A Novel Heat Shock Transcription Factor (ZmHsf08) Negatively Regulates Salt and Drought Stress Responses in Maize

Jing Wang, Li Chen, Yun Long, Weina Si *, Beijiu Cheng * and Haiyang Jiang *

Abstract: Heat shock transcription factors (HSFs) play important roles in plant growth, development, and stress responses. However, the function of these transcription factors in abiotic stress responses in maize (Zea mays) remains largely unknown. In this study, we characterized a novel HSF transcription factor gene, ZmHsf08, from maize. ZmHsf08 was highly homologous to SbHsfB1, BdHsfB1, and OsHsfB1, and has no transcriptional activation activity. The expression profiles demonstrated that ZmHsf08 was differentially expressed in various organs of maize and was induced by salt, drought, and abscisic acid (ABA) treatment. Moreover, the overexpression of ZmHsf08 in maize resulted in enhanced sensitivity to salt and drought stresses, displaying lower survival rates, higher reactive oxygen species (ROS) levels, and increased malondialdehyde (MDA) contents compared with wild-type (WT) plants. Furthermore, RT-qPCR analyses revealed that ZmHsf08 negatively regulates a number of stress/ABA-responsive genes under salt and drought stress conditions. Collectively, these results indicate that ZmHsf08 plays a negative role in response to salt and drought stresses in maize.

Keywords: maize; ZmHsf08; transcription factor; salt stress; drought stress

1. Introduction

Plants are prone to encountering environmental stresses during all aspects of plant growth and development, resulting in devastating damage to the survival and production of the plants. Among these environmental factors, drought and salt stress are the most important challenges limiting crop growth and grain yield [1,2]. On the one hand, salt and drought stress cause dehydration, which in its turn results in ionic and osmotic stress, destroying cellular homeostasis; on the other hand, salt and drought stress trigger oxidative stress, which produces reactive oxygen species (ROS) and provokes damages to membrane lipids, proteins, and DNA, resulting in plant wilting and even plant death [3,4].

Plants have developed sophisticated tolerance mechanisms to survive drought and salt stress. Many key factors are involved in drought and salt stress responses, including kinases [5,6], ion transporters [7,8], ROS [3,9], abscisic acid (ABA) [10,11], and transcription factors (TFs) [12,13]. A wealth of studies has demonstrated that many TF families, such as dehydration-responsive element-binding protein (DREB), WRKY, v-myb avian myeloblastosis viral oncogene homolog (MYB), basic leucine zipper (bZIP), and HSF, play a pivotal role in the transcriptional regulation of genes during abiotic stress responses [14–18]. TFs activate the transcription of stress-responsive genes by binding to their promoters, resulting in enhanced stress tolerance in plants [19]. HSFs are an important gene family of TFs, which are involved in plant growth, development, and stress response. Since the first plant HSF was isolated from tomato in the 1990s, a large number of plant HSFs have been identified and cloned [20]. For example, there are 21 HSFs in Arabidopsis that have been identified [21], 38 in soybean [22], 31 in maize [23], and 25 in rice [24]. Moreover, the maximum number of HSF families in plants was identified in wheat: at least 56 HSFs [25].
Generally, plant HSFs have conserved functional domains, including the N-terminal DNA binding domain (DBD), oligomerization domain (OD or HR-A/B), nuclear localization signal (NLS), and nuclear export signal (NES) [21,26]. In addition, plant HSFs are classified into three classes (HSFA, HSFB, and HSFC) based on the sequence characteristics of the OD domain. Interestingly, the HSFA have short activator motifs (AHA motifs) located in their C-terminal domains, and they function as transcription activators, while class B and C HSFs lack the activation domain [18,21]. For the past few years, many studies reported the critical roles of HSFA in plant response to various abiotic stresses. It has been determined in tomato and Arabidopsis that HsfA1s function as the master regulators in plant heat stress response (HSR) and are prerequisite for the activation of transcriptional networks [27–29]. The overexpression of AtHsfA1b enhanced the water productivity of transgenic plants, resulting in increased resistance to drought [30]. HsfA2s, the direct target genes of HsfA1s, were reported as key regulators in plant HSR and other abiotic stress responses [18,31]. Overexpression of HsfA2 enhanced thermotolerance of transgenic plants [32–35].

In contrast to HSFA, there was no evidence that HSFBs functioned as transcription activators on their own. In tomato, HsfB1 has been reported to act as a coactivator cooperating with HsfA1a or forming a functional triad with HsfA1a and HsfA2, regulating the expression of heat stress (HS)-responsive genes during plant HS responses [21,43,44]. Moreover, HsfB1 also cooperates with other transcriptional regulators to control target gene expression in abiotic stress responses. For example, ectopic expression of chickpea HSFB2 in Arabidopsis improved the drought tolerance of transgenic plants by increasing the expression of stress-responsive genes such as RD22, RD26, and RD29A [45]. Decreased drought and salt tolerances were observed in rice plants over-expressing OsHsfB2b [46]. A study of HSF interaction showed that HsfB1 and HsfB2b have the capability to form complexes in vivo and might be involved in complex regulatory networks of signaling processes and stress responses [47].

Recently, 31 ZmHSFs were identified in maize and divided into 16 subclass A, 10 subclass B, and 5 subclass C gene members [48]. Ectopic expression of maize ZmHsf05 (members of subclass A2) in Arabidopsis improve the heat tolerance of transgenic plants [49,50]. Furthermore, overexpression of a member of subclass A1 (ZmHsf06) in Arabidopsis enhances its salt stress tolerance [51]. However, the roles of ZmHSFs in plants’ salt and drought tolerance are largely unknown. In this study, we cloned ZmHsf08, a member of subclass B1, and analyzed its function in salt and drought stress responses. We found that ZmHsf08 was induced by NaCl and PEG treatments and localized to the nucleus. Notably, overexpression of ZmHsf08 in maize significantly enhanced sensitivity to both salt and drought stresses. Our results suggest that ZmHsf08 plays a negative role in regulation of salt and drought responses and serves as a gene resource for the improvement of maize against abiotic stresses in the future.

2. Results

2.1. Gene Isolation and Sequence Analysis of ZmHsf08

The coding sequence of ZmHsf08 was cloned from seedlings of the maize inbred B73. Sanger sequencing results showed that the coding sequence (CDS) of ZmHsf08 was 897 bp in length (Figure 1A). ZmHsf08 harbored 298 amino acids, including a conserved N-terminal DNA binding domain (DBD), an oligomerization domain (OD), a nuclear export signal (NES), and a nuclear location signal (NLS) (Figure 1B). Further multiple sequence alignments and homologous analysis revealed that ZmHsf08 exhibited high sequence similarity with other known HsfB1 proteins, homologous to the B1 subclass of ZmHsfs (Figure 1B,C). Most importantly, ZmHsf08 contained the core B3 repression domain
(BRD, K/SV/KLGVL/LTD), which is conserved in HsfB1 orthologs of five plant species (Figure 1B).

2.2. Subcellular Localization of ZmHsf08

To further analyze the subcellular localization of ZmHsf08, we constructed fusion proteins containing C-terminal and N-terminal green fluorescent protein (GFP), respectively (Figure 2A). The recombinant protein was transiently expressed in maize protoplasts. The p1305-GFP and PMDC43, used as the control vectors, were expressed in the nucleus and cytoplasm. Both ZmHsf08-GFP and GFP-ZmHsf08 fluorescence signals were only concentrated in the nucleus, indicating that ZmHsf08 was a nucleus-localized protein (Figure 2B).

2.3. Tissue-Specific and Stress-Induced Expression Profiles of ZmHsf08

To investigate the tissue-specific expression of ZmHsf08 in maize, we investigated the expression profiles of ZmHsf08 in six tissues (roots, stem, leaves, corn-silk, tassels, and bract) by RT-qPCR (Figure 3A). The results indicated that the ZmHsf08 constitutively expressed in all surveyed six tissues under the non-stress condition.

To explore the putative function of ZmHsf08 in the responses to environmental stresses, we also assessed the expression profiles of ZmHsf08 by RT-qPCR under different stress treatments, including NaCl, PEG, and exogenous ABA. As shown in Figure 3B, the expression level of ZmHsf08 was downregulated to different degrees after exogenous ABA treatment. Under drought stress, the ZmHsf08 transcript level was decreased after 0 h (Figure 3C). Similarly, ZmHsf08 expression was down-regulated following NaCl treatment (Figure 3D). These expression patterns indicated that ZmHsf08 may function in response to abiotic stresses through the ABA-mediated pathway.
2.4. Overexpression of ZmHsf08 Reduces Salt and Drought Tolerance in Maize

It is suggested that ZmHsf08 may play an important role in salt and drought stresses according to the expression pattern results. To investigate the function of ZmHsf08 in maize, we generated ZmHsf08-overexpressing plants (OE8-1 and OE8-2). The transcript level of ZmHsf08 in the transgenic lines were examined by RT-qPCR. As shown in Figure 4B, significantly increased ZmHsf08 expression levels were observed in OE8-1 and OE8-2 plants.
Figure 3. Expression patterns of ZmHsf08. (A) RT-qPCR analysis of ZmHsf08 expression in six tissues (roots, stem, leaves, cornsilk, tassels and bract). (B–D) Expression analysis of ZmHsf08 under ABA, PEG, and NaCl treatments. The three-leaf stage seedlings were treated with ABA, PEG, and NaCl, and the expression levels of ZmHsf08 were detected by RT-qPCR. Bars represent means ± SD (n = 3 repeats).

To determine whether ZmHsf08 is involved in plant salt stress tolerance, we performed a salt stress tolerance assay in transgenic lines and wild-type (WT) plants. Before stress treatments, there was no obvious difference in phenotype between the transgenic lines and WT plants. After being subjected to 200 mM NaCl treatment for 2 weeks, the most leaves of WT plants remained green, whereas the leaves of OE8-1 and OE8-2 plants were severely wilted (Figure 4A). Meanwhile, the survival rates of OE8-1 and OE8-2 plants were significantly decreased compared with the WT (Figure 4C). In addition, ZmHsf08 expression in the transgenic lines OE8-1 and OE8-2 was downregulated under stress conditions (Supplementary Figure S1). We also analyzed the physiological responses to salt stress in the WT plants and two transgenic maize lines. DAB staining showed that significantly more reddish-brown precipitate accumulated in the leaves of transgenic plants than that of WT under salt stress treatment, indicating higher H$_2$O$_2$ levels in transgenic lines (Figure 4D). Additionally, the contents of MDA were measured in WT and transgenic maize seedlings before and after salt stress treatment. As shown in Figure 4E, the accumulation of MDA in transgenic lines increased significantly compared with that in WT plants under both control and salt conditions. These results indicated that the overexpression of ZmHsf08 in maize enhances sensitivity to salt stress.
To confirm the role of ZmHsf08 in maize drought response, we performed a drought stress experiment in soil by withholding water. After drought for 2 weeks, the ZmHsf08-overexpressing plants exhibited more severe wilt phenotype than WT plants, and almost all their leaves were severely dehydrated and curled (Figure 5A). To examine whether the dehydration phenotype could be recovered, we further performed a re-watering experiment. After re-watering for 3 days, the WT plants had grown fresh new leaves, and about 75% of them showed growth recovery; however, only about 20% of the over-expressing plants could recover growth after re-watering (Figure 5A,B). In consistency with the phenotype, the leaves of ZmHsf08 transgenic plants accumulated higher H$_2$O$_2$ levels and had higher MDA content compared to WT plants, indicating that more damage was induced in transgenic plants (Figure 5C,D). Additionally, we performed a water loss assay of detached leaves using WT and ZmHsf08-overexpressing plants. The rate of water loss from the transgenic plants was faster than that from the WT plants, suggesting that overexpression of ZmHsf08 promoted water loss of leaves (Figure 5E). These findings demonstrated that overexpression of ZmHsf08 results in decreased drought stress tolerance in maize.
2.5. Expression of Stress-Responsive Genes Were Altered in ZmHsf08-Overexpressing Plants

To further understand the regulation mechanisms of ZmHsf08 in salt and drought stress responses, we assayed the expression levels of several known stress-responsive genes in WT and transgenic plants under control and stress conditions, such as ZmDREB2A (dehydration-responsive element-binding protein 2A), ZmNCED (9-cis-epoxycarotenoid dioxygenase), ZmERD1 (early responsive to dehydration 1), ZmRD20 (RESPONSIVE TO DESICATION 20), and ZmRAB18 (RESPONSIVE TO ABA 18). The RD20 gene is often used as a stress marker gene and played an important role in plant drought tolerance [52,53]. Overexpression of LEA (LATE EMBROGENESIS ABUNDANT PROTEIN), which encodes a highly hydrophilic protein, enhances plant tolerance to drought stress [54,55]. Constitutive expression of a vacuolar Na⁺/H⁺ antiporter gene, AtNHX3 (SODIUM/HYDROGEN EXCHANGER3), in sugar beet (Beta vulgaris) improved high salinity tolerance in transgenic plants [56]. NCED and ZEP (zeaxanthin epoxidase) are key enzymes for ABA biosynthesis, and overexpression of the two genes enhanced abiotic tolerance in transgenic plants [57,58].
Before salt stress treatment, the expression levels of the analyzed stress-responsive genes, including \textit{ZmDREB2A}, \textit{ZmABF2}(ABRE-BINDING FACTOR 2), \textit{ZmZEP}, and \textit{ZmNHX3}, were suppressed in transgenic plants compared with WT plants, while \textit{ZmNCED}, \textit{ZmERD1}, \textit{ZmDR20}, and \textit{ZmRAB18} expression levels in transgenic plants were higher than in WT plants. However, the expression levels of most analyzed genes (\textit{ZmDREB2A}, \textit{ZmERD1}, \textit{ZmNHX3}, \textit{ZmRAB18}, and \textit{ZmLEA2}) were obviously decreased in transgenic plants compared with that in WT plants under salt stress (Figure 6). There was a similar pattern of repressed expression of stress-responsive genes in transgenic plants following drought stress treatment (Figure 7). The expression levels of the analyzed genes (except \textit{ZmDREB2A}) were higher in transgenic plants compared with WT plants under control conditions to some extent; however, under drought stress, the expression levels of these genes in transgenic plants also dramatically decreased compared with those in WT plants (Figure 7). These results suggested that \textit{ZmHsf08} plays a negative role in regulating these stress/ABA-responsive genes under salt and drought stresses.

![Figure 6](image_url)

**Figure 6.** Expression patterns of stress-responsive genes in WT and \textit{ZmHsf08}-overexpressing plants under normal condition and NaCl treatment. Three-leaf stage maize seedlings were treated with 200 mM NaCl for 7 days. The expression levels of stress-responsive genes during salt stress were analyzed by RT-qPCR. Values are means ± SD. Bars represent means ± SD (\(n = 3\) repeats). Significant differences (Student’s \(t\) test): *, \(p < 0.05\), **, \(p < 0.01\).
Figure 7. Expression levels of stress-responsive genes in WT and ZmHsf08-overexpressing plants under normal condition and drought treatment. Three-leaf stage maize seedlings were withheld water for 7 days. The expression levels of stress-responsive genes during drought stress were analyzed by RT-qPCR. Values are means ± SD. Bars represent means ± SD (n = 3 repeats). Significant differences (Student’s t test): *, p < 0.05, **, p < 0.01.

2.6. Homomeric Interaction of ZmHsf08

Since ZmHsf08 is an HSFB protein and has a core BRD sequence, we speculated that it would have no transactivation activity. To confirm this hypothesis, we performed a yeast transactivation assay. The full-length ZmHsf08 CDS was cloned into the pGBK-T7 vector, and the recombinant construct was transformed into yeast AH109 (Figure 8A). As expected, the results showed that ZmHsf08 has no transcriptional activity in yeast (Figure 8B).
A previous study suggested that HsfBs (HsfB1 and HsfB2b) can form homologous interactions, and are involved in the regulatory networks of stress responses through forming complexes with other proteins [47]. To determine if ZmHsf08 forms homodimers, we performed a bimolecular fluorescence complementation (BiFC) assay. It was observed that ZmHsf08 interacted with itself; in addition, the homodimers of ZmHsf08 were located in the nucleus (Figure 8C). The interactions were further examined by a yeast two-hybrid (Y2H) assay. The full-length ZmHsf08 CDS was cloned into the pGAD-T7 and pGBK-T7 vectors to generate the ZmHsf08-AD and ZmHsf08-BD proteins, respectively (Figure 8A). The ZmHsf08-AD was cotransformed into yeast with ZmHsf08-BD; as we expected, the Y2H assay also showed that ZmHsf08 interacts with itself (Figure 8D).

3. Discussion

Abiotic stress, including salt and drought stress, has adverse effects on the growth and yield of crop plants. Maize, an important crop for livestock and humans, is threatened by salt and drought stress. It is known that transcription factors (TFs) play important roles in mediating abiotic stress responses by regulating the expression of stress-responsive genes [13,19]. Among these TFs, HSF TFs are an important family of regulatory proteins with diversified functions. In recent years, the functions of HSF genes involved in diverse abiotic stress responses have been reported in soybean, tomato, Arabidopsis, wheat, and rice [22,27,29,39,41,46]. In this study, we identified a class B HSF gene, ZmHsf08, from maize and analyzed its functions in response to salt and drought stress.

ZmHsf08 is a typical HSF transcription factor with conserved domains, including a DNA binding domain (DBD), an intermediate OD (HR-A/B) region, one nuclear localization signal (NLS), and one nuclear export signal (NES). The sequence analysis revealed that ZmHsf08 had a close homologous relationship with HsfB1 proteins from other species (Figure 1B), suggesting that ZmHsf08 belongs to the HsfB1 subgroup. We found that ZmHsf08 contained the NLS sequence (KKRR), which suggests a putative nuclear targeting domain; Protein–protein interactions were examined by yeast cell growth on the SD/-Leu/-Trp/-His/-Ade plate.

**Figure 8.** Homodimerization of ZmHsf08. (A) Schematic representation of the structures of ZmHsf08-AD and ZmHsf08-BD. (B) The transcriptional activation activity assay in yeast cells. (C) Bimolecular fluorescence complementation (BiFC) assay of the homodimerization of ZmHsf08 in maize protoplasts. Different combinations of the nYFP and cYFP fusion constructs were cotransformed into maize protoplasts, and the fluorescence signals were examined with a confocal microscope. (D) Yeast two-hybrid (Y2H) analysis of the self-interaction of ZmHsf08. AD, GAL4 activation domain; BD, GAL4 DNA binding domain; Protein–protein interactions were examined by yeast cell growth on the SD/-Leu/-Trp/-His/-Ade plate.
of ZmHsf08, and the subcellular localization assay determined that ZmHsf08 is a nuclear localized protein (Figure 2). In addition, ZmHsf08 had no transcriptional activity in yeast as a result of the conserved B3 repression domain (BRD) at the C-terminus of ZmHsf08 sequence (Figure 1B), which is consistent with the features of class B HSFs [52,53]. These results demonstrated that ZmHsf08 is a novel HsfB gene in maize.

Many studies have reported that the Hsf genes are involved in salt and drought stress responses. For example, the Arabidopsis plants overexpressing AtHsfA6a and AtHsfA7b exhibited enhanced tolerance against salt and drought stresses [42,54]. The ectopic expression of tomato HSEA3 in Arabidopsis increased salt hypersensitivity in transgenic plants [37]. The overexpression of OsHsfA7 in rice demonstrated that OsHsfA7 acted as a positive regulator in salt and drought tolerance [41]. On the contrary, OsHsfB2b functioned as a negative regulator in response to salt and drought stresses in rice [46]. However, the biological functions of most HsfB proteins in maize remains largely elusive. Interestingly, the expression profiling revealed that ZmHsf08 expression was down-regulated after treatment with PEG, NaCl, and ABA (Figure 2B–D), suggesting that ZmHsf08 may play a role in abiotic-stress responses in maize.

Our study demonstrated that ZmHsf08 is involved in salt and drought stress response. Overexpression of ZmHsf08 in maize increased sensitivity to salt and drought stress, with worse growth performance and a significantly decreased survival rate under stress treatments (Figures 4 and 5). Salt and drought stress impose osmotic stress, which leads to the excess generation of reactive oxygen species (ROS), resulting in damage to plant cellular physiology and biochemistry [55,56]. H$_2$O$_2$ and O$^-$ are two ROS indices that are involved in abiotic stress signaling. Therefore, the accumulation of H$_2$O$_2$ was revealed using diaminobenzidine (DAB) staining under normal and stress conditions using maize leaves. In our study, the accumulation of H$_2$O$_2$ contents was obviously increased in ZmHsf08 transgenic plants compared to WT plants under salt and drought stress conditions (Figures 4D and 5C), implying that the overexpressing ZmHsf08 in maize apparently aggravated oxidative damage by generating high ROS levels during stress treatments. This result was further confirmed by malonaldehyde (MDA) content, which is an indicator of lipid peroxidation and cell membrane damage. The MDA level in the transgenic plants was significantly higher than in WT plants in response to drought and salt stress treatments (Figures 4E and 5D), indicating that ZmHsf08 increases cell membrane injury under stress conditions. Therefore, ZmHsf08 may play a negative role in plant abiotic stress responses.

To adapt to various abiotic stresses, especially salt and drought stresses, plants develop different response strategies, including modulation of the expression of stress-responsive genes [4,12]. In this study, we found that the expression levels of stress-responsive genes including ZmERD1, ZmRD20, ZmRAB18, ZmNCED, ZmNHX3, ZmZEP, and ZmA-LEA were higher in transgenic plants than those in WT plants under control conditions (Figures 6 and 7). However, under drought stress, the expression levels of these stress-responsive genes were markedly decreased in transgenic plants compared with those in WT plants (Figure 7). Similarly, the stress-responsive genes ZmERD1, ZmRD20, ZmRAB18, ZmNCED, and ZmLEA also showed decreased transcript levels in transgenic plants compared with WT plants under salt conditions (Figure 6). These results indicated that ZmHsf08 negatively regulates salt and drought stress responses, perhaps through the repressed expression of stress-responsive genes. It was reported that HsfBs have the capability to form homodimers in vivo and may be involved in complex regulatory networks of signaling processes and stress responses. The BiFC and Y2H assays indicated that ZmHsf08 forms homodimer in vivo. Therefore, we speculate that the ZmHsf08 homodimers alone or interacting with other TFs to regulate the expression of stress-responsive genes in stress responses. This hypothesis needs to be studied in depth in the future.

In conclusion, we cloned and identified a novel HsfB member, ZmHsf08, from maize. Overexpression of ZmHsf08 in maize resulted in increased sensitivity to salt and drought stresses, which were associated with higher ROS levels and higher MDA contents. Furthermore, RT-qPCR analyses indicated that ZmHsf08 negatively regulates the expression of...
stress/ABA-responsive genes in response to salt and drought stresses (Figures 6 and 7). Our findings provide new information to better understand the function of ZmHsf08 in plant abiotic stress responses.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

The maize inbred line B73 (seeds stored in our laboratory) was used for ZmHsf08 gene cloning and expression analysis. The maize inbred line KN5585 (WT) and transgenic ZmHsf08 plants were provided by Weimi Biotech Co., Ltd. and used for salt and drought stress experiments. Maize seeds were surface sterilized and germinated at 28 °C for 3 days (dark). Then, seedlings with primary root were sown in pots with soil and grown in a greenhouse (14 h/10 h of light/dark; 30 °C/25 °C of day/night) until they reached the three-leaf stage.

4.2. Sequence Alignment and Phylogenetic Analysis

Sequences of ZmHsf08 and other HsfB members from different plant species (Oryza sativa, Sorghum bicolor, Brachypodium distachyon, and Arabidopsis thaliana) were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) (accessed on 6 May 2021). The amino acid sequences of different HsfB1 proteins were aligned by ClustalX software. The conserved motifs of these proteins, including DNA binding domain (DBD), oligomerization domain (OD), nuclear export signal (NES), and nuclear location signal (NLS), were defined by SMART (http://smart.embl-heidelberg.de/) (accessed on 8 January 2021).

A phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0. The neighbor-joining method was performed with 1000 bootstrap replicates.

4.3. Expression Analysis of ZmHsf08

The roots, stems, and leaves form seedlings of B73 at the three-leaf stage, and immature cornsilk, tassels, and bract from B73 plants in the V13 stage were sampled for tissue-specific expression analysis. For stress treatments, the B73 seedlings at the three-leaf stage were either watered with 20% (w/v) PEG6000, watered with 200 mM NaCl, or watered with 0.1 mM ABA. The leaves of seedlings under each treatment were collected at the designated time points (0, 1, 3, 6, 12, and 24 h), and the samples were immediately frozen in liquid nitrogen. Total RNA was extracted using Total RNA Extraction Reagent (Vazyme), and cDNA was synthesized using HiScript III RT SuperMix (Vazyme). RT-qPCR was performed using AceQ qPCR SYBR Green Master Mix (Vazyme) on the Thermo Scientific PikoReal 96 RT-PCR instrument. ZmGAPDH was used as internal control for maize. All the primers used for RT-qPCR are listed in Supplementary Table S2.

4.4. Subcellular Localization

The coding sequences (CDS) of ZmHsf08 without or with a stop codon were cloned by PCR and then constructed into p1305-GFP and PMDC43 vectors (stored in our laboratory), respectively, for subcellular localization. The primers are shown in Supplementary Table S1. The recombined vectors ZmHsf08-GFP and GFP-ZmHsf08, or the empty vectors p1305-GFP and PMDC43, were transformed into the maize protoplasts according to the previous description [59,60]. The RFP vector, a marker for nucleus localization, was cotransformed with these constructs into maize protoplasts. After being cultured in multi-well plates for 18–24 h in the dark, the protoplasts were observed under a confocal laser scanning microscope (LSM710; Zeiss).

4.5. Transactivation Activity and Two-Hybrid Assays in Yeast

The full-length CDS of ZmHsf08 was cloned into a pGAD-T7 or pGBK-T7 vector, respectively. For the transactivation activity assay, ZmHsf08-BD reconstructed plasmid was transformed into the yeast strain AH109. In addition, the transcriptional activation activity was examined by spot assay and X-gal staining. For the two-hybrid assays, recombined vec-
tors were transformed in pairs in the yeast Y2HGold cells. The interaction between the two proteins was examined by spot assay. The primers are listed in Supplementary Table S1.

4.6. BiFC Assay

The coding sequence of ZmHsf08 without a stop codon was cloned into pUC-SPYCE and pUC-SPYNE vectors, respectively, generating Hsf08-cYFP and Hsf08-nYFP constructs. The primers used in the assay are listed in Supplementary Table S1. Different combinations of cYFP and nYFP constructs were cotransformed with RFP into maize protoplasts. The isolation and transformation of maize protoplasts were performed according to the previous protocol [59,60]. The protoplasts were cultured for 18–24 h in the dark, and then were observed under a confocal laser scanning microscope (LSM710; Zeiss).

4.7. Salt and Drought Stress Experiment

For the salt stress tolerance test, the three-leaf stage maize seedlings (the transgenic lines and the WT plants) were irrigated with 200 mM NaCl solution for two weeks. Then, the phenotypes and survival rates of plants were measured.

For drought stress tolerance test, the WT and transgenic plants seedlings at three-leaf stage did not receive watering for 14 days until the plants withered. After drought for 14 days and re-watering for 3 days, the recovered conditions of the WT and transgenic lines were recorded. The survival rates of plants were measured after re-watering for 3 days under normal conditions.

4.8. DAB Staining and MDA Content Measurement

After salt and drought treatment for 10 days, leaves of the WT and transgenic plants were stained with diaminobenzidine (DAB) to detect the accumulation of H₂O₂. Leaves of the same location of maize seedlings were collected and immersed in DAB solution (1 mg/mL, pH 3.8). All the samples were incubated overnight at room temperature in darkness. The leaves were then bleached by boiling in bleach solution (ethanol: acetic acid: glycerol, 3:1:1, v/v/v) for 20 min to remove the chlorophyll before imaging.

The three-leaf stage seedlings of WT and transgenic plants were treated with 200 mM NaCl or watering was stopped for 10 days and then the leaves were collected. The MDA was extracted from 0.1 g maize leaf samples and measured according to the protocol from NanJing JianCheng Bioengineering Institute.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22111922/s1.

Author Contributions: Investigation, J.W. and L.C.; Data curation, J.W. and Y.L.; Funding acquisition, B.C. and H.J.; Writing—original draft, J.W.; Writing—review and editing, J.W. and W.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China, grant number 31771805.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.
References

1. Morton, M.J.L.; Awlia, M.; Al-Tamimi, N.; Saade, S.; Pailles, Y.; Negrao, S.; Tester, M. Salt stress under the scalpel—Dissecting the genetics of salt tolerance. Plant J. 2019, 97, 148–163. [CrossRef]

2. Sallam, A.; Alqudah, A.M.; Dawood, M.F.A.; Baenziger, P.S.; Borner, A. Drought Stress Tolerance in Wheat and Barley: Advances in Physiology, Breeding and Genetics Research. Int. J. Mol. Sci. 2019, 20, 3137. [CrossRef]

3. Gill, S.S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 2010, 48, 909–930. [CrossRef] [PubMed]

4. Nover, L.; Bharti, K.; Doring, P.; Mishra, S.K.; Ganguli, A.; Scharf, K.D. Arabidopsis and the heat stress transcription factor world: ABA-dependent and ABA-independent signaling in response to osmotic stress. J. Exp. Bot. 2017, 18, 1214. [CrossRef] [PubMed]

5. Sarkar, T.; Thankappan, R.; Mishra, G.P.; Nawade, B.D. Advances in the development and use of DREB for improved abiotic stress tolerance in transgenic crop plants. Front. Plant Biol. 2016, 7, 571. [CrossRef] [PubMed]

6. Balzer, G.; Dartevelle, T.; Godon, C.; Laugier, E.; Meisrimler, C.; Teulon, J.M.; Creff, A.; Bissler, M.; Brouchoud, C.; Hagege, A.; et al. Low phosphate activates STOP1-ALMT1 to rapidly inhibit root cell elongation. Nat. Commun. 2017, 8, 15300. [CrossRef] [PubMed]

7. Busoms, S.; Paajanen, P.; Marburger, S.; Bray, S.; Huang, X.Y.; Poschenrieder, C.; Yant, L.; Salt, D.E. Fluctuating selection on migrant adaptive sodium transporter alleles in coastal Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA 2018, 115, E12443–E12452. [CrossRef] [PubMed]

8. Zang, X.; Geng, X.; Wang, F.; Liu, Z.; Zhang, L.; Zhao, Y.; Tian, X.; Ni, Z.; Yao, Y.; Xin, M.; et al. Overexpression of wheat ferritin gene TaFER-5B enhances tolerance to heat stress and other abiotic stresses associated with the ROS scavenging. BMC Plant Biol. 2017, 17, 14. [CrossRef]

9. Yoshida, T.; Mogami, J.; Yamaguchi-Shinozaki, K. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. Curr. Opin. Plant Biol. 2014, 21, 133–139. [CrossRef]

10. Sah, S.K.; Reddy, K.R.; Li, J. Abscisic Acid and Abiotic Stress Tolerance in Crop Plants. Front. Plant Sci. 2016, 7, 571. [CrossRef] [PubMed]

11. Golldack, D.; Luking, I.; Yang, O. Plant tolerance to drought and salinity: Stress regulating transcription factors and their functional significance in the cellular transcriptional network. Plant Cell Rep. 2011, 30, 1383–1391. [CrossRef]

12. Singh, D.; Laxmi, A. Transcriptional regulation of drought response: A tortuous network of transcriptional factors. Front. Plant Sci. 2015, 6, 895. [CrossRef] [PubMed]

13. Sarkar, T.; Thankappan, R.; Misra, G.P.; Nawade, B.D. Advances in the development and use of DREB for improved abiotic stress tolerance in transgenic crop plants. Physiol. Mol. Biol. Plants 2019, 25, 1323–1334. [CrossRef]

14. Song, H.; Wang, P.F.; Hou, L.; Zhao, S.Z.; Zhao, C.Z.; Xia, H.; Li, P.C.; Zhang, Y.; Biao, X.T.; Wang, X.J. Global Analysis of WRKY Genes and Their Response to Dehydration and Salt Stress in Soybean. Front. Plant Sci. 2016, 7, 9. [CrossRef]

15. Wang, X.; Niu, Y.; Zheng, Y. Multiple Functions of MYB Transcription Factors in Abiotic Stress Responses. Int. J. Mol. Sci. 2021, 22, 6125. [CrossRef]

16. Ma, H.; Liu, C.; Li, Z.; Ran, Q.; Xie, G.; Wang, B.; Fang, S.; Chu, J.; Zhang, J. ZmbZIP4 Contributes to Stress Resistance in Maize by Major abiotic stresses and their role in regulation of heat shock protein genes. J. Exp. Bot. 2014, 65, 539–557. [CrossRef] [PubMed]

17. Li, P.S.; Khurana, N.; Agarwal, P.; Khurana, P. Heat shock factors in rice (Oryza sativa L.): Genome-wide expression analysis during reproductive development and abiotic stress. Mol. Genet. Genom. 2011, 286, 171–187. [CrossRef]

18. Xue, G.P.; Sadat, S.; Drenth, J.; McIntyre, C.L. 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. 2006, 57, 177–189. [CrossRef]
27. Mishra, S.K.; Tripp, J.; Winkelhausen, S.; Tschiersch, B.; Theres, K.; Nover, L.; Scharf, K.D. In the complex family of heat stress transcription factors, HSF1a has a unique role as master regulator of thermotolerance in tomato. *Genes Dev.* 2002, 16, 1555–1567. [CrossRef] [PubMed]

28. Yoshida, T.; Ohama, N.; Nakajima, J.; Kidokoro, S.; Mizoi, J.; Nakashima, K.; Maruyama, K.; Kim, J.M.; Seki, M.; Todaka, D.; et al. Arabidopsis HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Mol. Genet. Genom.* 2011, 286, 321–332. [CrossRef]

29. Liu, H.C.; Liao, H.T.; Charng, Y.Y. The role of class A1 heat shock factors (HSFA1s) in response to heat and other stresses in *Arabidopsis*. *Plant Cell Environ.* 2011, 34, 738–751. [CrossRef]

30. Bechtold, U.; Albihalal, W.S.; Lawson, T.; Fryer, M.J.; Sparrow, P.A.C.; Richard, F.; Persad, R.; Bowden, L.; Hickman, R.; Martin, C.; et al. Arabidopsis HEAT SHOCK TRANSCRIPTION FACTOR A1b overexpression enhances water productivity, resistance to drought, and infection. *J. Exp. Bot.* 2013, 64, 3467–3481. [CrossRef] [PubMed]

31. Schramm, F.; Ganguli, A.; Kiehlmann, E.; Englisch, G.; Walch, D.; von Koskull-Doring, P. The heat stress transcription factor HsfA2 serves as a regulatory amplifier of a subset of genes in the heat stress response in Arabidopsis. *Plant Mol. Biol.* 2006, 60, 759–772. [CrossRef] [PubMed]

32. Charng, Y.Y.; Liu, H.C.; Liu, N.Y.; Chi, W.T.; Wang, C.N.; Chang, S.H.; Wang, T.T. A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in *Arabidopsis*. *Plant Physiol.* 2007, 143, 251–262. [CrossRef]

33. Xin, H.; Zhang, H.; Chen, L.; Li, X.; Yuan, X.; Hu, X.; Cao, L.; He, X.; Yi, M. Cloning and characterization of HsfA2 from *Lilium longiflorum*. *Plant Cell Rep.* 2010, 29, 875–885. [CrossRef] [PubMed]

34. Gong, B.H.; Yi, J.; Wu, J.; Sui, J.J.; Khan, M.A.; Wu, Z.; Zhong, X.H.; Seng, S.S.; He, J.N.; Yi, M.F. LIHsFA1, a novel heat stress transcription factor in *lily (Lilium longiflorum)*, can interact with LIHsFA2 and enhance the thermotolerance of transgenic *Arabidopsis italiana*. *Plant Cell Rep.* 2014, 33, 1519–1533. [CrossRef] [PubMed]

35. Gu, L.; Jiang, T.; Zhang, C.X.; Li, X.D.; Wang, C.M.; Zhang, Y.M.; Li, T.; Dirk, L.M.A.; Downie, A.B.; Zhao, T.Y. Maize HSFA2 and HSFB2 antagonistically modulate rafinose biosynthesis and heat tolerance in *Arabidopsis*. *Plant J.* 2019, 100, 128–142. [CrossRef] [PubMed]

36. Yokotani, N.; Ichikawa, T.; Kondou, Y.; Matsu, M.; Hirochika, H.; Iwabuchi, M.; Oda, K. Expression of rice heat stress transcription factor OsHsfA2e enhances tolerance to environmental stresses in transgenic *Arabidopsis*. *Plant Bio.* 2008, 227, 957–967. [CrossRef] [PubMed]

37. Li, Z.J.; Zhang, L.L.; Wang, A.X.; Xu, X.Y.; Li, J.F. Ectopic Overexpression of Heat Shock Factor Gene HsfA3 Increases Galactinol Levels and Oxidative Stress Tolerance in *Arabidopsis*. *PLoS ONE* 2002, 16, 1555–1567. [CrossRef] [PubMed]

38. Song, C.; Chung, W.S.; Lim, C.O. Overexpression of Heat Shock Factor Gene HsfA3 Increases Galactinol Levels and Oxidative Stress Tolerance in *Arabidopsis*. *Mol. Cells* 2016, 39, 477–483. [CrossRef]

39. Poonia, A.K.; Mishra, S.K.; Sirohi, P.; Chaudhary, R.; Kanwar, M.; Germain, H.; Chauhan, H. Overexpression of wheat transcription factor (TaHsfA6b) provides thermotolerance in barley. *Planta* 2020, 252, 53. [CrossRef]

40. Yue, G.P.; Drenth, J.; McIntyre, C.L. TaHsfA6f is a transcriptional activator that regulates a suite of heat stress protection genes in *Arabidopsis*. *Plant Cell Environ.* 2011, 286, 321–332. [CrossRef]

41. Liu, A.L.; Zou, J.; Liu, C.F.; Zhou, X.Y.; Zhang, X.W.; Luo, G.Y.; Chen, X.B. Over-expression of OsHsfA7 enhanced salt and drought tolerance in transgenic rice. *BMB Rep.* 2013, 46, 31–36. [CrossRef] [PubMed]

42. Zang, D.D.; Wang, J.X.; Zhang, X.; Liu, Z.J.; Wang, Y.C. Arabidopsis heat shock transcription factor HSFA7b positively mediates salt stress tolerance by binding to an E-box-like motif to regulate gene expression. *J. Exp. Bot.* 2019, 70, 5355–5374. [CrossRef] [PubMed]

43. Fragkostefanakis, S.; Roth, S.; Schleiff, E.; Scharf, K.D. Prospects of engineering thermotolerance in crops through modulation of heat stress transcription factor and heat shock protein networks. *Plant Cell Environ.* 2015, 38, 1881–1895. [CrossRef]

44. Hahn, A.; Bublak, D.; Schleiff, E.; Scharf, K.D. Crosstalk between Hsp90 and Hsp70 Chaperones and Heat Stress Transcription Factors in Tomato. *Plant Cell* 2011, 23, 741–755. [CrossRef]

45. Ma, H.; Wang, C.T.; Yang, B.; Cheng, H.Y.; Wang, Z.; Mizoi, J.; Ren, C.; Qu, G.H.; Zhang, H.; Ma, L. CarHsfB2, a Class B Heat Shock Transcription Factor, Is Involved in Different Developmental Processes and Various Stress Responses in Chickpea (*Cicer arietinum* L.). *Plant Mol. Biol.* 2016, 34, 1–14. [CrossRef]

46. Jiang, J.H.; Ran, J.; Zou, J.; Zhou, X.Y.; Liu, A.L.; Zhang, X.W.; Peng, Y.; Tang, N.; Luo, G.Y.; Chen, X.B. Heat shock factor OsHsfB2b negatively regulates drought and salt tolerance in rice. *Plant Cell Rep.* 2013, 32, 1795–1806. [CrossRef] [PubMed]

47. Li, M.; Doll, J.; Weckermann, K.; Oecking, C.; Berendzen, K.W.; Schoff, F. Detection of in vivo interactions between Arabidopsis class A-HSFs, using a novel BiFC fragment, and identification of novel class B-HSF interacting proteins. *Eur. J. Cell Biol.* 2010, 89, 126–132. [CrossRef]

48. Zhang, H.M.; Li, G.L.; Fu, C.; Duan, S.N.; Hu, D.; Guo, X.L. Genome-wide identification, transcriptome analysis and alternative splicing events of Hsf family genes in maize. *Sci. Rep.-UK* 2020, 10, 8073. [CrossRef]

49. Jiang, Y.L.; Zheng, Q.Q.; Chen, L.; Liang, Y.N.; Wu, J.D. Ectopic overexpression of maize heat shock transcription factor gene ZmHsfB4 confers increased thermo and salt-stress tolerance in transgenic Arabidopsis. *Acta Physiol. Plant.* 2017, 40, 9. [CrossRef]
50. Li, G.L.; Zhang, H.N.; Shao, H.B.; Wang, G.Y.; Zhang, Y.Y.; Zheng, Y.J.; Zhao, L.N.; Guo, X.L.; Sheteiwy, M.S. 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.* 2019, 283, 375–384. [CrossRef]

51. Li, H.C.; Zhang, H.N.; Li, G.L.; Liu, Z.H.; Zhang, Y.M.; Zhang, H.M.; Guo, X.L. Expression of maize heat shock transcription factor gene ZmHsf06 enhances the thermotolerance and drought-stress tolerance of transgenic Arabidopsis. *Funct. Plant Biol.* 2015, 42, 1080–1091. [CrossRef]

52. Alexandre, C.; Moller-Steinbach, Y.; Schonrock, N.; Gruissem, W.; Hennig, L. Arabidopsis MSI1 Is Required for Negative Regulation of the Response to Drought Stress. *Mol. Plant* 2009, 2, 675–687. [CrossRef]

53. Aubert, Y.; Vile, D.; Pervent, M.; Aldon, D.; Ranty, B.; Simonneau, T.; Vavasseur, A.; Galaud, J.P. RD20, a Stress-Inducible Caleosin, Participates in Stomatal Control, Transpiration and Drought Tolerance in Arabidopsis thaliana. *Plant Cell Physiol.* 2010, 51, 1975–1987. [CrossRef] [PubMed]

54. Park, S.C.; Kim, Y.H.; Jeong, J.C.; Kim, C.Y.; Lee, H.S.; Bang, J.W.; Kwak, S.S. Sweetpotato late embryogenesis abundant 14 (IbLEA14) gene influences lignification and increases osmotic- and salt stress-tolerance of transgenic calli. *Planta* 2011, 233, 621–634. [CrossRef] [PubMed]

55. Bao, F.; Du, D.L.; An, Y.; Yang, W.R.; Wang, J.; Cheng, T.R.; Zhang, Q.X. Overexpression of Prunus mume Dehydrin Genes in Tobacco Enhances Tolerance to Cold and Drought. *Front. Plant Sci.* 2017, 8. [CrossRef] [PubMed]

56. Liu, H.; Wang, Q.Q.; Yu, M.M.; Zhang, Y.Y.; Wu, Y.B.; Zhang, H.X. Transgenic salt-tolerant sugar beet (Beta vulgaris L.) constitutively expressing an Arabidopsis thaliana vacuolar Na+/H+ antiporter gene, AtNHX3, accumulates more soluble sugar but less salt in storage roots. *Plant Cell Environ.* 2008, 31, 1325–1334. [CrossRef] [PubMed]

57. Iuchi, S.; Kobayashi, M.; Taji, T.; Naramoto, M.; Seki, M.; Kato, T.; Tabata, S.; Kakubari, Y.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. *Plant J.* 2001, 27, 325–333. [CrossRef]

58. Park, H.Y.; Seok, H.Y.; Park, B.K.; Kim, S.H.; Goh, C.H.; Lee, B.; Lee, C.H.; Moon, Y.H. Overexpression of Arabidopsis ZEP enhances tolerance to osmotic stress. *Biochem. Biophys. Res. Commun.* 2008, 375, 80–85. [CrossRef] [PubMed]

59. Yoo, S.D.; Cho, Y.H.; Sheen, J. Arabidopsis mesophyll protoplasts: A versatile cell system for transient gene expression analysis. *Nat. Protoc.* 2007, 2, 1565–1572. [CrossRef] [PubMed]

60. Cao, J.M.; Yao, D.M.; Lin, F.; Jiang, M.Y. PEG-mediated transient gene expression and silencing system in maize mesophyll protoplasts: A valuable tool for signal transduction study in maize. *Acta Physiol. Plant.* 2014, 36, 1271–1281. [CrossRef]