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ORIGINAL ARTICLE

ZmHSFA2d positively regulates maize seedling heat and drought tolerance by modulating photosystem protein synthesis

Yongyan Cao[1] · Chunyu Zhang[1] · Xuanxuan Chen[1] · Tuo Zeng[1,2] · Hongcheng Wang[1] · Xuye Du[1] · Xun Wu[3,4] · Bin Zhu[1] · Lei Gu[1,2]

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Abstract

Key message **ZmHSFA2d enhances maize heat and drought tolerance by safeguarding photosynthesis homeostasis, and its expression is directly activated by ZmbHLH124.**

Abstract Heat stress significantly influences plant growth, development, and productivity. Nevertheless, many maize (*Zea mays*) heat shock factors (HSFs) remain uncharacterized. Here, we explored the role of ZmHSFA2d in thermotolerance. *ZmHSFA2d* transcript levels were notably elevated under heat shock. ZmHSFA2d localized to the nucleus and displayed transactivation in yeast. Under heat shock, *ZmHSFA2d*-overexpressing maize seedlings exhibited greater thermotolerance, increased antioxidant enzyme activities, and reduced reactive oxygen species (ROS) and malondialdehyde (MDA) contents compared to control lines; however, these effects were reversed following the CRISPR/Cas9-mediated knockout (KO) of *ZmHSFA2d*. RNA-Seq analysis revealed that the expression of genes encoding ribosomal proteins, including those associated with ribosome assembly and translation progress, was significantly decreased in *ZmHSFA2d*-KO plants. Quantitative proteomic analysis further indicated that compared to control, the expression of key proteins [chlorophyll a/b binding proteins and NAD(P)H dehydrogenase (NDH) complex subunits] in photosystem was significantly decreased in *ZmHSFA2d*-KO lines. Furthermore, using Y1H, EMSA, Dual-Luciferin assays, and maize mesophyll protoplast expression, we found that ZmbHLH124, which is responsive to both heat and drought stress, directly upregulated the transcript levels of *ZmHSFA2d*. Additionally, ZmHSFA2d positively modulating maize drought tolerance, and compare to WT, ribosomal protein genes in *ZmHSFA2d*-KO plants also notably decreased under drought stress. Overall, our results indicated that the ZmHSFA2d positively regulates maize heat and drought tolerance by modulating the protein synthesis. This study contributes to the understanding of the role of HSFs in maize thermotolerance and offers useful genetic resources for the breeding of multiple stress-tolerant maize varieties.

Introduction

Temperature is an important environmental factor that regulates multiple aspects of plant growth and development. Recent studies have revealed that the ELF3 (EARLY FLOWERING 3) protein in the Evening Complex (EC), a core component of the *Arabidopsis* biological clock, contains a prion-like structural domain (PrD) that can rapidly switch between active and inactive states through phase transitions at high temperatures, thereby enabling the plant to sense changes in the external temperature (Jung et al. 2020). Plants respond to heat stress by activating a set of defense mechanisms collectively called the heat stress response (HSR), which is essential for counteracting the negative effects of elevated temperatures (Gong et al. 2020). During the plant HSR, the expression of some heat stress proteins (HSPs) and

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Yongyan Cao and Chunyu Zhang have contributed equally to this work.

✉ Bin Zhu
Zhugg130@126.com

✉ Lei Gu
201808009@gznu.edu.cn

[1] School of Life Sciences, Guizhou Normal University, Guiyang 550025, China

[2] Guizhou Key Laboratory of Forest Cultivation in Plateau Mountain, Guiyang 550025, China

[3] Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences, Guiyang 550006, China

[4] Ministry of Agriculture and Rural Affairs Key Laboratory of Crop Genetic Resources and Germplasm Innovation in Karst Region, Guiyang 550006, China

heat-tolerance genes is elevated via the activity of heat shock transcription factors (HSFs) (Scharf et al. 2012).

The HSF family is divided into A, B, and C classes based on the structural characteristics of their oligomerization domain (OD)/HR-A/B motif. HSFA family proteins typically contain five conserved structural domains—a DNA-binding domain (DBD); an OD domain; a nuclear localization signal (NLS); a nuclear translocation signal (NES), and a transcriptional activation domain (AHA) (Scharf et al. 2012). HSFB and HSFC family members lack the AHA and NES domains (Rabindran et al. 1993). HSF proteins have been identified in numerous plants, including 21 in *Arabidopsis thaliana*, 25 in rice (*Oryza sativa*), and 56 in wheat (*Triticum aestivum*) (Scharf et al. 2012).

Members of the *Arabidopsis* HSFA1 family (AtHSFA1a, AtHSFA1b, AtHSFA1d, and AtHSFA1e) are key regulators of the HSR (Liu et al. 2011; Nishizawa-Yokoi et al. 2011; Yoshida et al. 2011; Ohama et al. 2016). AtHSFA1s activate many key genes (*HsfA7s*, *HsfA2*, *HsfBs*, *MBF1c*, and *DREB2A*) that regulate the expression of downstream chaperone proteins and reactive oxygen species (ROS)-associated genes (Yoshida et al. 2011; Ohama et al. 2017). AtHSFA2 is also an important regulator of the HSR in *Arabidopsis* (Charng et al. 2007). The overexpression of the *TaHSFA2-10* gene of wheat in *Arabidopsis* transgenic lines markedly improves basal and acquired heat tolerance (Guo et al. 2020). AtHSFA4a is activated by MPK4 and plays an important role in *Arabidopsis* in response to both high salt and high-temperature stresses (Andrasi et al. 2019). The *AtHSFA6b* gene plays an important function in the resistance of *Arabidopsis* to heat stress and is an essential regulator of the thermotolerance acquisition pathway in this plant (Huang et al. 2016). In apple (*Malus domestica*), the overexpression of *MdHSFA8a* significantly increased the flavonoid content in transgenic apple leaves and enhanced the drought tolerance of the plants (Wang et al. 2020). Under normal conditions, AtHSFB1 and AtHSFB2b repress the expression of related heat stress response genes (*AtHsfA2* and *AtHsfA7a*), while under heat stress, they are essential for regulating the expression of HSPs (Ikeda et al. 2011). In soybean (*Glycine max*), the overexpression of *GmHSFB2b* resulted in the accumulation of flavonoids in the transgenic lines, which improved the salt tolerance of the plants (Bian et al. 2020). HSFB and C family members are considered to lack transcriptional activation activity owing to the absence of AHA and NES structural domains. Recent studies have found that a C-subfamily wheat HSF protein, TaHSFC2a-B, can directly interact with heat shock elements (HSEs) on the promoters of the *TaHSP70d* and *TaGalSyn* genes and activate their transcription. Additionally, *TaHSFC2a-B*-overexpressing wheat lines display significantly increased heat tolerance compared to WT (Hu et al. 2018). Based on the latest maize B73 reference genome, maize contains 31 HSF-coding genes (Zhang et al. 2020a). The overexpression of *ZmHSF17* in maize enhanced the heat tolerance of the plants by positively modulating membrane lipid synthesis (Zhang et al. 2024). Meanwhile, it was reported that ZmHSF08 and ZmHSF11 negatively regulate plant heat, drought, and salt tolerance (Wang et al. 2021; Qin et al. 2022). Although numerous *HSF* genes are predicted to exist in the maize genome, few have been functionally characterized.

HSFs activate plant heat stress signaling pathways by promoting the transcription of downstream target genes. Recent studies have revealed that under heat stress, ZmsHSP17.4, a cytoplasmic protein, is phosphorylated by ZmCDPK7, which activates its molecular chaperone function, thereby enhancing the heat tolerance of maize (Zhao et al. 2020). When plants are subjected to high-temperature stress, mitochondria and chloroplasts produce an excess of ROS, which can lead to cell death (Gong et al. 2020). Ascorbate peroxidases (APXs) and catalases (CATs) are key enzymes involved in intracellular ROS scavenging. In *Arabidopsis*, mutations in the genes encoding these enzymes significantly reduce plant heat tolerance (Vanderauwera et al. 2011). Although some HSF target genes are known to exert important functions under heat stress in maize, the roles of maize *HSF* genes in response to high-temperature stress remain unclear, as do the underlying regulatory mechanisms.

In China, maize is widely planted given its importance as a food crop and industrial raw material. However, extreme summer temperatures due to climate change have seriously impacted the growth, yield, and quality of maize seedlings. In this study, we identified a maize *HSF* gene, *ZmHSFA2d*, along with its upstream regulator ZmbHLH124. Further indicated that ZmHSFA2d positively modulates the maize heat stress response by influencing photosynthesis homeostasis, and it also regulates maize drought stress tolerance. Our study may provide candidate genes for the breeding of multi-stress tolerance maize varieties.

Materials and methods

Plant material and growth conditions

The maize B73 and B104 inbred lines were used in this research. Seeds were first surface-sterilized in 75% ethanol for 1 min, washed three times with distilled water, and then dark-cultivated in distilled water at 26 °C for 1 day. Germinated seeds were transplanted to nutrient soil and cultivated in an incubator under a 16 h (photon flux density of 8000 LUX) /8 h light/dark photoperiod at 28/24 °C with ~ 60% relative humidity. At the three-leaf stage, maize B73 seedlings were subjected to heat or dehydration stress. For heat stress, the seedlings were placed in a growth chamber at 42 °C for 0, 2, 4, or 8 h. For dehydration stress, the seedlings were removed from the soil for 0, 2, 4, or 8 h. Three pots of seedlings were used for each treatment, with five seedlings per pot. The maize leaves were harvested, immediately frozen in liquid nitrogen, and stored at − 80 °C until use.

Sequence analysis of ZmHSFA2d

The ZmHSFA2d sequence was aligned to those of *Arabidopsis* and rice HSFA2 proteins using DNAMAN 5.2.2 software. The accession numbers of protein sequence were as follows: OsHSFA2a (UniProt ID. F4MG08), OsHSFA2b (UniProt ID. Q6VBB2), OsHSFA2c (UniProt ID. Q338B0), OsHSFA2d (UniProt ID. Q8H7Y6), OsHSFA2e (UniProt ID. Q6F388), AtHSFA2 (UniProt ID. D7LCR0), and ZmHSFA2d (UniProt ID. B8A239). Phylogenetic trees were constructed for the relevant HSFA2 protein sequences based on the neighbor-joining method in MEGA 5.05. Evolutionary relationships were inferred using the neighbor-joining method.

Generation of *ZmHSFA2d* overexpression and knockout maize lines

To construct the *ZmHSFA2d* overexpression vector, the full-length coding sequence (CDS) of *ZmHSFA2d* was amplified from the leaves of seedlings after 2 h of heat stress. The amplicons were ligated into the *pEGPubi* vector (backbone: *pCAMBIA3300*) harboring the *bialaphos resistance* (*BAR*) gene and maize *Ubiquitin* promoter (Ubi), yielding *pEGOEPubi-UBI*:*ZmHSFA2d*. This expression vector was then transformed into the B104 line using *Agrobacterium*-mediated transformation (Kang et al. 2022). The CTAB method was used for the isolation of genomic DNA (Porebski et al. 1997). The genotypes of *ZmHSFA2d* overexpression lines were detected by PCR using *ZmHSFA2d*- and *BAR*-specific primers. Three independent transgenic lines with relatively high *ZmHSFA2d* expression levels (OE-*ZmHSFA2d*-1, -2, and -3) were obtained, and the T3 generation plants were used for further analyses. Primer information is provided in Table S1.

CRISPR/Cas9-based genome editing (Char et al. 2017) was used to generate *ZmHSFA2d* knockout (KO) lines. Two 20-bp segments within the CDS (first exon; targets 1 and 2) (Table S1) of *ZmHSFA2d* were inserted into the sgRNA-Cas9 expression vector (*pEGCas9PUbi*) under the control of the *OsU6a* or *OsU6b* promoter. Transformation into the B104 line (Kang et al. 2022) and genomic DNA isolation (Porebski et al. 1997) were performed as described above. A 706-bp DNA fragment was amplified from the T0 lines using sequence-specific primers (Table S1) to determine the knockout type. The *Cas9* sequence in T1 plants was detected using *Cas9*-specific primers (Table S1). Lines free of *Cas9* were retained, and their genotypes were determined.

Maize protoplast transformation

Maize protoplast preparation and transformation were performed following a previously published protocol (Gu et al. 2019, 2024b). The normal group was incubated at 25 °C for 17 h; the heat treatment group was placed at 25 °C for 7 h and then incubated at 42 °C for 10 h before being harvested for luciferase activity assay using the Dual-Luciferase Reporter Assay System (Promega Inc.) and for total RNA extraction.

ZmHSFA2d subcellular localization and transcriptional activation activity

For subcellular localization analysis, the CDS of *ZmHSFA2d* was PCR-amplified without the stop codon and inserted upstream of *GFP* in the *pROKII* empty vector (Gu et al. 2023, 2024b). This recombinant plasmid and a nuclear localization signal-containing mCherry (D53-RFP) (NLS-mCherry) reporter plasmid (Zhou et al. 2013) was co-transformed into maize mesophyll protoplasts. After culturing at 25 °C for 17 h, ZmHSFA2d, NLS-mCherry, or bright field signals were detected using a laser scanning confocal microscope (Andor Revolution WD).

For the construction of vectors for expressing the *ZmHSFA2d* gene in yeast, the CDS of *ZmHSFA2d* was amplified and ligated into the *pGBKT7* vector. The ZmHSFA2d transcriptional activation activity assay was performed as previously described (Gu et al. 2023).

Reverse transcription-quantitative PCR (RT-qPCR) and Western blot

Total RNA was extracted from the leaves of maize lines subjected or not to heat stress for 2 h or 4 h. Total RNA isolation and RT-qPCR were performed following a previously published protocol (Gu et al. 2024b). The gene-specific primers used for qPCR are listed in Table S1.

For Western blot analysis of ZmHSFA2d, the CDS of *ZmHSFA2d* was amplified and inserted into the *pET28a* vector (Takara, Japan). The expression of the ZmHSFA2d-6 × His-tagged fusion protein was induced for 4 h at 37 °C using 1 mM IPTG. The cells were then harvested, and total protein was extracted by sonication and purified using a 5-mL HisTrap HP Ni–NTA column (GE Healthcare Life Sciences Inc., Piscataway, NJ, USA) (Li et al. 2017). A polyclonal antibody against ZmHSFA2d was generated as previously described (Li et al. 2017). Total protein extraction and Western blot analysis were performed according to a recently published protocol (Gu et al. 2023). The antibody for ZmGAPDH was purchased from CWBIO (http://www.cwbiotech.com/). Gel images were captured with a ChemiDoc MP imager (Bio-Rad Inc., Hercules, CA, USA).

Transcriptome sequencing and proteomics

For RNA-Seq analysis, the B104 and knockout lines were subjected to heat stress (42 °C) for 4 h, and the leaves were collected. Total RNA was extracted using an RNA extraction kit from VAZYME (Nanjing, China). RNA purity and integrity were assessed using the NanoDrop 2000 and Agient2100/LabChip GX analyzers (Agilent Technologies, CA, USA). A total of 1 μg of RNA from each sample were used for library generation. RNA-Seq was performed on the Illumina platform (PE150 module). Differentially expressed genes (DEGs) were determined using DESeq2 (Love et al. 2014). A False Discovery Rate (FDR)-adjusted P-value of < 0.01 and $|\log2FC| \geq 1$ was used as criteria for screening DEGs. Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the DEGs were conducted using goatools (v.0.6.5) and KOBAS (v.2.1.1), respectively. Enrichment was considered significant at a P-value of < 0.05.

For data-independent acquisition (DIA) quantitative proteomic analysis, total protein was extracted from the same leaf samples as those used for RNA-Seq as previously reported (Isaacson et al. 2006). The protein samples were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA) as described by the manufacturer. For peptide sample preparation, approximately 100 μg of protein was reduced, alkylated, trypsin-digested, and quantified using a NanoDrop One (Thermo Fisher Scientific). Peptide desalting and quantification referred to a previously published report (Dong et al. 2024). Peptides were separated and analyzed using liquid chromatography (Vanquish Neo UHPLC system, Thermo Fisher Scientific) coupled with mass spectrometry (Orbitrap Astral mass spectrometer, Thermo Fisher Scientific). An ES906 chromatographic column (150 μm × 15 cm, Thermo Fisher Scientific) was used for peptide separation. The components of solvents A and B and the detailed peptide elution parameters were as previously reported (Dong et al. 2024). The original DIA data were collected (DIA mode, m/z range: 100–1700) and analyzed (selecting 6 peptides/protein and 3 daughter ions/peptide; analysis parameters: protein FDR ≤ 0.01, peptide FDR ≤ 0.01, peptide confidence $\geq 99\%$, XIC width ≤ 75 ppm) using Xcalibur 4.7 software (Thermo, USA) and Spectronaut 18/DIA-NN (Dong et al. 2024). FC ≥ 1.5 or ≤ 0.67 and a P-value of < 0.05 served as the criteria for identifying differentially expressed proteins (DEPs). GO annotation was conducted against the non-redundant protein database using InterProScan-5. The KEGG database was used to identify protein families and pathways. An enrichment pipeline was used to perform GO and KEGG enrichment analysis. Enrichment was considered significant at a P-value of < 0.05.

Determination of the phenotypes of transgenic maize plants

We first tested a 42 °C heat stress (data not shown). After 10 h, clear phenotypic differences emerged between B104 and the KO lines; prolonging the treatment likewise distinguished B104 from the OE lines. This heat shock regime was, therefore, adopted for all subsequent drought assays.

To test the heat stress tolerance of *ZmHSFA2d*-OE and -KO lines, maize plants at the three-leaf stage were incubated at 42 °C for 18 h (OE lines) or 10 h (KO lines). After heat shock treatment, the plants were grown under normal conditions for 4 (OE lines) or 6 (KO lines) days. The phenotypes of the first leaves and the H_2O_2 levels of the second leaves were evaluated before and after heat shock (Gu et al. 2024c). The third leaves were used for analyzing superoxide anion and malondialdehyde (MDA) contents and superoxide dismutase (SOD) and peroxidase (POD) activities. Antioxidant enzyme extraction and measurements were performed as previously reported (Gu et al. 2024a). To determine the survival rates after heat stress, maize plants at the three-leaf stage were incubated at 42 °C for 48 h (OE lines) or 36 h (KO lines). After heat treatment, the plants were grown under normal conditions for 4 (OE lines) or 6 (KO lines) days.

To test the drought stress tolerance of *ZmHSFA2d*-OE and -KO lines, maize plants at the three-leaf stage were deprived of water for 14 (OE lines) or 8 (KO lines) days. After drought treatment, the plants were rewatered for 4 days, and the survival rate was determined. For DAB (3, 3-diaminobenzidine) staining, leaves were stained in 1 mg/mL DAB (Boster) staining solution (Gu et al. 2024c). The physiological index (SOD, POD, MDA, and proline contents) was measured using the respective kits (Solarbio, Beijing, China) (Gu et al. 2024a). Plants that continued to grow after recovering were included in the survival rates. Three biological replicates were performed in physiological index measurements and four biological replicates in survival rates assay.

To test the Fv/Fm, B104, *ZmHSFA2d*-KO and *ZmHSFA2d* OE lines at the three-leaf stage were incubated at 42 °C for 10 h, then dark treatment for 0.5 h. A plant chlorophyll fluorescence imaging system (FC800-C/1010, PSI, Germany) was used to determine the Fv/Fm (represents the stability of the photosystem II).

Yeast one-hybrid (Y1H), EMSA, and Dual-Luciferin (LUC) reporter assays

To construct the Y1H assay bait vector, a 2000-bp fragment of the *ZmHSFA2d* promoter (upstream of the ATG) was inserted into the *pHIS2* vector using the primers listed in Table S1. The CDS of *ZmbHLH124* was amplified and subsequently ligated into the *pGADT7* vector. The Y1H assay was performed as previously described (Gu et al. 2024c).

For EMSA assay, the CDS of *ZmbHLH124* was amplified using primers listed in Table S1 and subsequently ligated into the *pET28a* vector for expression in the *Escherichia coli* BL21(DE3). The fusion protein was purified using the Ni–NTA beads (Smart-Lifesciences, Changzhou, China). Biotin-labeled probes containing E-box and the mutated E-box (Table S1) were synthesized according to the Biotin 3´ End DNA Labeling Kit (Thermo Fisher Scientific). EMSA was conducted with an EMSA kit (LightShift Chemiluminescent EMSA Kit, Thermo Fisher Scientific) followed the protocol.

To construct the LUC assay vectors, a 2000-bp *ZmHSFA2d* promoter fragment was amplified and cloned into pGreenII 0800-LUC (reporter). The *ZmbHLH124* open reading frame (ORF) (forward or reverse sequence) was inserted into pGreen II 62-SK (effector). Then, mixed vector (1 ml reporter and 4 ml effector) was co-transformation into the leaves of 3-week-old tobacco plants using the *Agrobacterium*-mediated method. The samples were collected for detection 72 h after injection. The LUC assay was performed following a previously reported protocol (Gu et al. 2024c). The luciferase activity was detected using the Promega Glomax 2020 luminometer.

Primers

All primers used in this work are listed in Table S1.

Statistical analysis

The data were analyzed using the Student's *t*-test in SPSS 19.0 (IBM SPSS Statistics, IBM Inc., Armonk, NY, USA). Pearson correlation analysis was performed using RStudio (v4.5.1).

Results

ZmHSFA2d identification and expression analysis

Through analysis of the published heat shock stress transcriptome data from maize B73 leaves (Long et al. 2023) and our unpublished data, we identified a maize *HSF* gene that strongly responded to heat stress. This gene contained a 1155-bp ORF encoding a 43.28-kDa protein with 384 amino acids that exactly matched the MaizeGDB reference sequence. Protein sequence analysis showed that the product of this gene displayed high sequence similarity to OsHSFA2d (68%, Fig. 1a) and was named ZmHSFA2d. Sequence alignment of the five OsHSFA2s (a–e), AtHSFA2, and ZmHSFA2d indicated that the DNA-binding domain (N-terminus) and the OD domain (HR-A and B) are conserved among these seven proteins (Fig. 1a). To further analyze the relationship among these proteins, we constructed a phylogenetic tree of the seven proteins (Fig. 1b) and noted that ZmHSFA2d was evolutionarily closer to the OsHSFA2s than to AtHSFA2 (Fig. 1b). To explore whether ZmHSFA2d is involved in the heat shock response in maize, RT-qPCR and Western blot analyses were performed for high-temperature conditions. As shown in Fig. 1c, high temperature induced the expression of *ZmHSFA2d* at both the mRNA and protein levels in maize seedlings.

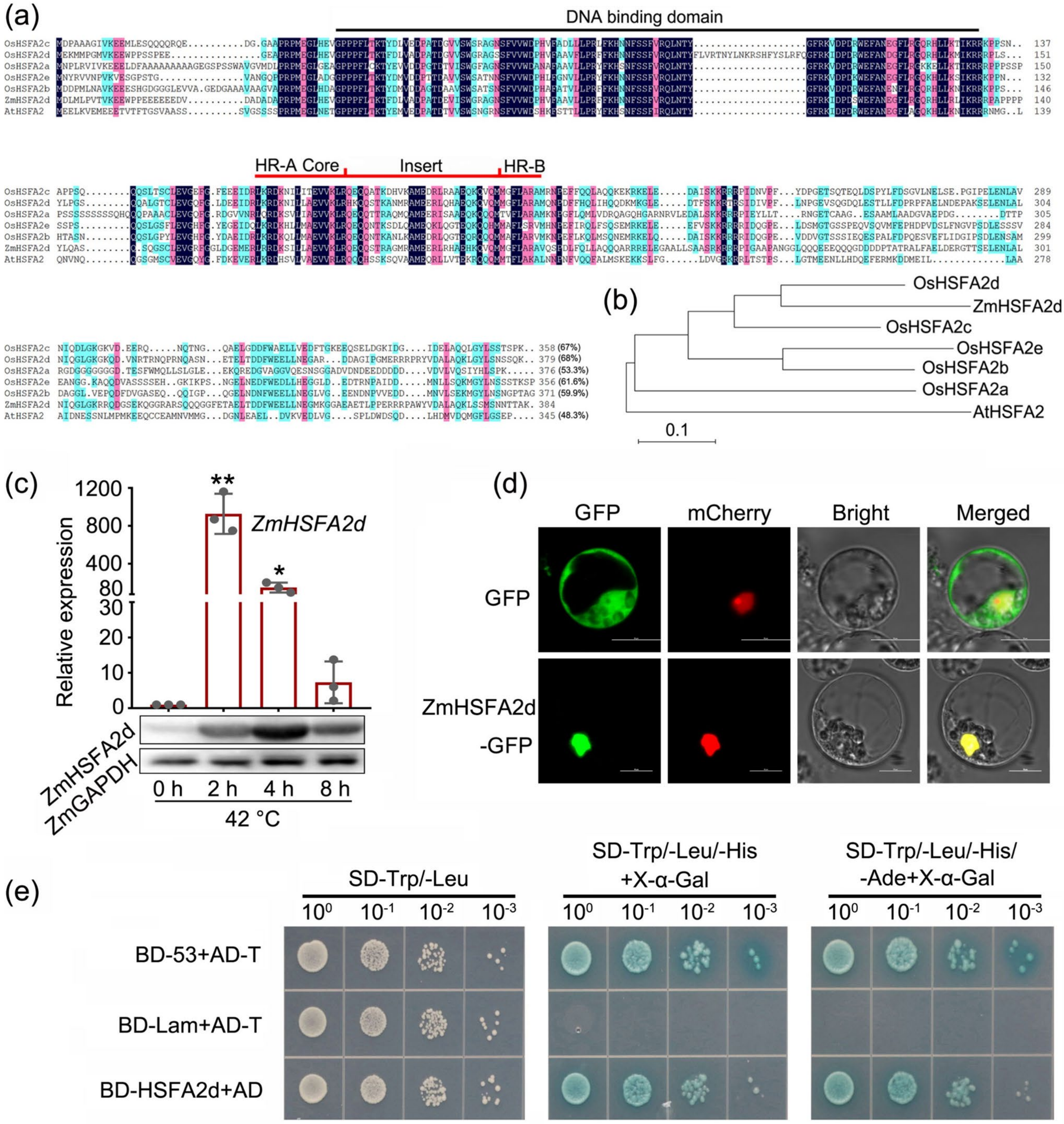
ZmHSFA2d is nucleus-localized and shows transcriptional activity in yeast

To determine the subcellular localization of ZmHSFA2d, *GFP* and *ZmHSFA2d:GFP* expression vectors were constructed and co-transformed with D53-RFP, a nuclear marker, into maize mesophyll protoplasts (Fig. 1d). Microscopic analysis showed that the control GFP signal was localized to both the cytosol and nucleus, whereas the ZmHSFA2d:GFP signal was only detected in the nucleus (Fig. 1d). To explore the transcriptional activity of ZmHSFA2d, the full-length CDS of the *ZmHSFA2d* gene was fused with the GAL4 DNA-binding domain (BD) (BD-HSFA2d) and co-transformed into yeast with the GAL4 activation domain (AD). As shown in Fig. 1e, like the positive control, yeast cells carrying both BD-HSFA2d and the GAL4 AD grew on SD selective medium without Trp, Leu, His, and Ade. This result indicated that ZmHSFA2d can activate the transcription of reporter genes in yeast.

The overexpression of *ZmHSFA2d* enhanced maize thermotolerance

To investigate the biological function of ZmHSFA2d, a *ZmHSFA2d*-OE vector (Fig. 2a) was generated and transformed into the maize B104 inbred line. Three independent T3 *ZmHSFA2d*-OE lines (OE-1, -2, and -3) were obtained, and the integration was identified by DNA-PCR using the relevant primers (Fig. 2a, b). The correct fragments (277 bp for *BAR* and 613 bp for *ZmHSFA2d*) were amplified from the OE lines but not from B104 (Fig. 2b), suggesting that *ZmHSFA2d* was stably integrated into the OE lines.

Under normal conditions, ZmHSFA2d mRNA and protein expression levels were markedly increased in the leaves of three constitutive *ZmHSFA2d* expression lines, but not those of the B104 line, as determined by RT-qPCR and Western blot (Fig. 2c, d). To establish whether the overexpression of *ZmHSFA2d* positively altered the basal heat stress tolerance in maize, a heat shock experiment was performed. The morphology of the three *ZmHSFA2d*-OE maize



seedlings at the three-leaf stage was similar to that of B104 plants when grown on nutrient soil under normal conditions (Fig. 2e). However, after heat shock treatment (42 °C for 18 h, followed by 4 days of recovery), all three *ZmHSFA2d*-OE plants displayed greater resistance to heat stress than the B104 controls. Additionally, the first leaves of the OE lines displayed markedly less damage than those of B104 control plants after heat shock (Fig. 2f), while the second and third leaves exhibited lower H_2O_2 and $O_2^{\cdot-}$ accumulation, respectively (Fig. 2f, g). Moreover, compared to control plants, the third leaves of the *ZmHSFA2d*-OE lines showed higher antioxidant enzyme (SOD and POD) activities and lower MDA contents (Fig. 2h–j), consistent with the marked reduction in H_2O_2 and $O_2^{\cdot-}$ contents observed in the leaves of the OE lines (Fig. 2f, g). We also evaluated the survival rates of B104 and *ZmHSFA2d* overexpression lines after heat shock. As shown in Fig. S1, after longer heat stress (42 °C for 48 h) followed by 4 days of recovery, the survival rates

◂**Fig. 1** Characteristic analysis of *ZmHSFA2d*. **a** Sequence alignment analysis showed that the DNA-binding domain and the HR-oligomerization core domains are conserved among HSFA2 proteins from different species. The conserved motifs are labeled in figure. The accession numbers of these HSFA2 protein are listed in the Materials and Methods section. Numbers in parentheses () represent the sequence similarity to ZmHSFA2d. Os: rice; Zm: maize; and At: Arabidopsis. **b** A phylogenetic tree was constructed based on full-length HSFA2 protein sequences from three species. The bar represents evolutionary distance. **c** Characterization of *ZmHSFA2d* gene and protein expression in heat shock-treated maize seedlings using RT-qPCR and Western blot, respectively. *ZmActin1* expression and ZmGAPDH protein were used as internal reference for normalization. Values are means ± SD; $n = 3$. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with wild type (WT) (Student's *t*-test). **d** The subcellular localization of ZmHSFA2d. *GFP* or *ZmHSFA2d:GFP* expression vectors were co-transformed with nuclear localization signal (NLS)-fused red fluorescent protein (RFP:mCherry) into maize mesophyll protoplasts. Scale bars = 10 μm. **e** The transcriptional activation activity of ZmHSFA2d. ZmHSFA2d fused with the GAL4 DNA-binding domain (BD) (BD-HSFA2d) was co-transformed into yeast with pGADT7 (activation domain, AD). The pGBKT7 vector fused with murine p53 protein (BD-53) or human lamin C protein (BD-Lam) co-transformed into yeast cells with pGADT7 fused with SV40 large T-antigen (AD-T) served as positive and negative controls, respectively. Yeast cells were grown on SD lacking tryptophan and leucine (SD – Trp/ – Leu) and then transferred to SD medium (containing X-α-Gal) without tryptophan, leucine, and histidine (SD – Trp/ – Leu/ – His) and SD medium (containing X-α-Gal) lacking tryptophan, leucine, histidine, and adenine (SD – Trp/ – Leu/ – His/ – Ade) for the evaluation of transcriptional activity

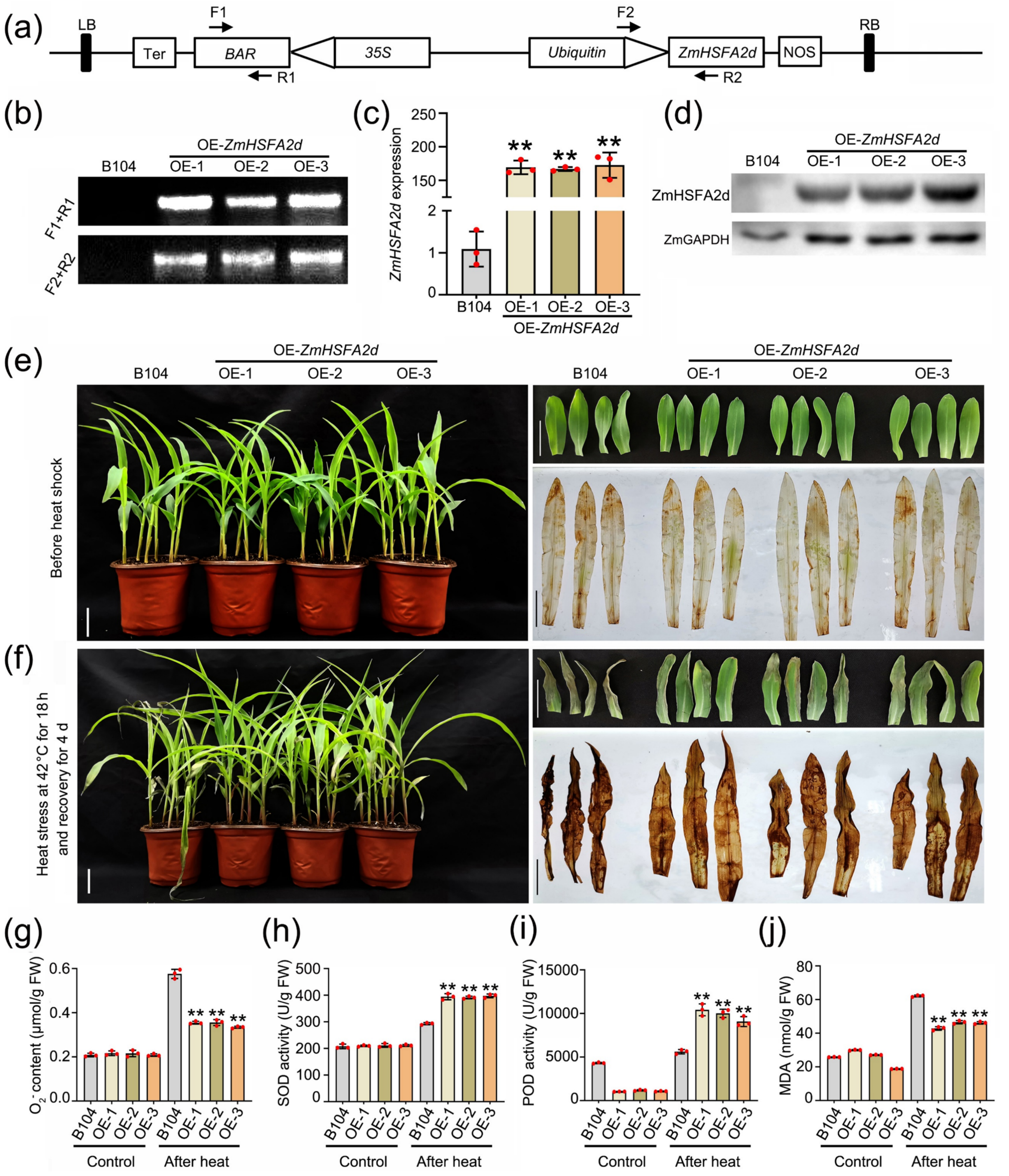
of the OE lines were significantly superior to that of control plants. These results indicated that the overexpression of *ZmHSFA2d* improved heat resistance in maize.

The knockout of *ZmHSFA2d* reduced maize thermotolerance

To determine whether the knockout of the *ZmHSFA2d* gene in maize decreased heat stress tolerance, we designed two single-guide RNAs (sgRNA1 and 2) targeting two sites located in the first exon of *ZmHSFA2d* (target 1: GGACCT GATGCTGCCGGTGACGG and target 2: CCAGGAGAT GACCTCGTCGGTGG) (Fig. 3a, b) to generate *ZmHSFA2d*-KO lines using CRISPR/Cas9 technology. We obtained two homozygous *Cas9*-free gene-edited lines, namely, *Zmhsfa2d-1* (1 bp insertion at target 1) and *zmhsfa2d-2* (1 bp insertion at target 2) (Fig. 3a, b). In both cases, the insertions resulted in premature termination of translation. Although *ZmHSFA2d* mRNA transcripts could also be detected in the *zmhsfa2d-1* and *zmhsfa2d-2* mutants after 4 h of heat shock, *ZmHSFA2d* protein accumulation in both mutants was notably reduced compared with that in NS controls, or was even undetectable, after 4 h of heat stress treatment (Fig. S2). To evaluate if the knockout of *ZmHSFA2d* negatively affected heat tolerance in maize, a heat shock experiment was performed. The morphology of the plants of two of the *ZmHSFA2d*-KO lines at the three-leaf stage was similar to that of the B104 controls under normal conditions (Fig. 3c, d). However, after exposure to 42 °C heat stress for 10 h followed by 6 days of recovery, plants from both KO lines exhibited more extensive damage than B104 control plants (Fig. 3c, d). After heat shock, the first leaves of the KO plants showed greater injury than those of the B104 plants. Additionally, compared with B104 controls, the second leaves of the KO plants accumulated more H_2O_2 (Fig. 3c, d). The third leaves had reduced antioxidant enzyme activities (SOD and POD) and increased MDA and $O_2^{\cdot -}$ accumulation (Fig. 3e–j). Furthermore, after longer heat shock (42 °C for 36 h) followed by 6 days of recovery, the survival rate of the WT was significantly superior to that of two KO plants (Fig. S3). These results revealed that the knockout of *ZmHSFA2d* reduced heat tolerance in maize.

ZmHSFA2d positively regulates maize heat stress tolerance by mediating ribosome biogenesis and assembly and then maintaining photosystem stability

To further evaluate the regulatory role of *ZmHSFA2d* under heat stress, we compared the leaf gene expression profile between B104 and *zmhsfa2d-2* mutant plants after 4 h of heat shock using RNA-Seq. An FDR-adjusted *P*-value < 0.01 and fold change ≥ 2 were used to screen DEGs. A total of 1207 DEGs were identified (Fig. 4a), with 694 being upregulated and 553 downregulated in the leaves of *zmhsfa2d-2* mutant plants compared to WT (Fig. 4a, Table S2). GO enrichment analysis indicated that the DEGs associated with 14 of the top 20 enriched terms were downregulated, with the three most enriched terms being translation, ribosomal small subunit assembly, and ribosomal large subunit assembly (Fig. 4b). KEGG pathway enrichment analysis revealed that the DEGs were mainly involved in ribosome and ribosome biogenesis in eukaryotes (the top two pathways); notably, the DEGs enriched in both terms were also downregulated (Fig. 4c). Except for the genes with unknown function, most of the downregulated DEGs (enrichment in top three terms of GO and top two terms KEGG) coded for components of ribosomal subunits (60S, 40S, 50S, and 30S) (Fig. 4d). To confirm the RNA-Seq data, RT-qPCR was performed to determine the expression level of two 60S ribosomal genes (*Zm00001d045578* and *Zm00001d010030*), two 40S ribosomal genes (*Zm00001d036567* and *Zm00001d028117*), 50S ribosomal gene (*Zm00001d008360*), and 30S ribosomal gene (*Zm00001d022386*) in WT and *zmhsfa2d-2* lines. As shown in Fig. S4a, the RT-qPCR results consistent with RNA-Seq analysis. Conversely, the expression of these 6 ribosomal genes was significantly increased in OE-1 under heat stress (Fig. S4b). These results suggested



that *ZmHSFA2d* may positively modulates ribosomal gene expression under heat shock.

Furthermore, we compared the leaf protein expression level between B104 and *zmhsfa2d-2* mutant plants after 4 h of heat shock (the sample sampling time as that used for RNA-Seq) using DIA proteomic analysis. A FC ≥ 1.5 or ≤ 0.67 and a *P*-value < 0.05 served as the DEP selection criteria. A total of 494 proteins were found to be differentially expressed between WT and *zmhsfa2d-2* leaves (Fig. 5a), of which 291 were upregulated while 203 were

◂**Fig. 2** ZmHSFA2d positively regulates maize heat stress tolerance. **a** Schematic representation of the constructs used for the overexpression (OE) of *ZmHSFA2d* in maize. The primers (F1, R1, F2, and R2) used in (**b**) are indicated. **b** Genotyping identification of control (B104) and OE maize plants (OE-*ZmHSFA2d*-1, -2, or -3) was conducted using the primers displayed in (**a**). **c** RT-qPCR analysis of the *ZmHSFA2d* expression levels in B104 and three *ZmHSFA2d*-OE maize lines under normal conditions. *ZmActin1* expression was used as internal reference for normalization. Values are means ± SD. $^{**}P < 0.01$, $n = 3$ (Student's *t*-test). **d** Western blot analysis of ZmHSFA2d levels in B104 and the three OE lines. ZmGAPDH protein was used as internal reference for normalization. **e**, **f** Photographs of B104 and the three OE plants before (**e**) and after (**f**) 18 h of heat stress treatment at 42 °C. Maize seedlings at the three-leaf stage were subjected to heat shock and then allowed to recover under normal conditions for 4 days. The phenotyping of the first leaves and the DAB staining of the second leaves before and after heat shock are shown on the right. Scale bar = 3 cm. (G–J) Determination of $O_2^{\cdot -}$ contents (**g**), SOD (**h**) and POD (**i**) activities, and malondialdehyde (MDA) concentrations (j) in the third leaves of each line before and after heat treatment. Values are means ± SD; $n = 3$. $^{**}P < 0.01$ compared with B104 (Student's *t*-test)

downregulated in *zmhsfa2d-2 versus* WT plants (Fig. 5a). GO enrichment analysis showed that the DEPs, especially those downregulated in the *zmhsfa2d-2* line, were significantly enriched in photosynthesis-related processes (Fig. 5b and Fig. S5). Photosynthesis-related pathways were also predominantly enriched in the KEGG analysis (Fig. 5c). Notably, the DEPs were found to be primarily involved in light-harvesting (chlorophyll *a*/*b* binding protein, also known as photosynthesis-antenna protein), photosystem I, and NAD(P)H dehydrogenase (NDH) complex (located in the photosynthetic chain) (Kouril et al. 2014), and were significantly downregulated in the leaves of mutant plants (Fig. 5d). Different from the protein levels, the relative expression levels of *ZmPnsB2*, *ZmNdhO*, *ZmNdhN*, *ZmNdhL*, *ZmChlab10(B6SZ08),* and *ZmChlab8(A0A8J8YQP7)* were not change between WT and KO line under heat stress (Fig. S6). Moreover, we also detected the stability of photosystem II between B104 and *zmhsfa2d-2* mutant/OE plants; as shown in Fig. S7a, compare to control, the photosystem II stability of mutant lines was significantly reduced. On the contrary, the photosystem II stability of OE-1 line was significantly increased (Fig. S7b). Besides the proteins related to photosynthesis, the expression levels of some ribosomal and ROS-scavenging proteins were also significantly decreased in the leaves of *zmhsfa2d-2* plants (Table S3). Overall, these results indicated that *ZmHSFA2d* positively influences protein synthesis, especially that related to photosynthesis, under heat stress.

ZmbHLH124 directly upregulated the expression of *ZmHSFA2d*

To explore upstream regulators of *ZmHSFA2d*, we analyzed a 2000-bp sequence of the promoter of this gene and found several E-boxes (CANNTG) (Fig. S8), which serve as binding sites for bHLH transcription factors (TFs) (Wei et al. 2021). A recent study indicated that the expression level of *ZmHSFA2d* (GRMZM2G165972) differed between WT and *ZmbHLH124* overexpression lines (Wei et al. 2021). To assess if ZmbHLH124 directly regulates the expression of *ZmHSFA2d*, we performed YIH, EMSA, and LUC assays (Fig. 6). As shown in Fig. 6a, yeast cells containing the combination of pGADT7-ZmbHLH124 and pHIS2-*ZmHSFA2dpro* could grow in 90 mM 3-AT, whereas the controls could not. This indicated that ZmbHLH124 can activate the promoter of *ZmHSFA2d* in yeast. Further EMSA analysis indicated that ZmbHLH124 could directly binding to the E-box located in *ZmHSFA2d* promoter (Fig. 6b). Meanwhile, when the *ZmbHLH124*-OE construct was co-transformed with *ZmHSFA2dpro* into tobacco leaves, LUC/REN activity was markedly increased compared with that of the control (p62-SK + *ZmHSFA2dpro*) (Fig. 6c). Further, LUC assay in maize mesophyll protoplasts also revealed that ZmbHLH124 could upregulated the expression of *ZmHSFA2d* under normal and heat stress (Fig. 6d). Overexpression of *ZmbHLH124* gene in maize mesophyll protoplasts led to the upregulation of the expression of *ZmHSFA2d*, while knockdown the expression of *ZmbHLH124* under heat stress result in downregulation of the expression of *ZmHSFA2d* (Fig. 6e). We found that in addition to drought stress (Wei et al. 2021), *ZmbHLH124* was also upregulated under heat stress, and its induction occurred earlier than that of *ZmHSFA2d* (Fig. S9 and Fig. 1c). These results indicated that ZmbHLH124 may directly upregulate *ZmHSFA2d* expression under heat stress.

ZmHSFA2d positively regulated drought stress tolerance in maize

ZmbHLH124 has been reported to positively modulate maize drought tolerance (Wei et al. 2021). To explore whether *ZmHSFA2d* also participates in maize drought stress responses, RT-qPCR was performed in maize seedlings under dehydration conditions. As shown in Fig. S10, drought stress also induced the expression of *ZmHSFA2d* gene in maize seedlings. Furthermore, overexpressing *ZmHSFA2d* in maize enhanced the survival rate of the OE lines after drought treatment (Fig. 7a, b), reduced H_2O_2 levels (Fig. 7c), increased SOD and POD activities (Fig. 7d, e), reduced the MDA content (Fig. 7f), and increased the proline content (Fig. 7g). In contrast, after drought treatment, the survival rate of *ZmHSFA2d*-KO plants was significantly lower than that of B104 (WT) plants (Fig. 8a, b). Moreover, compared to WT, the leaves of *ZmHSFA2d*-KO plants accumulated greater quantities of H_2O_2 (Fig. 8c). Meanwhile, the relative expression levels of ribosomal genes in Figure S4 also significantly decreased in KO line under drought stress (Fig. S11).

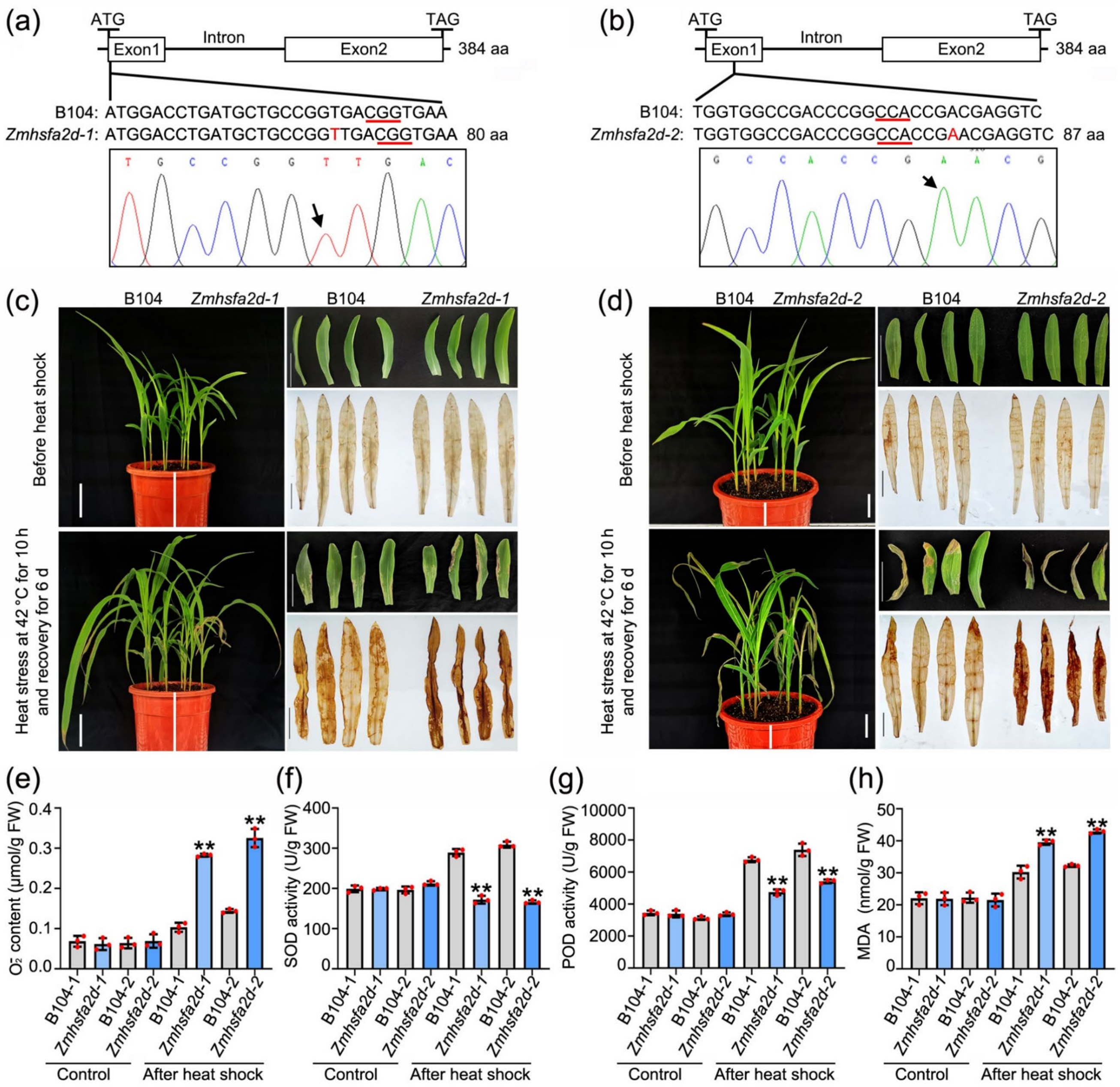


Fig. 3 CRISPR/Cas9-mediated knockout of *ZmHSFA2d* decreases maize heat stress tolerance. **a**, **b** Schematic representation of the gene structure of *ZmHSFA2d* and the mutation sites of the two *ZmHSFA2d* gene knockout mutants (*zmhsfa2d-1* and *zmhsfa2d-2*). **c**, **d** Photographs of B104, *zmhsfa2d-1* (**c**), and *zmhsfa2d-2* (**d**) knockout lines before and after 10 h of heat stress treatment at 42 °C. Maize seedlings at the three-leaf stage were subjected to heat shock treatment and then allowed to recover under normal conditions for 6 days. The phenotyping of the first leaves and the DAB staining of the second leaves before and after heat shock are shown on the right. Scale bar = 3 cm. **e**–**f** Determination of $O_2^{\cdot-}$ contents (**e**), SOD (**f**) and POD (**g**) activities, and malondialdehyde (MDA) concentrations (h) in the third leaves of each line before and after heat treatment. Values are means ± SD; $n = 3$. $^{**}P < 0.01$ compared with B104 (Student's *t*-test)

Pearson correlation analysis further indicated that there is an obvious positive correlation (Fig. S12) between the survival rate of *ZmHSFA2d-2* (Fig. 8b) and the downregulation of these genes. These results indicated that ZmHSFA2d may also positively regulates maize drought stress tolerance through influencing the protein translation.

Discussion

The function of ZmHSFA2d

HSFs have been characterized in numerous plant species (Bakery et al. 2024). However, many of the *HSF* genes that

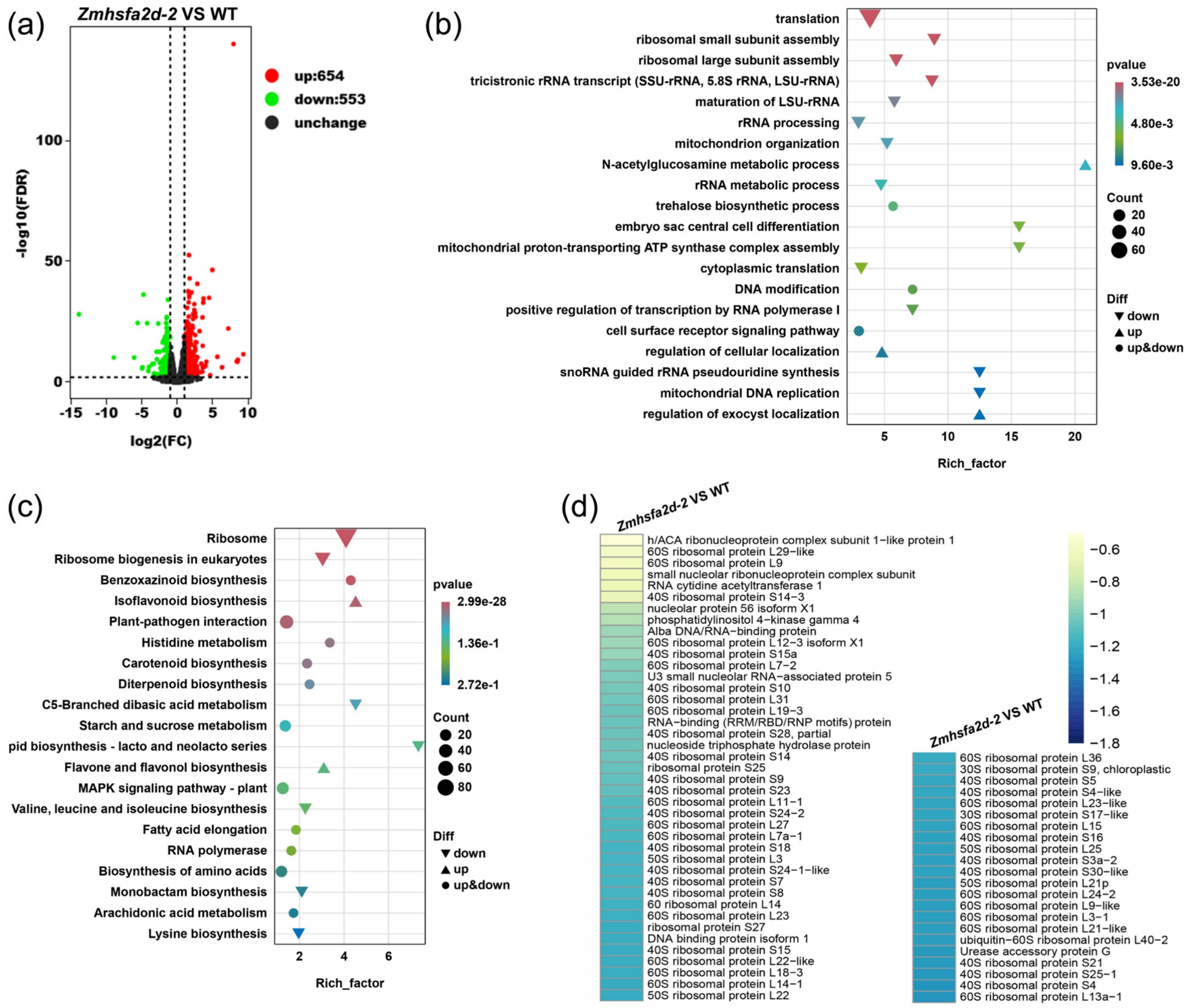


Fig. 4 RNA-Seq analysis of the wild-type (WT) and *zmhsfa2d-2* knockout (KO) lines under heat stress. **a** A volcano plot of the differentially expressed genes (DEGs) between *zmhsfa2d-2* mutant leaves and WT leaves after 4 h of heat shock. **b** The top 20 terms identified in the Gene Ontology (GO) enrichment analysis of the DEGs. **c** The top 20 pathways identified in the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DEGs. **d** A heatmap of the downregulated genes (top three terms of GO and top two terms of KEGG) in *zmhsfa2d-2 versus* the WT. The colors indicate relative Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values (*zmhsfa2d-2 vs* WT)

are predicted to exist in the maize genome remain to be functionally characterized. In this study, Zm00001eb004670 from the inbred line B73, previously known as *ZmHSFA6b* (https://www.uniprot.org/uniprotkb/B8A239/entry) and *ZmHSF01/13* (Li et al. 2020; Zhang et al. 2020b), was experimentally characterized in maize. We renamed this gene *ZmHSFA2d* as it is evolutionarily close to *OsHSFA2d* (Fig. 1a, b). The overexpression of *ZmHSFA2d* from the inbred line H21 (named *ZmHSF01*) in *Arabidopsis* significantly increased its thermotolerance (Zhang et al. 2020b). Here, compared to WT plants, *ZmHSFA2d*-OE maize plants displayed greater heat stress tolerance (Fig. 2), while *ZmHSFA2d*-KO plants exhibited greater sensitivity to heat (Fig. 3). These results indicated that the function of ZmHSFA2d is conserved between maize and *Arabidopsis*.

In addition to playing an important role under heat stress, HSFs have also been reported to be involved in responses to other stress types. Upregulating the expression of *ZmHSF08* (B class member) in maize increased the sensitivity of the plants to both drought and salt stress (Wang et al. 2021). In contrast, *ZmHSF28*-overexpressing maize plants display significantly enhanced tolerance to drought, an effect that is exerted via the modulation of hormone- and ROS-related pathways (Liu et al. 2023). Similarly, the overexpression

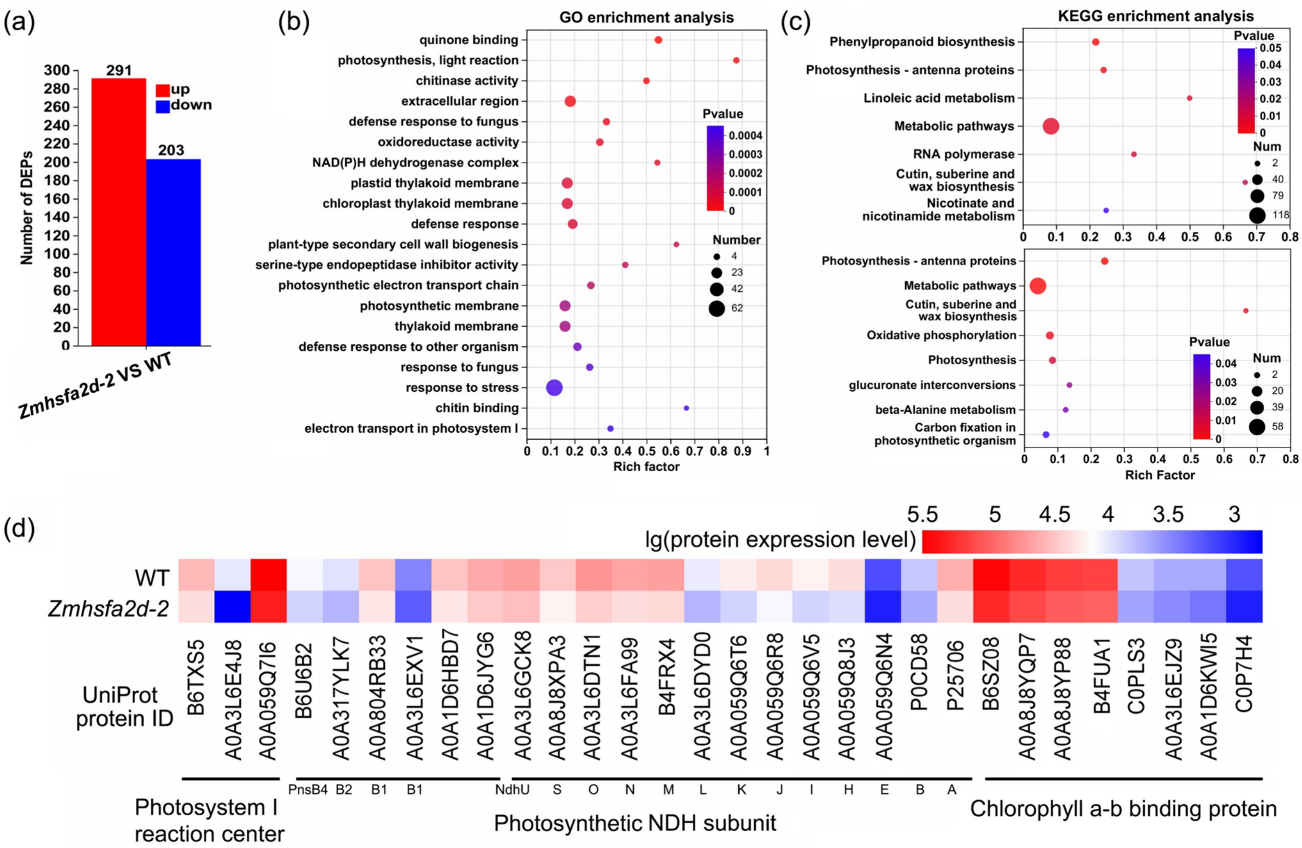


Fig. 5 Data-independent acquisition (DIA) quantitative proteomic analysis of the wild-type (WT) and *zmhsfa2d-2* lines under heat stress. **a** The differentially expressed proteins (DEPs) between *zmhsfa2d-2* mutant leaves and WT leaves after 4 h of heat shock. **b** The top 20 terms from the Gene Ontology (GO) enrichment analysis of the DEPs. **c** Top panel: The seven pathways (P-value < 0.05) identified in the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DEPs. Bottom panel: The eight pathways (P-value < 0.05) identified in the KEGG enrichment analysis of the downregulated proteins (*zmhsfa2d-2 vs*. WT). **d** A heatmap of the downregulated proteins (involved in photosynthesis) in *zmhsfa2d-2* compared with the WT. The colors indicate relative protein expression levels

of *ZmHSF06* in *Arabidopsis* also conferred greater drought tolerance to the plants (Li et al. 2015). It was recently shown that ZmHSF21 positively modulates the cold stress response of maize seedlings by influencing lipid metabolism homeostasis (Gao et al. 2024). In this work, we found that ZmHSFA2d expression was induced by drought (Fig. S9) and that ZmHSFA2d also participates in maize drought responses. We further observed that the overexpression of *ZmHSFA2d* in maize enhanced the drought tolerance of the plants, whereas its knockdown elicited the opposite phenotype (Fig. 7, 8). Due to global warming, the frequency of combined drought and heat stress has risen significantly over recent years (Chang et al. 2024); however, only a few genes have been reported to provide resistance to both abiotic stresses. Recent research has shown that ONAC023 may play a regulatory role under combined drought and heat stress in rice at both the seedling and reproductive stages. Under stress conditions, ONAC023 translocate to the nucleus and positively regulates water transport and ROS homeostasis, thereby conferring tolerance to heat and drought stresses in rice (Chang et al. 2024). Like ONAC023, ZmHSFA2d may also confer tolerance to drought and heat in maize, making it a promising candidate gene for breeding multi-stress-resistant maize varieties.

The regulatory mechanisms of ZmHSFA2d

Ribosomes function in protein synthesis by binding mRNA and translating the genetic code into amino acids. Ribosomes typically comprise approximately 50–80 ribosomal proteins (Scarpin et al. 2023). Under cold stress, *Flammulina velutipes* mycelia accumulate the ribosomal proteins L1/L4 and the 60S ribosomal subunit protein L36, which may enhance the tolerance of the cells to cold conditions (Liu et al. 2017). Soybean ribosomal protein S3 is involved in the seedling flood stress response by modulating protein homeostasis (Wang et al. 2024). Under drought stress, a greater number of ribosomal proteins were downregulated in

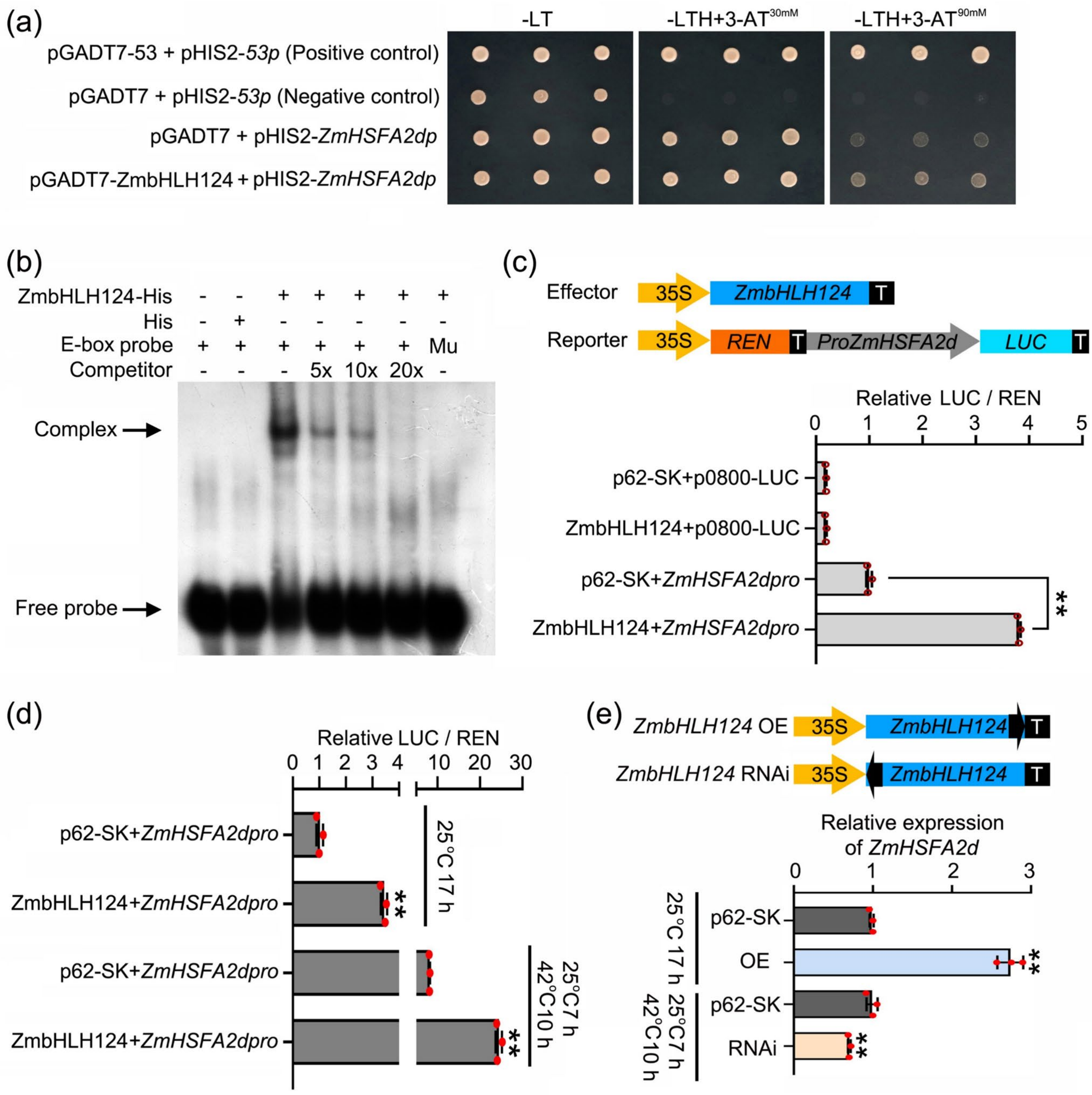


Fig. 6 ZmbHLH124 induces the expression of *ZmHSFA2d*. **a** Yeast one-hybrid (Y1H) assay. Yeast Y187 strains co-transformed with pGADT7-ZmbHLH124 and pHIS2-*ZmHSFA2dpro*, pHIS2-*ZmHSFA2dpro* and pGADT7 (self-activating), or negative and positive controls were grown on SD medium without leucine and tryptophan (−LT) and then transferred to SD medium lacking leucine, tryptophan, and histidine (SD-LTH) and supplemented with 30 or 90 mM 3-AT. **b** Binding of ZmbHLH124 to the E-box element-containing *ZmHSFA2d* promoter fragment in vitro. EMSA was performed by incubating the E-box element (biotin-labeled and unlabeled competitor probe) or mutated (Mu) probe with a recombinant ZmbHLH124-His-tagged protein. **c** ZmbHLH124 upregulated *ZmHSFA2d* expression in tobacco leaves. Effector (ZmbHLH124), *ZmbHLH124* overexpressing vector. Reporter (*ZmHSFA2dpro*), *LUC* expression was under the control of the promoter (−2000 bp) of *ZmHSFA2d*. p62-SK: pGreenII 62-SK vector. p0800-LUC: Dual-LUC vector. Each vector combination (labeled in the figure) was co-transformed into tobacco leaves. The LUC/REN ratio of the *ZmHSFA2dpro*/p62-SK combination was used as a control. Values are means ± SD, $n = 3$. $^{**}P < 0.01$ compared with the control (Student's *t*-test). **d** ZmbHLH124 upregulated *ZmHSFA2d* expression under normal and heat stress in maize mesophyll protoplast. Vector name showed in c. The LUC/REN ratio of the *ZmHSFA2dpro*/p62-SK combination under normal was used as a control. Values are means ± SD, $n = 3$. $^{**}P < 0.01$ compared with the p62-SK (Student's *t*-test). **e** RT-qPCR analysis of *ZmHSFA2d* expression in maize mesophyll protoplast transformed with p62-SK, effector (*ZmbHLH124* OE), or ZmbHLH124 RNAi construct. Values are means ± SD, $n = 3$. $^{**}P < 0.01$ compared with the p62-SK (Student's *t*-test)

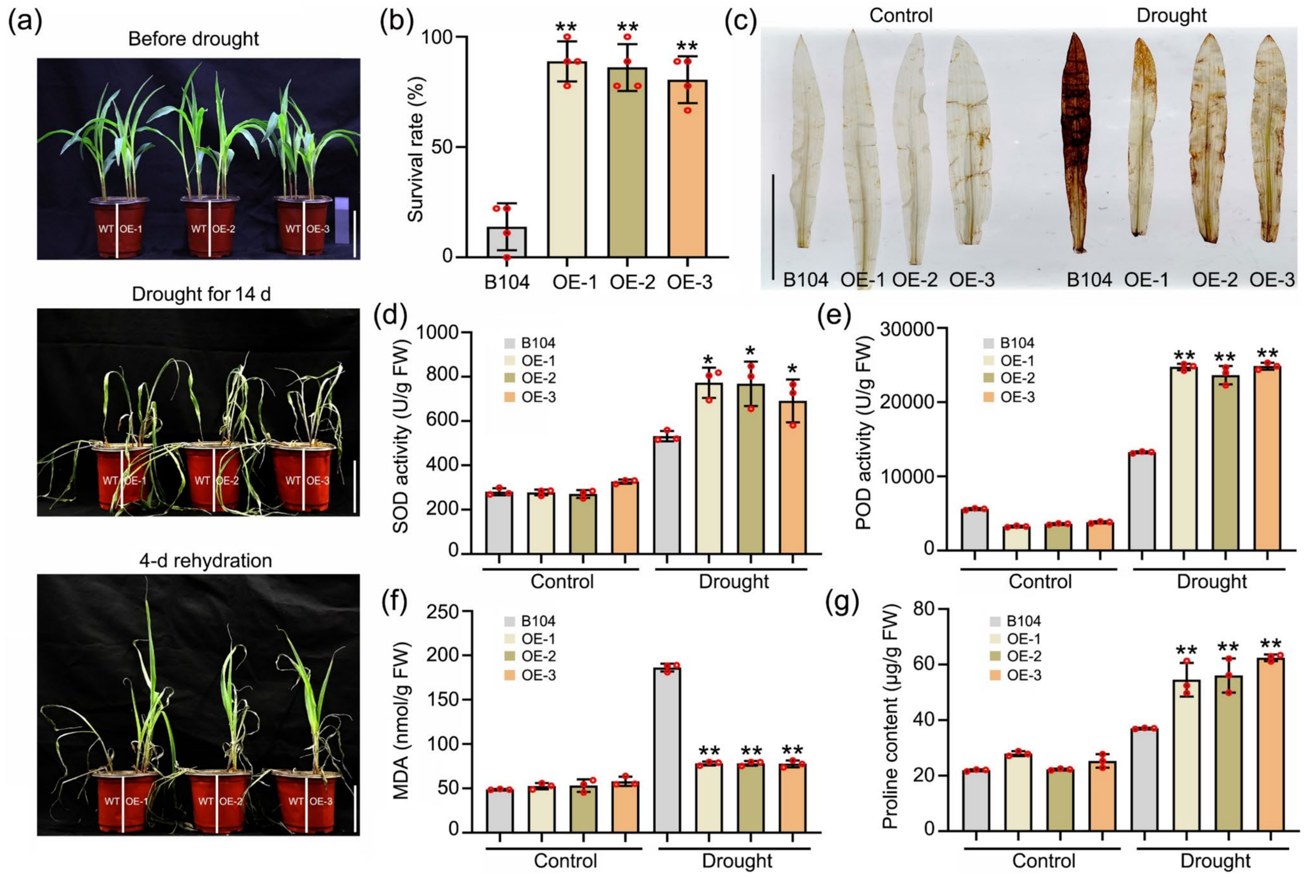


Fig. 7 The overexpression of *ZmHSFA2d* in maize enhanced the drought tolerance of the plants. **a** Photographs of B104 (wild type, WT) and three *ZmHSFA2d* overexpression (OE) lines (OE-1, -2, and -3) before drought treatment and after 14 days of drought treatment followed by 4 days of rehydration. Scale bar = 7.5 cm. **b** The survival rates of the WT and OE lines after rewatering. Values are means ± SD; $n = 4$. $^{**}P < 0.01$ compared with WT (Student's *t*-test). **c** DAB staining of the second leaves of each line before and after drought treatment. Scale bar = 7.5 cm. **d–g** Determination of SOD (**d**) and POD (**e**) activities and the malondialdehyde (MDA) (**f**) and proline (**g**) contents of each line before and after drought treatment. Values are means ± SD; $n = 3$. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with WT (Student's *t*-test)

drought-sensitive alfalfa than in the drought-tolerant variety (Quan and Liu 2024). Through phenotypic and transcriptome association analysis, Frey et al. (Frey et al. 2015) identified 39 heat tolerance-related genes in the seedling stage in maize, including those encoding ribosomal proteins (L4/L1 family member and 40S ribosomal protein 23). A recent study indicated that ribosomal protein genes may play essential roles in yeast and ryegrass heat shock responses (Chen et al. 2023). In the present study, we found that the knockout of *ZmHSFA2d* in maize resulted in a significant downregulation of the expression of ribosomal protein-encoding genes (Fig. 4 and Table S2) under heat shock. One explanation may be that the loss of *ZmHSFA2d* disrupts protein synthesis under heat stress, resulting in reduced maize plant thermotolerance. Proteomic analysis further confirmed that the levels of 60S ribosomal proteins and those of the 50S ribosomal protein L3 were significantly decreased (Table S3). Because RNA and protein levels under heat shock were analyzed at the same time point, the DEGs and DEPs may not show a strong correlation. The DEPs associated with photosynthesis were enriched and notably downregulated in the *ZmHSFA2d*-KO lines (Fig. 5 and Table S3). In line with this, it was recently reported that heat shock sustainably influenced the expression patterns of photosynthesis-related genes (Chen et al. 2023). Light-harvesting chlorophyll *a*/*b* binding protein is an integral thylakoid membrane protein that forms part of photosystems I and II, where it acts in the capture, distribution, and transmission of light energy (Quan and Liu 2024); it is also involved in plant abiotic stress responses (Peng et al. 2019). Drought-tolerant alfalfa accumulates greater quantities of chlorophyll *a*/*b* binding proteins than drought-sensitive lines (Quan and Liu 2024). The NAD(P)H dehydrogenase (NDH) complex is the largest complex in the photosynthetic electron transport chain and also interacts with photosystem I (Peng et al. 2008; Peng and Shikanai 2011). The NDH complex comprises

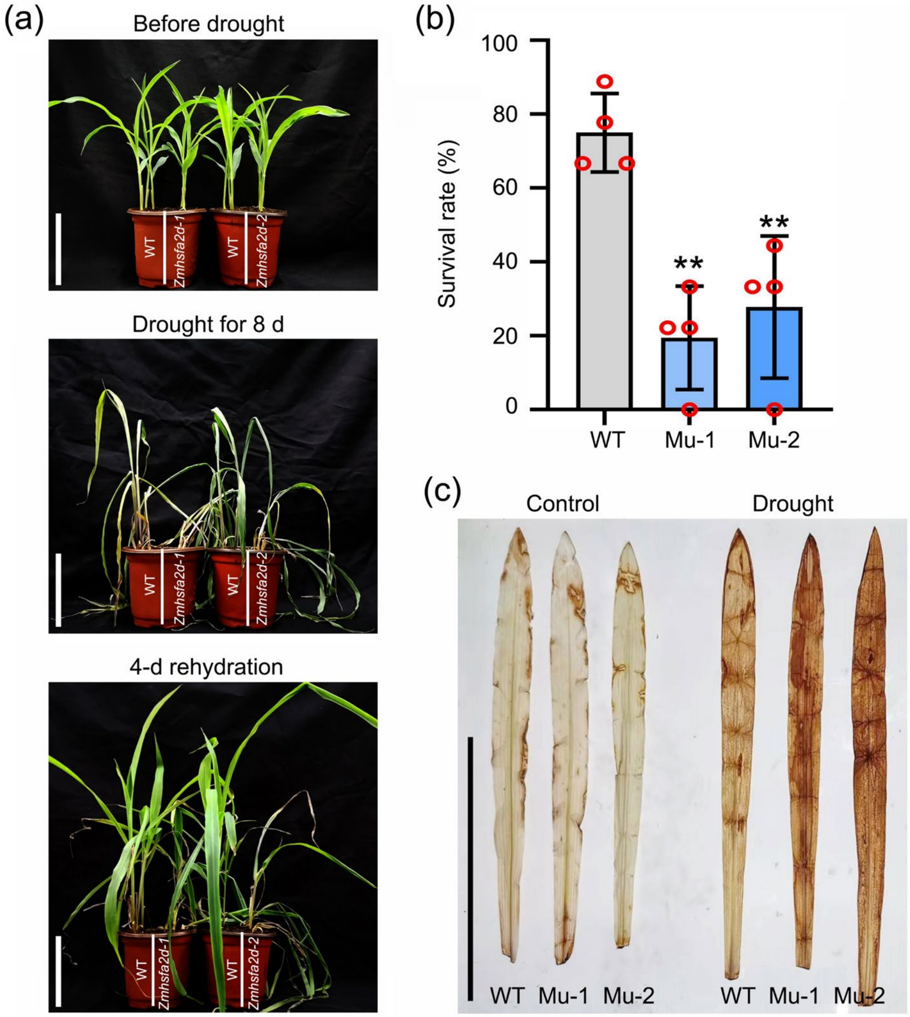


Fig. 8 The knockout of *ZmHSFA2d* decreases maize drought tolerance. **a** Photographs of B104 (wild type, WT) and two *ZmHSFA2d* knockout (KO) lines (*zmhsfa2d-1* and *zmhsfa2d-2*) before drought treatment and after 8 days of drought treatment followed by 4 days of rehydration. Scale bar = 7.5 cm. **b** The survival rates of the WT and KO lines after rewatering. Values are means ± SD; $n = 4$. $^{**}P < 0.01$ compared with WT (Student's *t*-test). **c** DAB staining of the second leaves of each line before and after drought treatment. Scale bar = 7.5 cm. Mu-1: *zmhsfa2d-1* and Mu-2: *zmhsfa2d-2*

six subcomplexes—SubA (containing NdhH-K and NdhL-O), SubM (containing NdhA-G), SubB (PnsB1-B5), SubL (PnsL1-L5), and SubE (NdhS-V) (Ifuku et al. 2011). In the tobacco *ndhC-ndhK-ndhJ* mutant, photosynthetic electron transport is inhibited under heat stress, leading to an increase in cellular ROS levels (Wang et al. 2006). In our work, compared to WT, the levels of chlorophyll *a*/*b* binding protein and the subunits of the NDH complex (PnsB1, B2, B4 and NdhA, B, E, H–O, U, S) (Fig. 5, Table S3) were significantly decreased in *zmhsfa2d-2* mutants. Furthermore, compare to B104, the photosystem II stability of *zmhsfa2d-2* lines was significantly reduced (Fig. S7a). This indicated that photosynthesis was impaired, leading to an increase in the sensitivity of *ZmHSFA2d*-KO plants to heat shock. In addition to affecting the accumulation of photosynthesis-related proteins, in *zmhsfa2d*-KO lines, the levels of proteins involved in ROS scavenging and the stress response, such as SOD (protein ID: B6TIS2), GST4 (protein ID: B6TIS2), 17.5 kDa HSP (protein ID: B6U175), and two 14–3-3-like proteins (Wang et al. 2023) (protein IDs: A0A3L6EZ34 and B6U284), were also significantly reduced (Table S3). In this study, we were unable to validate the proteomic results by Western blot owing to the absence of specific antibodies; this constitutes a methodological limitation. Overall, these findings suggested that ZmHSFA2d positively regulates maize heat shock tolerance by modulating protein synthesis, especially that related to photosynthesis.

It has been demonstrated that ZmbZIP60 from the maize inbred line W22 can upregulate the expression of *ZmHSFA2d* (*ZmHSF13*) under high-temperature conditions (Li et al. 2020). bHLH, one of the largest plant TF families, plays an important role in plant stress response (Feller et al. 2011). Here, we found several E-boxes (bHLH binding site) located in the *ZmHSFA2d* promoter (Fig. S8), and through Y1H, EMSA, LUC assays and maize mesophyll protoplast expression, confirmed that ZmbHLH124 can directly bind to the promoter of *ZmHSFA2d* and upregulate its expression (Fig. 6). Further analysis indicated that, in addition to drought (Wei et al. 2021), *ZmbHLH124* is also induced under heat stress (Fig. S9), and *ZmHSFA2d* is also responsive to drought stress (Fig. S10). These results indicated that ZmbHLH124 may positively regulate the expression of *ZmHSFA2d* under heat and drought conditions. Recent study indicates CsSPT (a bHLH TF) positively regulates cucumber heat stress response through recruiting photosynthesis components and modulating the expression of *HSF* genes (Liang et al. 2024); suggesting that the bHLH-HSF regulatory module might evolutionary conservation. Comprehensive functional validation of ZmbHLH124 under maize heat shock, such as through stable genetic transformation, is still necessary in the future research.

The decline in photosynthetic efficiency leading to increase the level of ROS (such as H_2O_2); H_2O_2 may act as a potential signal molecule in chloroplast retrograde response (CRR) through moving from chloroplast to nucleus, and then directly modulating the transcription process of genes (Exposito-Rodriguez et al. 2017). ZmbHLH124-ZmHSFA2d pathway may involve in CRR. *ZmbHLH124* might respond to H_2O_2 and then upregulates *ZmHSFA2d*; the active ZmHSFA2d may positively influence photosystem protein synthesis, thereby enhancing maize stress (heat and drought) tolerance; however, this possibility requires further experimental verification.

There is greater focus on the heat stress tolerance of maize during reproductive stages than that during vegetative growth. So, our further study will focus on how ZmHSFA2d affects pollen viability, fertilization, and grain filling under heat stress.

Conclusion

In this study, a maize HSF *ZmHSFA2d* gene, which responds to drought and heat stress, was functionally characterized in maize. The overexpression of *ZmHSFA2d* in maize significantly enhanced both drought and heat tolerance, while the knockout of *ZmHSFA2d* elicited the opposite phenotype. Further analysis indicated that ZmHSFA2d confers heat tolerance in maize by modulating protein synthesis, especially that relating to photosynthesis. Moreover, we found that ZmbHLH124 upregulates the expression of *ZmHSFA2d* under both drought and heat stress, implying that the ZmbHLH124-ZmHSFA2d regulatory module may positively modulate the response of maize to these abiotic stresses. This study advances our understanding of the molecular mechanism underlying the role of ZmHSFs in the response of maize to heat shock and drought and may provide excellent genetic resources for maize breeding.

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Author contributions LG and BZ conceived research plans and designed experiments. YC, CZ, and XC conducted experiments. LG, YC, and CZ wrote the draft. TZ, HW, XD, and XW analyzed the data. LG, YC, and BZ reviewed and edited this article.

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Data availability The RNA-Seq data can be downloaded from NCBI-SRA, PRJNA1017655, and the proteomics data can be downloaded from iProX (http://www.ipox.org), project ID: IPX0009669000. The other data will be made available on request.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval The experiments were performed in compliance with the current laws of China.

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