genetic transformation and heat tolerance identification of yeast positive strain. Under normal conditions, no significant phenotype difference was found between the two kinds of transgenic yeast cells (pYES2-ZmHsf01 and pYES2 control). The growth of the two groups cells were both inhibited after heat-treatment at 50°C for 15 min, but the growth potential of *ZmHsf01*-expressing cells was better than that of control cells (Fig. 5). These results demonstrated that *ZmHsf01* improved the thermotolerance of transgenic yeast cells.

Transcription activation activity of ZmHsf01 in yeast

Based on the domain analysis, ZmHsf01 has the AHA domain which exists in class A members. ZmHsf01was constructed into pGBKT7 vector. The yeast strains transformed with fusion vector pGBKT7-ZmHsf01 grew well and turned blue like the positive control groups in SD/-Trp/-His/-Ade/X-α-gal culture medium (Fig. 6). The results showed that ZmHsf01 has transcription activated activity in yeast cells.

ZmHsf01 rescued the thermotolerance defects of Arabidopsis athsfa2 mutant

Charng et al. reported that the *Arabidopsis* mutant *athsfa2* (SALK_008978) has the thermotolerance defects (*Charng et al., 2007*). We used the *athsfa2* mutant to investigate the thermotolerance of *ZmHsf01*. Three *ZmHsf01*/*athsfa2* complementary lines (2_10, 3_1 and 4_11) were selected in this experiment, which expressed different levels of *ZmHsf01* by semi RT-PCR (Fig. 7D). Seedlings of the three *ZmHsf01*/*athsfa2* complementary lines, *athsfa2* and WT were photographed (Fig. 7A) and chlorophyll content (Fig. 7C) was measured under normal conditions or heat stress (Fig. 7B). Compared with WT, the thermotolerance of *ZmHsf01*/*athsfa2* was better than that of *athsfa2* or WT (Fig. 7B) and chlorophyll content of *ZmHsf01*/*athsfa2* was higher than that of *athsfa2* or WT (Fig. 7C). The results showed that *ZmHsf01* can partially or completely rescue the thermotolerance defects of *athsfa2*.

The functional identification of ZmHsf01 in thermotolerance of Arabidopsis

To further investigate the thermotolerance of *ZmHsf01* in *Arabidopsis*, three *ZmHsf01*-overexpressing lines (26_26, 28_4 and 36_7) which displayed different expression levels by semi RT-PCR (Fig. 8D) were used for analysis of basal and acquired thermotolerances. Five-day-old seedlings of WT and the three *ZmHsf01*-overexpressing lines growing on the same MS medium were exposed to special heat stress regimes (Fig. 8B), and then recovered to grow at 22°C for another 8 days (Fig. 9B). Under the normal conditions, no obvious phenotypic changes were observed between the *ZmHsf01*-overexpressing lines and WT (Fig. 8A and Fig. 9A). After heat shock, the WT seedlings became wilt, but the three *ZmHsf01* over-expressing lines still retained green (Fig. 8B). Chlorophyll content in different lines and WT were measured. Without heat treatment, no remarkable difference in chlorophyll contents was found among WT and three transgenic lines (Fig. 8C and Fig. 9C). After basal and acquired HS treatment, chlorophyll content of all genotypes decreased, but chlorophyll content of the three *ZmHsf01*-overexpressing lines was higher than that of WT (Fig. 8C and Fig. 9C).

Overexpression of ZmHsf01 in Arabidopsis affected the expression of AtHsps

It was proved that Hsp genes can be induced and accumulated in cells to enhance the resistance of the *Arabidopsis* plants upon heat stress(*Wan et al. 2016*). To test the role of *ZmHsf01* in *Hsp* expression, qRT-PCR was performed using *ZmHsf01* over-expressing line 36_7. Five-day-old seedlings of WT and the three *ZmHsf01*-overexpressing lines growing on the same MS medium were exposed to special heat stress regimes. Some *AtHsps* including *AtHsp18.2*, *AtHsp21*, *AtHsa32*, *AtERDJ3A*, *AtHsp70b*, *AtHsp70T*, *AtHsp90.1* and *AtHsp101* were detected after the basal and acquired heat treatments (Fig. 10). These results displayed that the expression levels of *AtHsps* in *ZmHsf01*-overexpressing line 36_7 were higher from 1.1 to 2.5 times than that in WT after HS treatment (Fig. 10). These results indicated that *ZmHsf01* can regulate the expression of *AtHsps* to improve the thermotolerance of transgenic *Arabidopsis*.

Discussion

In Hsfs studies, the A1 and A2 subclasses were treated as the main research objects in *Arabidopsis* and tomato (*Nakai, 2016; Scharf et al., 1998; Ogawa et al., 2007; Liu & Charng, 2013*). With the completion of many crops genome sequencing in recent years, the number of Hsfs family member in more and more species was speculated and genetic analyzed. Function analysis of other subclass members is being developed gradually (*Ma et al., 2016; Hu et al., 2018*). Relatively, the research on *Hsfs* in field crops started late. Many TFs family, such as WRKY,MYB,DREB and HSF, have enormous potential in terms of improving the resistance of maize (*Kimotho et al., 2019*). At least 25 *Hsfs* were identified in maize in 2011 and the number of *ZmHsfs* reached 30 in 2016 (*Lin et al., 2011; Guo et al., 2016*). Xue et al. reported that at least 56 *Hsfs* exist in wheat (*Xue et al., 2014*), but our recent analysis results suggested a larger amount (*Duan et al., 2019*). The large number of Hsfs family members in plant mean the complexity and diversity of Hsfs family.

In *Arabidopsis*, A1 subclass *Hsfs* were considered to be the master regulator in the process of sense stress and activate the downstream genes (*Liu et al., 2013*). Unlike A1 subclass, the genes in A2 subclass belong to heat-induced factors, they were induced to express after heat stress (*Schramm et al., 2006; Mittal et al., 2009*). The expression of *ZmHsf01* increased first and then decreased after 30mins in leaves and roots under HS. ZmHsf01 contains the typical domains of HsfA class including DBD, HR-A/HR-B, NLS and AHA motif. ZmHsf01-GFP fusion protein was localized to the nuclei, which is consistent the structure of ZmHsf01 including the motif of NLS. This change trend of expression levels and these typical characteristics in ZmHsf01 was same to the other two

reported ZmHsfA2s (*Li et al., 2019; Jiang et al., 2018*). The conservative domain among these HsfA members means the similarity of gene function. ABA, low temperature or NaCl treatment induced the up-regulation of *ZmHsf04* significantly (*Li et al., 2019*). But *ZmHsf01* was up-regulated in roots after ABA or H_2O_2 treatment. The expression of *OsHsfA2d* increased four to six fold under salt or PEG stress, and hold the line under 4°C (*Liu et al., 2010*). The abiotic stresses assays suggested that *HsfA2s* are induced by various environmental stresses, but each member of HsfA2 has a different response to different abiotic stress.

Previous studies on *Arabidopsis* have shown that AtHsfA2 sustained the expression of Hsp genes in the recovery stage and the process of the acquired thermotolerance (*Charng et al., 2007*). There is only one *HsfA2* in *Arabidopsis*. Compared with WT, the *athsfa2* mutant were more sensitive to heat stress (*Li et al., 2005; Charng et al., 2007*). *FaHsfA2c* from tall fescue restored the heat sensitive deficiency of *athsfa2* mutant in *Arabidopsis* (*Wang et al., 2017*). *ZmHsf05*, another member of *HsfA2*, could complement the lack of thermotolerance of *athsfa2* mutant, too (*Li et al., 2019*). In this study, *ZmHsf01* rescued the thermotolerance phenotypes of *athsfa2* mutant and *ZmHsf01*-overexpressing *Arabidopsis* seedlings improved the basal and acquired thermotolerance compared with WT. These results demonstrated that *ZmHsf01* has the thermotolerance in plant response to heat stress and plays similar functions to that of *ZmHsf05* in terms of the thermotolerance.

As molecular chaperones, Hsps belong to multigene families participate in various biological process of protein folding, refolding, co-degradation of the denature protein and normal growth development (*Kuang et al., 2017; Queitsch et al., 2000*). The known “refolding machines”, consisting of *Hsp70* and *ERDJ3A*, work on the refolding of denatured protein upon heat stress and alleviating the stress damage (*Ma et al., 2015*). The Hsf/Hsp network which includes activating downstream responses and feedback suppress the Hsfs played a vital role in HS and other stresses (*Frangkostefanakis et al., 2015*). In *ZmHsf04* and *Zmhsf05* transgenic *Arabidopsis*, the expression levels of all detected *AtHsps* were higher than that in WT after HS (*Li et al., 2019; Jiang et al., 2018*). After heat treatment, the transcript levels of some *Hsps* in *ZmHsf01* over-expressed lines are higher than that of WT to some extent, such as *AtHsp18.2*, *AtHsp21*, *AtHsp70b*, *AtHsp70T*, *AtHsp90* and *AtHsp101*. These results indicated that ZmHsf01 may improve the thermotolerance of plants by regulating the expression of *Hsps*.

As the member of *HsfA2* subclass, *ZmHsf01* perhaps participate in thermotolerances by activate multiple related genes expression. The obvious induced expression of *ZmHsf01* maybe means the key position response to heat stress. Our present study proved that heat treatment may be helpful to accumulate various Hsps to improve plant

316 thermotolerance. Each member of *Hsfs* family plays different role in HS signal transduction and regulation of
317 downstream genes. It will be of considerable interest to test the interaction between different Hsfs and gene
318 regulatory mechanism in transgenic maize.

319 **Conclusion**

320 *ZmHsf01* was cloned from maize inbred line H21. ZmHsf01 was highly conserved compared to its homologs in
321 other plants. *ZmHsf01* from maize rescued thermotolerant defects of *athsfa2* in *Arabidopsis thaliana*. Ectopic
322 expression of *ZmHsf01* in *Arabidopsis thaliana* increased thermotolerance to heat stress as compared with WT.

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329 providing *athsfa2* mutant seeds and Dr. Rongmin Chen (School of Medicine, Yale University, USA) for polishing
330 the language and grammar.

331 **Conflict of interest**

332 The authors declare that there is no any conflict of interest regarding the paper.

333

334 **References**

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Figures and legends

Figure 1 - Amino acid sequence alignment between ZmHsf01 and homologs from different species. DBD, the conserved DNA binding domain; HR-A/HR-B, two hydrophobic heptad repeats; NLS, nuclear localization signal; NES, nuclear export signal; AHA, aromatic, large hydrophobic and acidic amino residues. The accession numbers of proteins in NCBI as follows: SbHsfA2d from *Sorghum bicolor*, XP_002468465.1; DoHsfA2d from *Dichanthelium oligosanthes*, OEL38242.1; SiHsfA2d from *Setaria italica*, XP_004985605.1.

Figure 2 - Subcellular localization of ZmHsf01. GFP, green fluorescence of GFP; DAPI, blue fluorescence of DAPI; Bright, bright field; Merged, merged images.

Figure 3 - Expression patterns of *ZmHsf01* in tissues and under heat stress. A, Expression levels of *ZmHsf01* in different tissues and organs under the normal growth conditions. B and C, Expression levels of *ZmHsf01* in leaves and roots of the maize seedlings after 42°C heat shock. There are three biological repeats for each sample and the data are mean± standard error.

Figure 4 - Expression patterns of *ZmHsf01* under ABA and H_2O_2 treatment. A and B, Expression levels of *ZmHsf01* in leaves and roots of the maize seedlings after 200 μM ABA treatment. C and D, Expression levels of *ZmHsf01* in leaves and roots of maize seedlings after 10 mM H_2O_2 treatment. There are three biological repeats for each sample and the data are mean± standard error.

Figure 5 - Thermotolerance assays of yeast cell harboring pYES2 or pYES2-ZmHsf01 after 50°C heat shock. A, culture under normal conditions; B, culture under normal conditions after HS at 50°C for 45 min.

Figure 6 - Transcription activation analysis of ZmHsf01 in yeast. Yeast cells transformed with pGBKT7 vector was set as the Negative control, and yeast cells co-transformed with pGBKT7-53 and pGADT7-T vectors were set as the positive control. The group of ZmHsf01 was the yeast cells transformed with the fusion vector pGBKT7-ZmHsf01.

Figure 7 - The thermotolerance phenotypes of deletion mutant and three restoration lines of *Arabidopsis* seedlings were showed. A, Seedlings of WT, deletion mutant and three restoration lines (2_10, 3_12 and 4_11) growing on the MS plate under normal conditions were used as a control. B, Seedlings of all genotypes under HS at 44°C for 70 min and recovered at normal conditions for 8 days. C, Chlorophyll contents of seedlings under normal conditions and HS treatments. D, Semi qRT-PCR assay of the *ZmHsf01* transcript. E, Schematic representation of the HS regimes.

Figure 8 - Overexpressing *ZmHsf01* improved the basal thermotolerances in *Arabidopsis* seedlings. A, Seedlings of WT and three overexpressing lines (26_26, 28_4 and 36_7) growing on the MS plate under normal conditions used as a control of basal thermotolerance. B, Seedlings of all genotypes upon basal HS at 45°C for 50 min and recovered at normal conditions for 8 days. C, Chlorophyll contents of seedlings under the normal conditions and the basal HS treatments. D, Semi qRT-PCR analysis of the *ZmHsf01* transcript in different lines. The expression levels of *AtActin2* were used as the control. E, Schematic representation of the basal HS regimes.

Figure 9 - Overexpressing *ZmHsf01* improved the acquired thermotolerances in *Arabidopsis* seedlings. A, Seedlings of WT and three overexpressing lines (26_26, 28_4 and 36_7) growing on the MS plate under normal conditions used as a control of acquired thermotolerance. B, 5-day-old seedlings of all genotypes under acquired HS at 37°C for 60 min and recovered at normal conditions for 2 days and treated at 46°C for 60 min and recovered at normal

conditions for 8 days. C, Chlorophyll contents of seedlings under the normal conditions and the acquired HS treatments. D, Schematic representation of the acquired HS regimes.

Figure 10 - The expression levels of heat-related Hsp genes in WT and *ZmHsf01* over-expressing lines 36_7 after HS treatment. qRT-PCR was performed about the *Arabidopsis* genes *AtHsp18.2*, *AtHsp21*, *AtERDJ3A*, *AtHsfa32*, *AtHsp70b*, *AtHsp70T*, *AtHsp90* and *AtHsp101* upon BT and AT heat stress. Set the expression level of WT samples was 1. The reference gene *Atactin8* (At1g49240) was used as an internal control to normalize the loading of different samples. Data were means ± SD from three biological experiments.

Figure 11 - The CDS and amino acid sequences of *ZmHsf01*

Table 1 - The qPCR primers of *Zea mays* L. and *Arabidopsis thaliana*

Table 1(on next page)

TABLE1

The qPCR primers of *Zea mays* L. and *Arabidopsis thaliana*

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1 **Table1**

Gene Name	Forward	Reverse
ZmHsf01	AGAACCTGGCGCTCAACA	TCAGCAGCTCCTCCCAAA
AtHsp18.2	GCAGATTAGCGGAGAGAGGA	CCTTCACTTCTTCCATCTTTGC
AtHsp21	AAGTCCGCTACACCGTTCTC	CCAACAATCCGAAAGGAGAG
AtERDJ3A	CTCCTGTTTGTATCATTGGTGC	TGTGTCCTGAGAACCTGTGG
AtHsa32	GCGAAGTTGGTTGAGTGGTT	GGAGGAACTGAGAACAGATTGG
AtHsp70b	TCCGCTTAGCCTTGGACTT	ACGCCTGGTTGATTGTCTG
AtHsp70T	TGATTGAGGTGAGGATGCC	CCACTTCAACGACAAACCC
AtHsp90.1	CCCTCTCTTCTTCATAAATCAACA	CCATCGCAACGAACTTTG
AtHsp101	TGTCTTCAACACTCTGCTCCA	CACTTCCATTGTTACTTTCCCAG
AtActin8	CTCTCAATCGCATACACCAGC	ATCCACTAAGCACTTGCCTCA

2

Figure 1

FIG1

Amino acid sequence alignment between ZmHsf01 and homologs from different species. DBD, the conserved DNA binding domain; HR-A/HR-B, two hydrophobic heptad repeats; NLS, nuclear localization signal; NES, nuclear export signal; AHA, aromatic, large hydrophobic and acidic amino residues. The accession numbers of proteins in NCBI as follows: SbHsfA2d from *Sorghum bicolor*, XP_002468465.1; DoHsfA2d from *Dichanthelium oligosanthes*, OEL38242.1; SiHsfA2d from *Setaria italica*, XP_004985605.1.

Fig. 1.

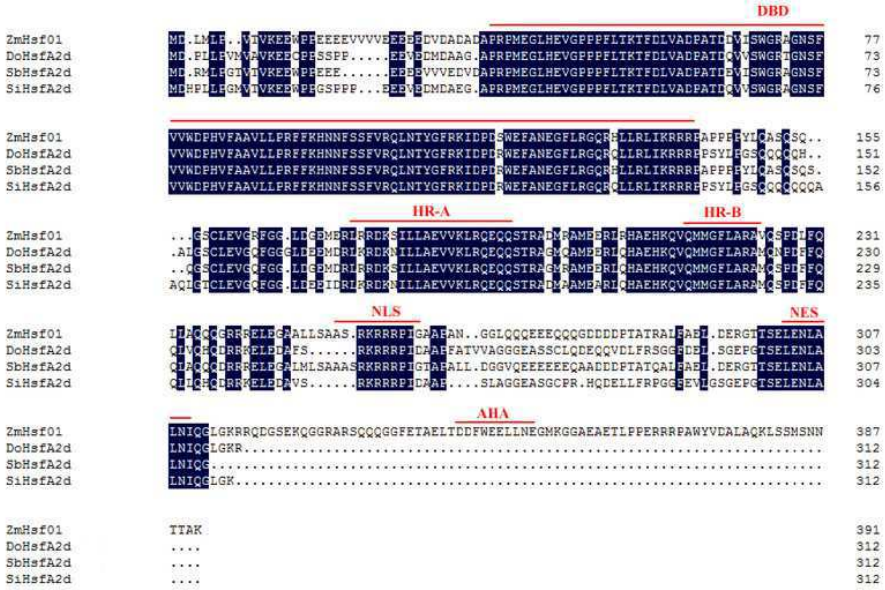

Figure 2

FIG2

Subcellular localization of ZmHsf01. GFP, green fluorescence of GFP; DAPI, blue fluorescence of DAPI; Bright, bright field; Merged, merged images.

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Fig. 2.

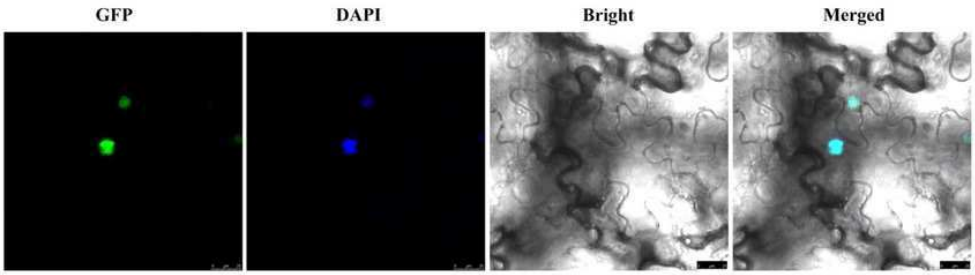

Figure 3

FIG3

Expression patterns of *ZmHsf01* in tissues and under heat stress. A, Expression levels of *ZmHsf01* in different tissues and organs under the normal growth conditions. B and C, Expression levels of *ZmHsf01* in leaves and roots of the maize seedlings after 42°C heat shock. There are three biological repeats for each sample and the data are mean± standard error.

Fig. 3.

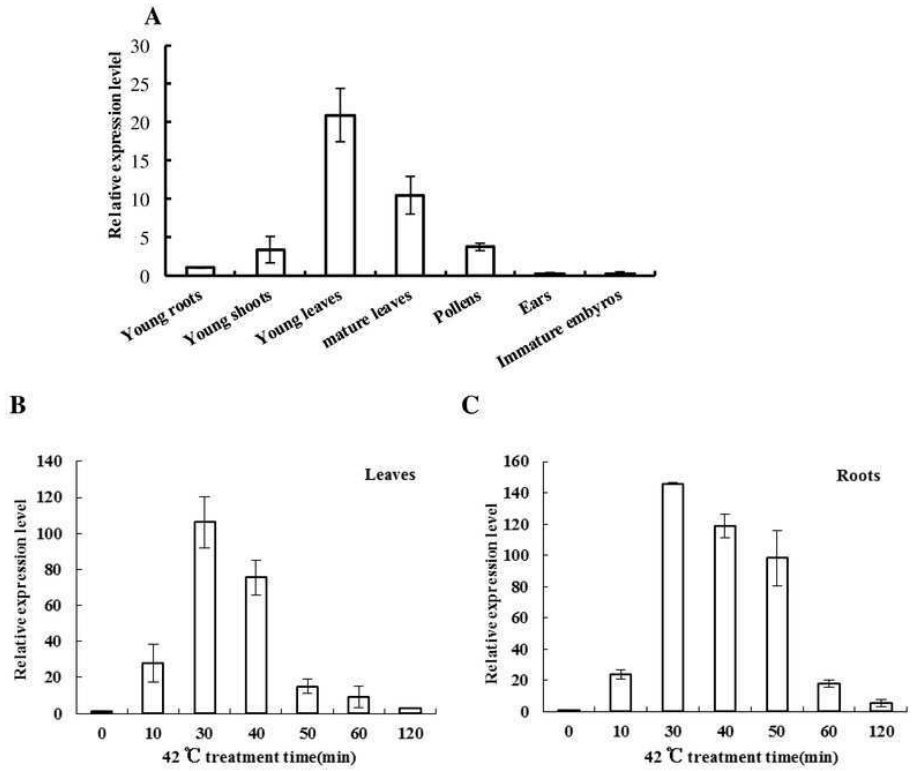

Figure 4

FIG4

Expression patterns of *ZmHsf01* under ABA and H_2O_2 treatment. A and B, Expression levels of *ZmHsf01* in leaves and roots of the maize seedlings after 200 μM ABA treatment. C and D, Expression levels of *ZmHsf01* in leaves and roots of maize seedlings after 10 mM H_2O_2 treatment. There are three biological repeats for each sample and the data are mean± standard error.

Fig. 4.

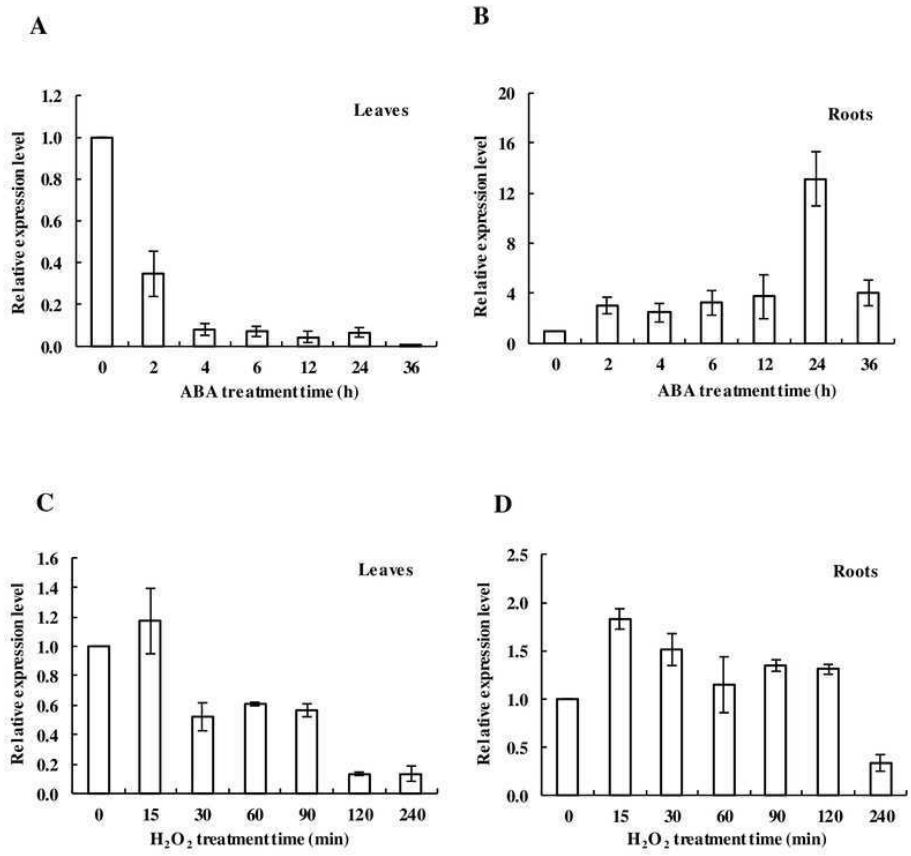

Figure 5

FIG5

Thermotolerance assays of yeast cell harboring pYES2 or pYES2-ZmHsf01 after 50°C heat shock. A, culture under normal conditions; B, culture under normal conditions after HS at 50°C for 45 min.

Fig. 5.

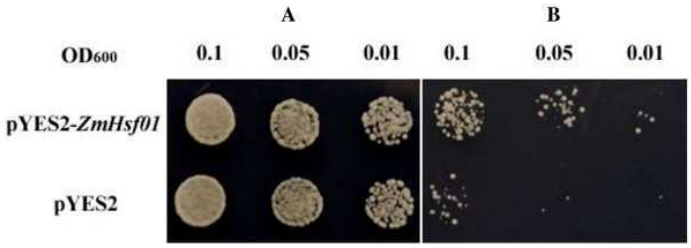

Figure 6

FIG6

Transcription activation analysis of ZmHsf01 in yeast. Yeast cells transformed with pGBKT7 vector was set as the Negative control, and yeast cells co-transformed with pGBKT7-53 and pGADT7-T vectors were set as the positive control. The group of ZmHsf01 was the yeast cells transformed with the fusion vector pGBKT7-ZmHsf01.

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Fig. 6.

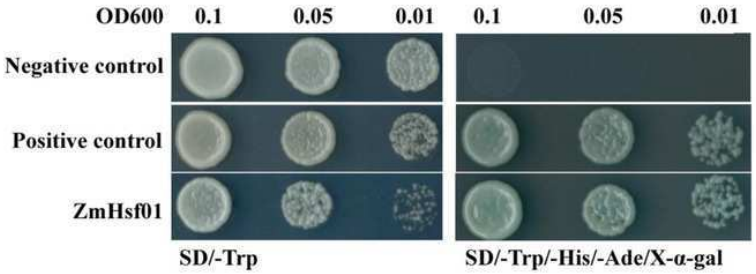

Figure 7

FIG7

The thermotolerance phenotypes of deletion mutant and three restoration lines of *Arabidopsis* seedlings were showed. A, Seedlings of WT, deletion mutant and three restoration lines (2_10, 3_12 and 4_11) growing on the MS plate under normal conditions were used as a control. B, Seedlings of all genotypes under HS at 44°C for 70 min and recovered at normal conditions for 8 days. C, Chlorophyll contents of seedlings under normal conditions and HS treatments. D, Semi qRT-PCR assay of the *ZmHsf01* transcript. E, Schematic representation of the HS regimes.

Fig. 7.

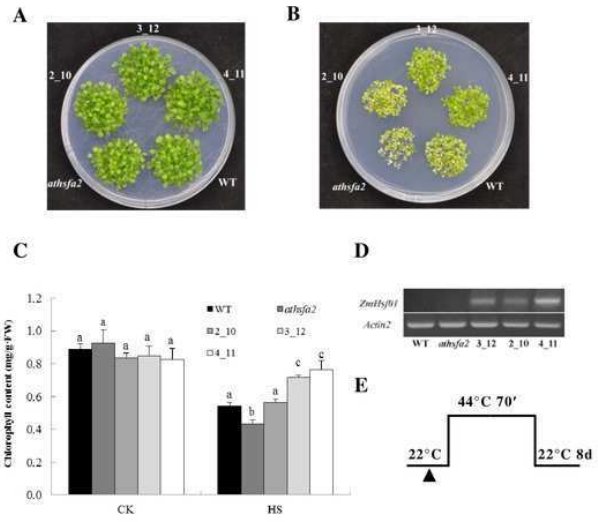

Figure 8

FIG8

Overexpressing *ZmHsf01* improved the basal thermotolerances in *Arabidopsis* seedlings. A, Seedlings of WT and three overexpressing lines (26_26, 28_4 and 36_7) growing on the MS plate under normal conditions used as a control of basal thermotolerance. B, Seedlings of all genotypes upon basal HS at 45°C for 50 min and recovered at normal conditions for 8 days. C, Chlorophyll contents of seedlings under the normal conditions and the basal HS treatments. D, Semi qRT-PCR analysis of the *ZmHsf01* transcript in different lines. The expression levels of *AtActin2* were used as the control. E, Schematic representation of the basal HS regimes.

Fig. 8.

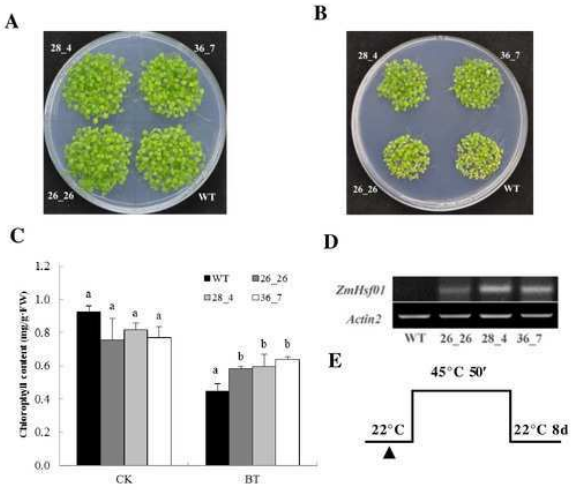

Figure 9

FIG9

Overexpressing *ZmHsf01* improved the acquired thermotolerances in *Arabidopsis* seedlings. A, Seedlings of WT and three overexpressing lines (26_26, 28_4 and 36_7) growing on the MS plate under normal conditions used as a control of acquired thermotolerance. B, 5-day-old seedlings of all genotypes under acquired HS at 37°C for 60 min and recovered at normal conditions for 2 days and treated at 46°C for 60 min and recovered at normal conditions for 8 days. C, Chlorophyll contents of seedlings under the normal conditions and the acquired HS treatments. D, Schematic representation of the acquired HS regimes.

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Fig. 9.

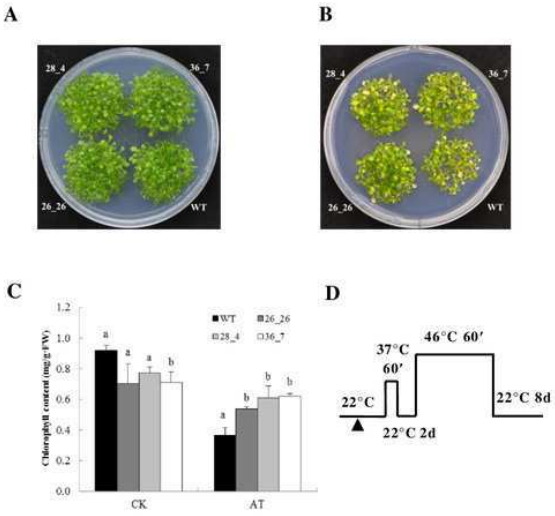

Figure 10

FIG10

The expression levels of heat-related Hsp genes in WT and *ZmHsf01* over-expressing lines 36_7 after HS treatment. qRT-PCR was performed about the *Arabidopsis* genes *AtHsp18.2*, *AtHsp21*, *AtERDJ3A*, *AtHsfa32*, *AtHsp70b*, *AtHsp70T*, *AtHsp90* and *AtHsp101* upon BT and AT heat stress. Set the expression level of WT samples was 1. The reference gene *Atactin8* (At1g49240) was used as an internal control to normalize the loading of different samples. Data were means ± SD from three biological experiments.

Fig. 10.

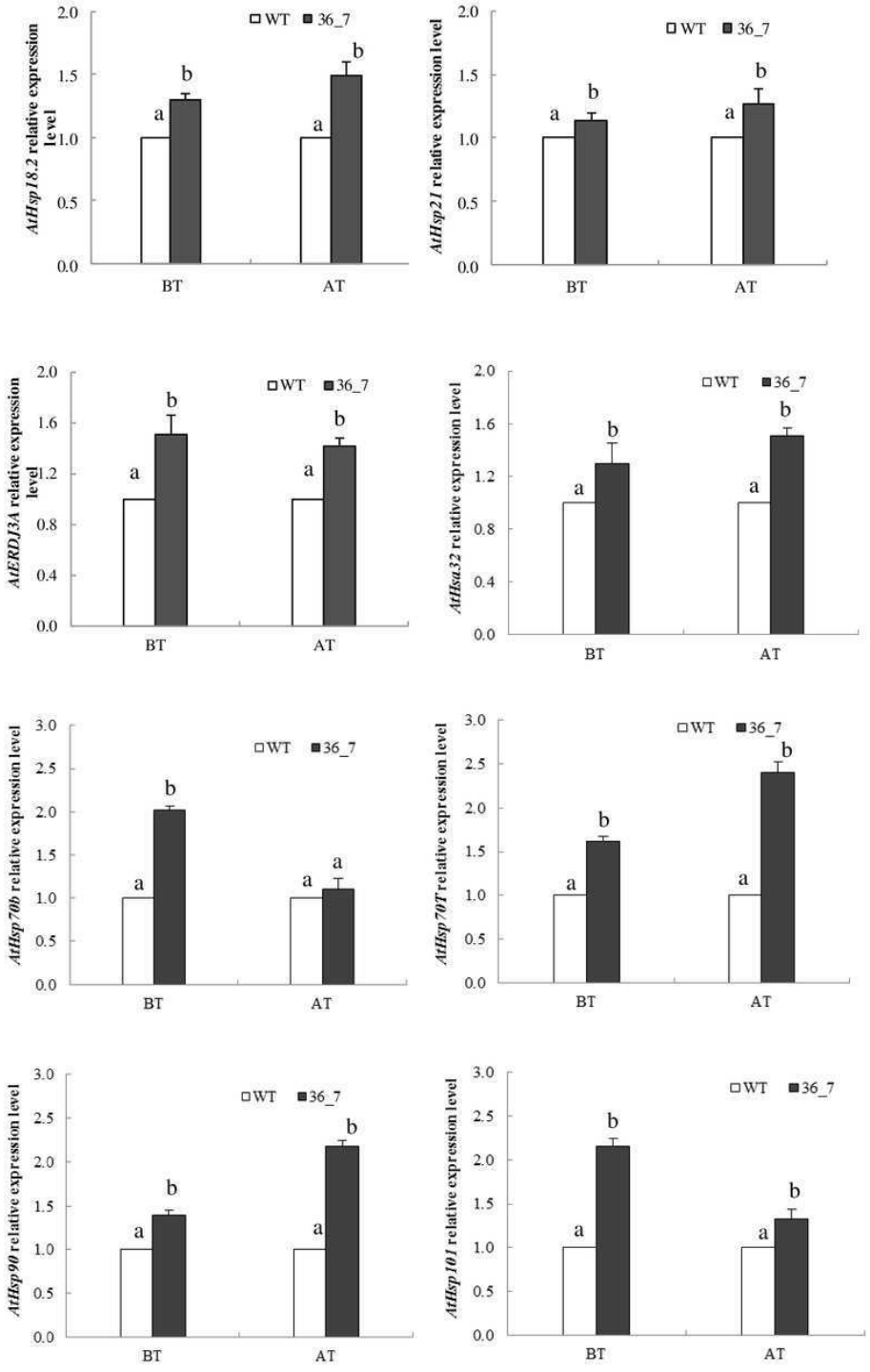

Figure 11

FIG11

The CDS and amino acid sequences of *ZmHsf01*

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Figure 11

```
1       ATGGACCTGATGCTGCCGGTGACGGTAAAGGAGGAGTGGCCTCCGGAGGAGGAGGAGGTG
1        M  D  L  M  L  P  V  T  V  K  E  E  W  P  P  E  E  E  E  V

61      GTGGTGGTGGAGGAGGAGGAGGAGGACGTGGACGCGGACGCGGACGCTCCGCGGCCGATG
21       V  V  V  E  E  E  E  E  D  V  D  A  D  A  D  A  P  R  P  M

121     GAGGGGCTGCACGAGGTCGGGCCACCGCCGTTCCTGACCAAGACGTTCGACCTGGTGGCC
41       E  G  L  H  E  V  G  P  P  P  F  L  T  K  T  F  D  L  V  A

181     GACCCGGCCACCGACGACGTCATCTCCTGGGGCCGCGCCGGCAACAGCTTCGTGGTCTGG
61       D  P  A  T  D  D  V  I  S  W  G  R  A  G  N  S  F  V  V  W

241     GATCCCCACGTGTTCGCCGCCGTGCTGCTCCCCAGGTTCTTTAAGCACAACAACTTCTCC
81       D  P  H  V  F  A  A  V  L  L  P  R  F  F  K  H  N  N  F  S

301     AGCTTTGTCCGCCAGCTGAACACCTATGGCTTCAGAAAGATCGACCCGGACAGCTGGGAG
101      S  F  V  R  Q  L  N  T  Y  G  F  R  K  I  D  P  D  S  W  E

361     TTCGCGAACGAAGGATTCCTGAGGGGCCAGAGGCATCTTCTCCGGCTGATCAAGCGTCGG
121      F  A  N  E  G  F  L  R  G  Q  R  H  L  L  R  L  I  K  R  R

421     AGGCCGGCGCCGCCGCCGCCGTACCTCCAGGCGTCGCAGTCGCAGGGGTCGTGCCTGGAG
141      R  P  A  P  P  P  P  Y  L  Q  A  S  Q  S  Q  G  S  C  L  E

481     GTGGGCCGGTTCGGGGGGCTGGACGGAGAGATGGAGCGGCTGAGGCGCGACAAAAGCATC
161      V  G  R  F  G  G  L  D  G  E  M  E  R  L  R  R  D  K  S  I

541     CTGCTGGCGGAGGTGGTGAAGCTGCGGCAGGAGCAGCAGAGCACGCGGGCGGACATGCGG
181      L  L  A  E  V  V  K  L  R  Q  E  Q  Q  S  T  R  A  D  M  R

601     GCCATGGAGGAGCGGCTGCGGCACGCGGAGCACAAGCAGGTGCAGATGATGGGGTTCCTG
201      A  M  E  E  R  L  R  H  A  E  H  K  Q  V  Q  M  M  G  F  L

661     GCGCGGGCGGTGCAGAGCCCGGACTTGTTCCAGCTGCTGGCCCAGCAGCAGGGCAGGCGG
221      A  R  A  V  Q  S  P  D  L  F  Q  L  L  A  Q  Q  Q  G  R  R

721     AGGGAGCTGGAGGGCGCGGCGCTGCTCTCCGCCGCCTCCCGGAAGCGGAGGCGGCCCATC
241      R  E  L  E  G  A  A  L  L  S  A  A  S  R  K  R  R  R  P  I

781     GGCGCCGCGCCGGCCAACGGCGGCTTGCAGCAGCAGGAGGAGGAGCAGCAGCAGGGCGAC
261      G  A  A  P  A  N  G  G  L  Q  Q  Q  E  E  E  Q  Q  Q  G  D

841     GACGACGACCCCACCGCCACGCGGGCGCTGTTCGCGGAGCTGGACGAGCGAGGGACCACG
281      D  D  D  P  T  A  T  R  A  L  F  A  E  L  D  E  R  G  T  T

901     TCGGAGCTGGAGAACCTGGCGCTCAACATCCAGGGGCTCGGCAAGCGCAGGCAGGACGGG
301      S  E  L  E  N  L  A  L  N  I  Q  G  L  G  K  R  R  Q  D  G

961     AGCGAGAAGCAGGGTGGCCGCGCGCGGAGCCAGCAGCAGGGCGGGTTCGAGACGGCGGAG
321      S  E  K  Q  G  G  R  A  R  S  Q  Q  Q  G  G  F  E  T  A  E

1021    CTCACCGACGACTTTTGGGAGGAGCTGCTGAACGAAGGGATGAAGGGCGGTGCCGAGGCT
341      L  T  D  D  F  W  E  E  L  L  N  E  G  M  K  G  G  A  E  A

1081    GAGACGCTGCCGCCGGAGAGGAGACGACCGGCTTGGTACGTCGACGCGCTGGCGCAGAAG
361      E  T  L  P  P  E  R  R  R  P  A  W  Y  V  D  A  L  A  Q  K

1141    TTGAGCTCCATGAGCAATAACACCACGGCGAAGTAG
381      L  S  S  M  S  N  N  T  T  A  K  *
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