Function analysis of *ZmZHD9*, a positive regulator in drought stress response in transgenic maize

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Abstract

Backgrounds: Drought stress is one of the major factors that affects maize yield. ZF-HD transcription factors have been proved to play pivotal roles in the regulation of plant growth, hormone conduction signaling and abiotic stress response. However, the molecular mechanism of *ZmZHD9*-mediated drought tolerance is not well understood.

Results: In the present study, we analyzed the functions of *ZmZHD9*, a member of the maize ZF-HD family. *ZmZHD9* is predominantly expressed in leaves, and was induced by drought, salinity, high temperature and abscisic acid (ABA). Subcellular localization indicated that *ZmZHD9* protein was localized in the nucleus. *ZmZHD9*-overexpressing plants showed increased tolerance to drought stress compared with wild-type plants, evaluated by higher RWC and proline content, higher SOD and POD activity, lower REL and MDA content in transgenic plants under drought stress. In addition, the expression of six stress-responsive genes were significantly higher in *ZmZHD9* transgenic plants than that in wild-type plants under drought stress.

Conclusion: These results demonstrate that *ZmZHD9* as a stress-responsive transcription factor which plays a positive regulatory role in response to drought.

Background

Transcription factors (TFs) are proteins that regulate the transcription of genes by binding specific regions within the promoter [1, 2], and play key roles in gene expression regulation in response to external or internal stimuli [3, 4]. Zinc finger homeodomain (ZF-HD) proteins are homeodomain (HD) proteins which also contain conserved zinc finger domains at N-terminus regions and consist of two zinc finger motifs, CH2C and C3H2 [5]. Phylogenetic analyses showed that ZF-HD proteins were divided into two subfamilies, ZHD and MIF (Mini Zinc finger), and MIF genes only possess zinc finger structure but lacking HD domains at C-terminal, which similar with ZHD proteins without introns [6]. Since the first ZF-HD TF was identified in the C4 plant *Flaveria bidentis* [7], a series of studies in plants have focused on ZF-HD TFs, including *Arabidopsis thaliana* [8], *Oryza sativa L.* [9], *Solanum lycopersicum* [10], *Fagopyrum tataricum* [11] and *Glycine max* [12].

ZF-HD genes play pivotal roles including the regulation of plant growth and development, hormone conduction and various environmental stresses [12, 13]. *Arabidopsis* ZF-HD gene *ZFHD1* was induced by drought, high salt, and ABA stress treatments, and its overexpression can enhance series of stress-induced gene expression and drought tolerance [14]. *Arabidopsis* ZF-HD protein *AtHB33* was negatively regulated by *ARF2* and involved in ABA response signal pathway. Over-expression *AtHB33* transgenic plants were sensitive to ABA, while transgenic plants reducing *AtHB33* by RNAi were more resistant to ABA [15]. Liu et al reported that overexpression of *TsHD1* can improve the heat stress resistance of *Thellungiella halophila* and retarded its vegetative growth slightly. The co-overexpression of *TsHD1* and

TsNAC1 highly improved heat and drought stress resistance by increasing the accumulation of heat shock proteins and enhancing the expression levels of drought stress response genes [16].

Although ZF-HD TFs have been identified in many species, less information regarding the ZF-HD genes in maize is available. As one of the most widely cultivated crops, maize frequently suffers from drought stress, especially in Huanghuai region, so screening new genes resistant to drought stress is of great practical significance. We carried out the transcriptome analysis using a drought-resistant maize line Yu882 as experimental material under drought stress and rewatering treatment, and identified many TFs and functional protein families. In this study, In this study, we have analyzed the expression of the *ZmZHD9* gene in different tissues of maize as well as under different abiotic stress. The subcellular localization and transactivation activity of ZmZHD9 protein were also determined. The *ZmZHD9*-overexpression transgenic maize plants were generated to analyze the function in response to drought stress. This study would provide a candidate gene in drought resistance for molecular breeding.

Results

Cloning and Bioinformatics Analysis of ZmZHD9 Gene

ZmZHD9 gene was cloned from Y882 and sequencing analysis showed that the open reading frame was 303 bp encoding a putative protein of 100 amino acids residues of 10.37 kDa with an isoelectric point of 8.93. Phylogenetic relationship analysis of *ZmZHD9* with 17 *Arabidopsis* ZF-HD TFs and 14 *Oryza sativa L.* ZF-HD TFs showed that *ZmZHD9* belongs subfamily of MIF (Mini Zinc finger). *ZmZHD9* was clustered into group with *OsMIF1, OsMIF3, AtMIF2* and *AtMIF2* (Fig. 1A). Multiple alignments showed that *ZmZHD9* proteins contained a Zinc finger domain at its N terminus (Fig. 1B), which suggested that *ZmZHD9 was a member of* subgroup MIF in maize.

Subcellular Localization and Transcriptional Activation Activity Analysis of ZmZHD9 protein

To detect the subcellular localization of the *ZmZHD9* protein, the ORF of *ZmZHD9* gene without the termination codon was cloned and the expression vector for pMDC83-*ZmZHD9*-GFP fusion protein was constructed. The recombinant vector pMDC83-*ZmZHD9*-GFP and pMDC83-GFP were introduced into *Nicotiana benthamiana* leaves and observed under a laser scanning confocal microscopy. As shown in Fig. 2, the pMDC83-*ZmZHD9*-GFP fusion protein was exclusively detected in the nucleus, whereas Green fluorescent protein (GFP) control distributed evenly in the nucleus and the cytoplasm, indicating that *ZmZHD9* gene encoded a nuclear localization protein.

To detect the transcriptional function of the *ZmZHD9* as a transcription factor in maize, we performed the yeast two-hybrid procedure to evaluate its transactivation activity. The complete ORF region of *ZmZHD9* was fused to the GAL4 DNA-binding domain in the pGBKT7 vector and transformed into Yeast strain AH109. As shown in Fig. 3, all transgenic yeasts grew well on SD/-Trp medium. The yeast cells harboring the pGBKT7-*ZmZHD9* plasmid grew as well as the positive control and exhibited positive for α-

galactosidase activity on SD/-Trp/-His/-Ade/X-α-gal medium, suggesting that the ZmZHD9 protein had transactivation activity to act as a transcriptional activator.

Stress-Related Regulatory Elements analysis in the Promoter of ZmZHD9

Cis-elements in promoter regions of genes always play crucial roles in stress responses. *Cis*-elements analysis showed that there were numerous stress-related regulatory elements such as TATA-box, ARE, low-temperature responsive (LTR), dehydration and salicylic acid (SA) and W box in the promoter region of 2000 bp upstream of the ATG start codon, indicating that *ZmZHD9* was involved in plant hormone-related signal transduction and response to abiotic stress (Table 1).

Table 1
Cis-element analysis of the *ZmZHD9* in promotor sequence

Cis-Element	Target Sequences	Number	Function
ABRE	ACGTG	2	ABA-responsive
ARE	AAACCA	4	antioxidant, drought and salt responsive
DRE core	GCCGAC	1	dehydration responsive
LTR	CCGAAA	1	low-temperature and salt responsive
TCA-element	CCATCTTTTT	2	salicylic acid responsive
W box	TTGACC	1	drought and salt responsive
TATA-box	TATATA	3	drought, cold and salt responsive
O_2-site	GATGACATGG	1	zein metabolism regulation
P-box	CCTTTTG	1	gibberellin responsive

Expression Profiling Analysis of ZmZHD9 in Different Tissues and Response to abiotic stress

Tissue-specific expression was performed by qRT-PCR and the result indicated that *ZmZHD9* showed high expression in leaf, root and ear, with weak expression detected in stem, and marginally expression observed in tassel (Fig. 4A). In addition, the expression patterns of *ZmZHD9* of leaves under various treatments, including drought, salt, high temperature and ABA were also investigated. Under drought treatment, the *ZmZHD9* transcript level was pronouncedly induced until reaching the highest level at 4 h, which greater than 2.6-fold of the initial level and followed by decreased at the last time point (Fig. 4B). *ZmZHD9* was also induced by salt treatment and reached the highest level by 1.8-fold at 24 h after treatment (Fig. 4C). After treatment with high temperature, the expression of *ZmZHD9* gradually decreased and reached the lowest level at 48 h, then sharply increased by 9-fold at 60 h than that 48 h (Fig. 4D). The expression of *ZmZHD9* was also increased by exogenous ABA treatment and reached the highest level at 12 h (Fig. 4E). These results indicate that *ZmZHD9* is highly expressed in leaves and that its expression is induced by abiotic stress, such as drought, high salinity and ABA.

Overexpression of ZmZHD9 Enhances Drought Tolerance in Transgenic Maize Plants

To investigate the function of *ZmZHD9*, we performed *Agrobacterium-mediated* transformation and obtained single-copy homozygous T_3 transgenic lines. Finally, three independent *ZmZHD9*-OE transgenic lines (OE8, OE13, and OE17) were selected for subsequent experiments. qRT-PCR analysis showed that the expression levels of *ZmZHD9* were higher in three transgenic plants than that in control plants (WT), respectively, indicating that the transgenic plants were successfully generated as designed (Fig. 5A). Under normal conditions, there was no visible morphological differences in phenotype between 3 transgenic lines (OE8, OE13, OE17) and control (WT), while after natural drought stress for 10 days, the leaves of WT plants appeared severe dehydration and wilting symptoms, whereas the leaves of *ZmZHD9*-OE transgenic plants had significantly delayed leaf rolling (Fig. 5B). the leaf relatively water content (RWC) and proline of three transgenic lines exhibited higher levels than that of WT plants (Fig. 5C and 5D), respectively. Moreover, the relative electrolyte leakage levels (REL) and malondialdehyde (MDA) contents of *ZmZHD9*-OE plants were lower than WT plants (Fig. 5E and 5F). Compared to the WT plants, antioxidant reductases activity of SOD and POD were significantly higher in *ZmZHD9*-OE plants (Fig. 5G and 5H). Taken together, the results indicated that the overexpression of *ZmZHD9* can regulate physiological processes that increase tolerance of transgenic maize plants to drought stress.

Expression Patterns Analysis of ZmZHD9 in Transgenic Plants under Different Abiotic Stress

The expression patterns of *ZmZHD9* gene under abiotic stress in transgenic plants were performed by qRT-PCR. Under drought and salt stress, the expression of *ZmZHD9* was upregulated in transgenic lines that was increased 2.2-fold and 3.3-fold as compared to those in the wild-type plants, respectively (Fig. 6A, 6B). In contrast, *ZmZHD9* was inhibited with much lower expression level under high temperature stress (Fig. 6C). Under ABA treatment, *ZmZHD9* was induced, with 2.7-fold increased expression level than that in wild-type (Fig. 6D). These results are consistent with the expression of *ZmZHD9* gene in non-transgenic plants, indicated that *ZmZHD9* play important role in response to abiotic stress.

Altered Expression of Drought Stress Responsive Genes in Transgenic Plants

To further investigate the pathway regulated by *ZmZHD9* under drought stress, transcript levels of six stress-responsive genes were verified in WT and transgenic maize plants under normal and drought stress. The transcript levels of *ZmPC5S1, ZmABI2, ZmED22, ZmSNC1, ZmDRE2A, and ZmLEA3* had no significant difference between WT and OE plants under normal conditions. Under drought stress, the expression level of *ZmDREB2A* were upregulated in *ZmZHD9* transgenic lines that was increased over 3-fold as compared in the wild-type plants (Fig. 7E). *ZmP5CS1, ZmSAC1*, and *ZmLEA3* were induced by *ZmZHD9* in transgenic plants with about 2.5-fold increased expression level in comparison to that of the WT plants (Fig. 7A, 7D, and 7F). Among all the detected genes, *ZmABI2* and *ZmRD22* were upregulated in *ZmZHD9* transgenic lines by less than 2-fold as compared to those in wild-type plants (Fig. 7B, 7C). All these results suggest that *ZmZHD9* may enhance maize resistance to drought through activate the expression of stress-responsive genes.

Discussion

ZF-HD genes play important role in plant growth, development and abiotic stress response. Significant progress has been made in identifying and characterizing ZF-HD genes in a number of plant species, but only a few maize ZF-HD TF genes were reported. To further study the function of the ZF-HD TFs in maize, we isolated and characterized *ZmZHD9* gene from the former transcriptome result and systemic analyses was conducted in this study. Structural analysis showed that *ZmZHD9* proteins only contained a Zinc finger domain at its N terminus, belonging to subgroup MIF. Tissue expression pattern analysis showed that *ZmZHD9* was highly expressed in leaves, and responsive to drought, high salinity, heat and ABA treatment (Fig. 2B-2E). C*is*-elements are key molecular switches involved in the transcriptional control of dynamic networks of stress-induced gene and play crucial role in response to abiotic stress, such as ABRE, ARE, DRE, LTR, W box and TATA-box [17–20]. In our study, ABRE, ARE, DRE, LTR and W box were detected in the *ZmZHD9* promoter region (Table 1). DREs (dehydration-responsive element) are well-known specific *cis*-elements that are regulated by ABA-independent drought-induced *OsDREB2* transcription factors [21, 22]. Thus, the drought-induced expression of *ZmZHD9* could be regulated in an ABA-independent manner.

With the development of genetic transformation technology, numerous studies have illustrated that manipulating TFs can enhance abiotic stress tolerance by activating stress response signal transduction pathways in transgenic plants (Cao et al. 2017; Jiang et al. 2018). To investigate the function of *ZmZHD9*, overexpression of *ZmZHD9* transgenic maize plants were obtained. Our finding showed that *ZmZHD9-OE* transgenic plants led to increased drought tolerance based on the physiological datum, such as MDA, RWC, REL, proline and enzyme activities. The malondialdehyde (MDA) content and relative electrolyte leakages (REL) are important index to evaluate the damage of plant cell membrane caused by osmotic stress [4, 23]. In this study, MDA content was lower from *ZmZHD9*-OE transgenic lines than wild-type plants under drought stress, indicating that *ZmZHD9*-OE can improve tolerance to oxidative stress caused by drought (Fig. 6F). And the REL exhibited the same result, keep a lower level in *ZmZHD9*-OE transgenic plants than in WT (Fig. 6E), illustrating that drought stress have less membrane damage to transgenic plants than wild-type plants. Previous studies have proved that proline content increased could enhance the concentration of cell protoplasm to maintain normal membrane function under abiotic stresses, which could be improved tolerance to environment stress [24, 25]. In the present study, the proline content in *ZmZHD9*-OE transgenic lines increased, and the increasing rate were significantly higher than wild-type plants under drought stress, the same with Tang's report [26].

Drought and salt stresses often lead to oxidative stress, reactive oxygen species (ROS) accumulation, leading to protein structure damage, membrane peroxidation[4, 27–29]. Scavenging ROS can reduce oxidative damage and improve the plants tolerance to abiotic stresses [30]. Previous study showed that under salt and drought stresses, the ROS scavenging enzyme genes such as SOD, glutathione peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX), and DHAR were systematically up-regulated in the overexpressing transgenic plants [31–33]. In the current study, the activities of SOD and POD in the *ZmZHD9*-OE lines were higher than in the wild-type plants under drought stress (Fig. 6G, 6H), which

contributed to the increased drought tolerance of transgenic maize plants. The results suggesting that *ZmZHD9* gene might protect the cell me membrane integrity by regulating the cellular levels of ROS to under drought stress.

Many reports have revealed that gene-overexpression in transgenic plants lead to induced expression of stress-responsive genes, which in turn leads to enhance tolerance to various stress [34, 35]. For example, *P5CS1* encodes a rate-limiting enzyme, which is upregulated at transcriptional level and necessary for proline accumulation under drought stress treatment [36]. The overexpression of *OsP5CS* in rice can promote the accumulation of proline and increase the resistance to abiotic stress [37]. Furthermore, DREB proteins belong to AP2/ERF TF family, which were indeed implicated responses to drought stress in plants [38]. In *Arabidopsis*, *DREB1A* and *DREB2A* specifically interact with dehydration-responsive element (C-repeat) involved in drought stress-responsive gene expression [21]. Subsequent studies showed that *ZmLEA3*, *ZmSNAC1* and *RD22* are stress-inducible genes that improve resistance to abiotic stress in transgenic plants [39–41]. Similarly, in our study, the expression of stress-responsive genes including *ZmP5CS1, ZmABI2, ZmED22, ZmSNC1, ZmDRE2A* and *ZmLEA3* were higher in transgenic lines than in wild-type plants under drought stress (Fig. 8). However, the expression of these genes were no significant differences under normal condition, despite the fact that constitutive promoter was to drive the gene expression. One possible to explanation is that other stress-responsive regulators are required to activate *ZmZHD9*-dependent stress responsive gene under drought stress. A similar observation has been reported in rice *OsMYB6* transgenic lines [26]. Taken together, *ZmZHD9* overexpression transgenic plants can enhance drought tolerance possible due to the reinforced expression of these stress-responsive genes.

Conclusions

In this study, we isolated and characterized the maize *ZmZHD9* gene, phylogenetic tree and sequence analyses confirmed that *ZmZHD9* belongs to MIF subgroup which contain Zinc finger domain at its N terminus. The promoter region of *ZmZHD9* gene contains multiple core elements responsive to abiotic stresses and hormones. qRT-PCR results showed that *ZmZHD9* genes was up-regulated in response to PEG, NaCL and ABA treatment. Subcellular localization assay showed that ZmZHD9 protein is localized in the nucleus. Over-expression of *ZmZHD9* in transgenic maize remarkably improved drought resistance. Moreover, over-expression of *ZmZHD9* enhanced the expression of stress-responsive genes indicating that *ZmZHD9* may as a stress-responsive transcription factor which plays a positive regulatory role in response to drought. These results suggested that *ZmZHD9* could act as a potential candidate gene for genetic engineering to improve drought and other abiotic stress.

Methods

Plant materials, Growth Condition and Abiotic Stress

Maize (*Zea mays L.* Yu882), tobacco (*Nicotiana benthamiana*) were used in this study. All seeds were provided by laboratory of Professor Li xia Ku of Henan Agricultural University. Seeds of Yu882 were selected and sown in soil and vermiculite mixture (3:1) in a growth chamber at 25 ± 2℃ under a long-day conditions, with 16 h/8h (light/dark) photoperiod cycles, 70% relative humidity and a light density of approximately 300 μmol/ (m^{-2}·s^{-1}). The nutrient solution was replaced every 2 days. When the seedlings have 3-fully expanded leaves, they were transferred into Hoagland nutrient solution with drought stress (20% PEG6000), salinity (200 mmol/L NaCL), ABA (5 μmol/L) and heat stress (37 °C) treatments. The second fully expanded leaves were sampled at 0, 4, 12, 24, 48 and 60 h were collected, frozen in liquid nitrogen immediately and stored at -80℃ for further expression analysis. Three plants from different treatment were used as biological replicates.

Gene Cloning and Sequence Analysis

Total RNA was extracted from leaves using Trizol reagent (TaKaRa, Dalian, China) following the manufacturer's instructions. RNA integrity and purity were analyzed by spectrophotometry and 1% agarose gel electrophoresis. 1 μg RNA was took for first-strand cDNA synthesis using the Prime Script™ RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. Based on the sequence of *ZmZHD9*, the RT-PCR primers (Additional file 1: Table S1) were designed to amplify the full length of *ZmZHD9* gene. The PCR product was cloned into the pMD19-T vector, and sequenced to confirm the accuracy.

The nucleotide and amino acid sequence of *ZmZHD9* were used to search its homologous genes and proteins by using the database of National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). DNAMAN software (Version 5.2.2.0; Lynnon Biosoft, USA) was used to alignment the amino acid sequences of *ZmZHD9* and its homologs. A phylogenic analysis of ZF-HD proteins from maize and other species were performed by MEGA 6.0 by the neighbor-joining method [42]. The online database *Expasy* (http://web.expasy.org/protparam/) was used to predicted molecular weight (MW) and isoelectric point (PI) of *ZmZHD9* protein [43]. The online website: PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to predict *Cis*-acting elements that respond to abiotic stresses in the promoter region (the 5□upstream regions 2000 bp) [44] .

Expression Profile Analysis by qRT-PCR

qRT-PCR analyses were performed according TB Green *PremixEx Taq™* II (TaKaRa, Dalian, China) manufacturer's instructions with following program: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. Quantitative RT-PCR was carried out with a CFX96 system (Bio-Rad, CA, USA). The *18S* (GenBank No. AF168884.1) gene was used as an internal control. The method of $2^{-\Delta\Delta CT}$ was used to calculate the relative expression level [45]. All qRT-PCR experiments contained three biological replicates. And all primers were list in Additional file 1: Table S1.

Subcellular Localization of ZmZHD9 Protein

The coding region of *ZmZHD9* was amplified by PCR using primers containing *Asc I* and *Spe I* site (Additional file 1: Table S1) and the PCR products were digested with *Asc I* and *Spe I* and then inserted

into the same enzymes digested pMDC83-GFP to product a fusion protein (pMDC83-*ZmZHD9*-GFP). The fusion vector was introduced into EHA105 and transiently expressed in leaves of *N. benthamiana* (1-month-old) according infiltration method [46]. The infected tobacco was cultured for 24 h in the dark and then transferred to a light incubator. The GFP-associated fluorescence of fusion constructs was detected after 48 h using a fluorescence microscopy (LSM 700, Carl Zeiss, and Jena, Germany).

Transactivation Activity of ZmZHD9 in Yeast

The coding region of *ZmZHD9* were amplified from cDNA templates from leaves sample using a pair of gene-specific primers (Additional file 1: Table S1) and then ligated into *EcoR I* and *BanH I*-digested pGBKT7 vector. The vector pGBKT7-*ZmZHD9*, pGADT7 empty vector (negative control) and pGBKT7-53 vector (positive control) were transformed into Yeast strain AH109 according the lithium acetate-mediated method, respectively. The transformed Yeast cells were examined on the solid medium plates of SD/-Trp and SD/-Trp/-Ade/-His/X-α-gal (5-Bromo-4-chloro-3-indolyl-α –D-galactoside) at 30℃ for 3-5d.

Generation of Transgenic Maize of ZmZHD9 Gene

The CDS of *ZmZHD9* was amplified using the primers containing the *Asc I* and *BamH I* sites (Additional file 1: Table S1). The PCR product and the expression vector pFGC5941 were double digested with *Asc I* and *BamH I*, and the vector pFGC5941-*ZmZHD9* was obtained with T4 DNA ligase. The expression vector was transferred into the *Agrobacterium tumefaciens* strain EHA105 for maize transformation by using *Agrobacterium*-mediated transformation method [47].

Drought stress Tolerance Analysis of ZmZHD9-overexpressing Transgenic Maize

Three transgenic line (OE8, OE13 and OE17) and wild-type (WT) plants were grown in pots containing soil and vermiculite mixture (3:1) in greenhouse. When seedlings were at the three-leaf stage, transgenic lines and WT plants stopped watering to practice natural drought stress for 10 days. The control groups were watered normally. Leaves from transgenic and WT plants were collected for qRT-PCR and physiological traits analysis. Drought stress treatment contained three biological replicates.

For other abiotic stress, the seedings of 3-fully expanding leaves were transferred into Hoagland nutrient solution with drought stress (20% PEG6000), salinity (200 mmol/L NaCL), ABA (5 μmol/L) and heat stress (37 °C) treatments. The second fully expanded leaves were sampled at 24 h were collected, frozen in liquid nitrogen immediately and stored at -80℃ for further expression analysis. Three plants from different treatment were used as biological replicates.

Physiological and Biochemical Analysis of Transgenic Maize

Leaves fresh weight (FW), dry weight (DW) and saturated weight (SW) were measured to calculate the relative water content (RWC) based on the formula: RWC = [(FW − DW)/ (SW − DW)] × 100%. The relative electrolyte leakage (REL) was measured referring to the method described by with some modifications [48]. Proline content was detected as described method by Zhao [49]. The malondialdehyde (MDA) content was detected according to the method reported by Xia [50]. The superoxide dismutase (SOD) and

peroxidase (POD) activities were estimated according to described method by Zhang [51]. All of the measurements have three replicates.

qRT-PCR Analysis of Stress-responsive Gene Expression Profiles in Transgenic Maize

To examine the expression of stress -responsive genes, total RNA was extracted from the leaves of WT and transgenic maize under normal condition and drought stress for 10d. Then, the first-strand cDNA was synthesized and used as template for qRT-PCR analysis. The stress -responsive genes including *ZmP5CS1* (BT083588.1), *ZmABI2* (EU956794.1), *ZmRD22* (BT040391.1), *ZmSNAC1* (JQ217429.1), *ZmLEA3* (Z29512.1) and *ZmDREB2A* (AB218833.1). The *18S* (GenBank No. AF168884.1) gene was used as an internal control. All relevant primers were listed in Additional file 1: Table S1.

Statistical Analysis

All values reported in this study were the means of three independent replicate measurements. Statistical significance of the differences was analyzed by SAS software using Duncan's multiple-range test with a significance level of 0.05 ($P < 0.05$).

Abbreviations

qRT-PCR: Quantitative real time polymerase chain reaction; ABA: abscisic acid; ROS: reactive oxygen species; RWC: relative water content; REL: relative electrolyte leakage; MDA: malondialdehyde; SOD: superoxide dismutase; POD: peroxidase; GPX: glutathione peroxidase; APX: ascorbate peroxidase; CAT: catalase

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PY, LR and LX designed the experiments. PY and QX performed the simulations and analyzed the corresponding results. PY and JX performed the experiments and analyzed the results. PY and GR wrote the paper. WL and TC supervised this whole process and reviewed this paper. All authors read and approved the final manuscript.

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Figures

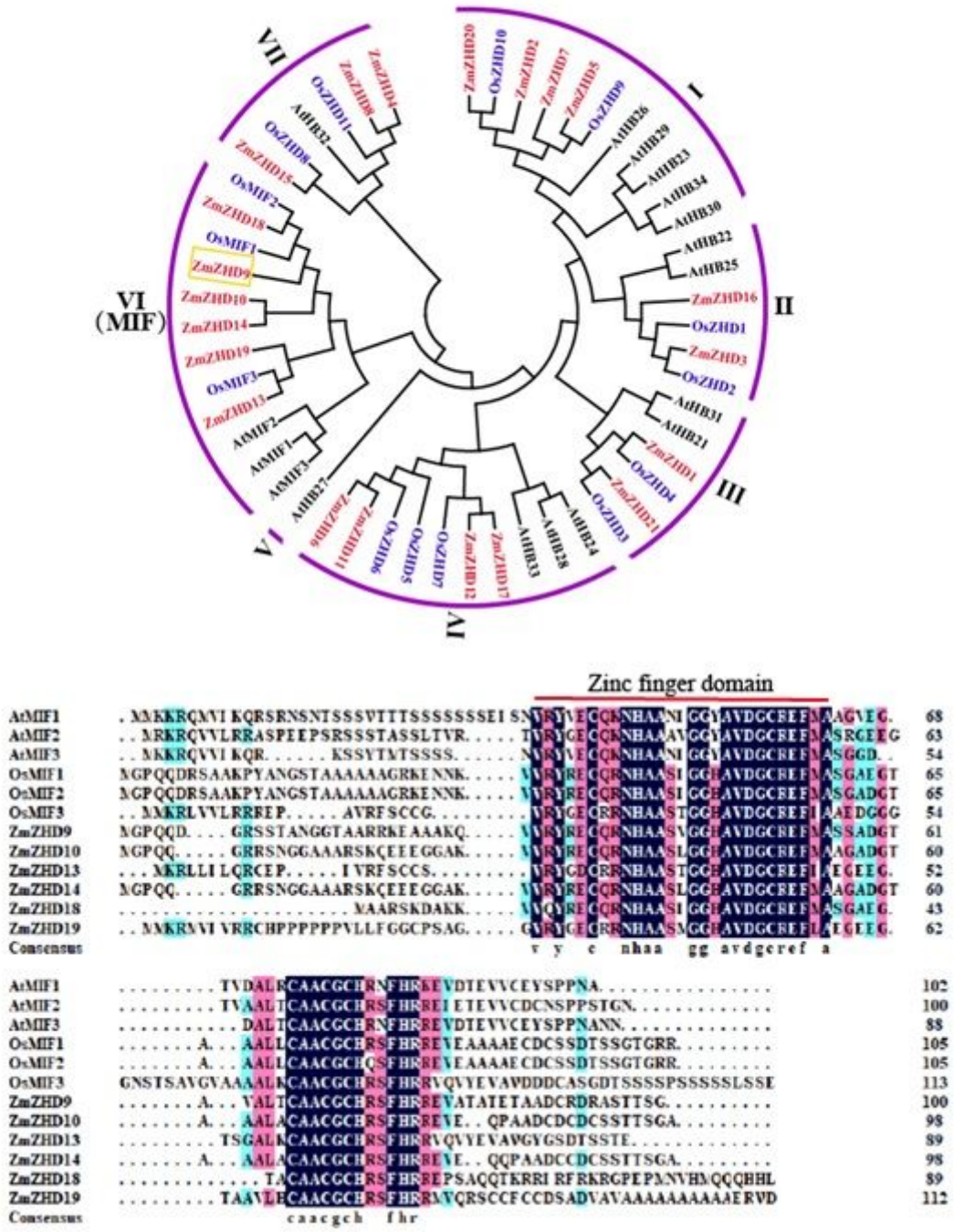

Figure 1

Sequence analysis of the ZmZHD9 protein. (A) Phylogenetic relationship of the ZmZHD with Arabidopsis (AtHB) and Oryza sativa L.(OsZHD) ZF-HD protein. The ZF-HD proteins of the Arabidopsis and Oryza sativa L. were downloaded from the NCBI. The neighbor-joining (NJ) phylogenetic tree was constructed

using MEGA 6.0 with the following parameters: bootstrap (1000 replicates), and pairwise deletion gaps. (B) Multiple sequence alignment of ZF-HD proteins among subgroup Ⅱ. Clustal Omega software was used to align the ZF-HD protein sequences using default parameters and the results were minor repaired by DNAMAN Version 9.0 software. The domains have been indicated.

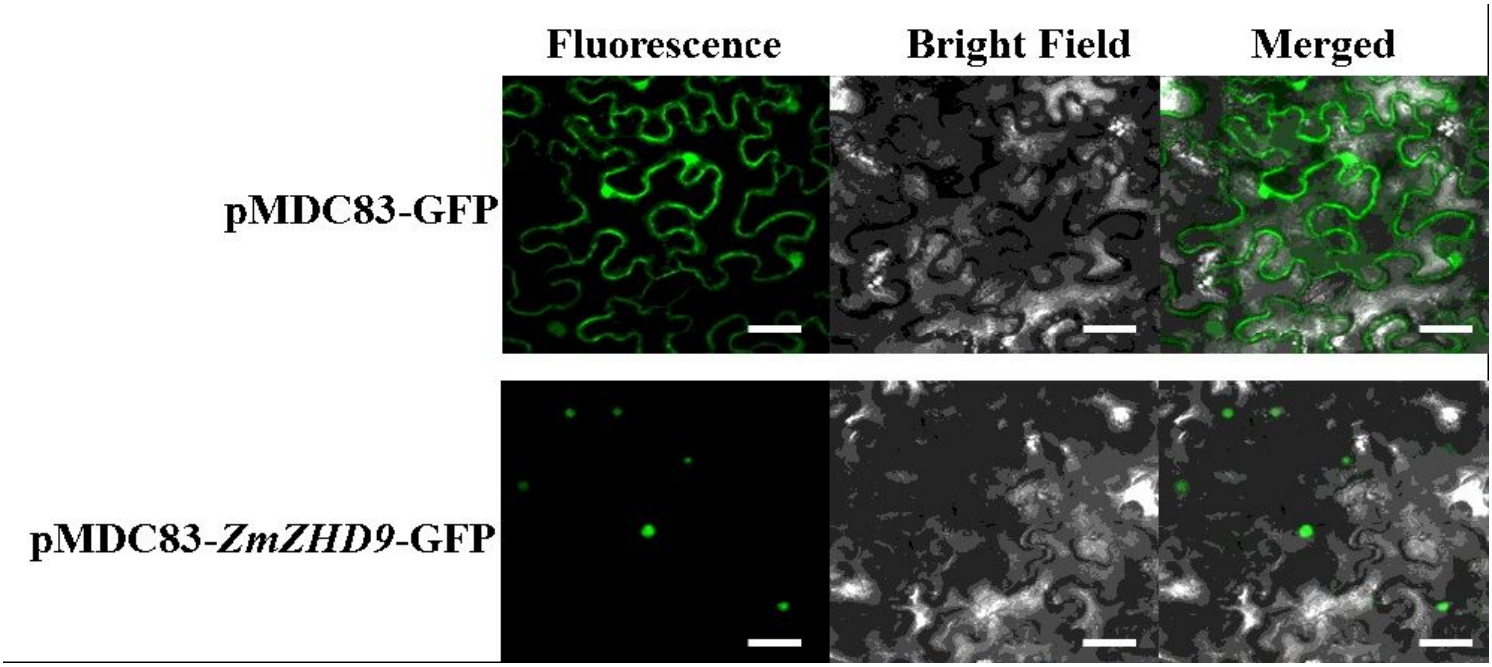

Figure 2

Subcellular localization of the ZmZHD9-GFP protein in Nicotiana benthamiana epidermal cells. The scale bars represent 20μm.

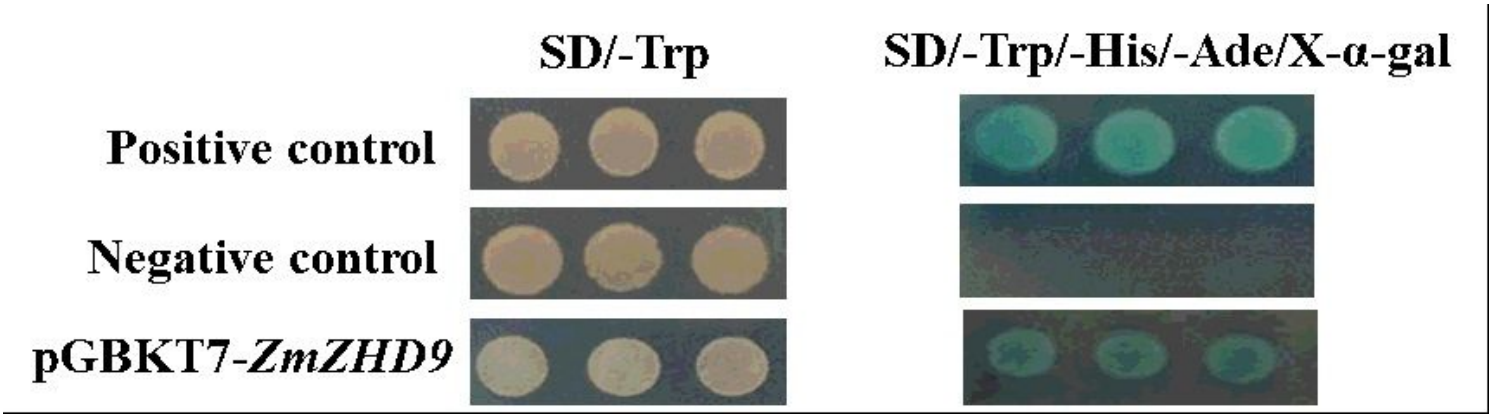

Figure 3

Transcriptional activation assays of ZmZHD9 in yeast cell. Growth of transformed yeast cells 3 days after spotting on selective mediums. Positive control, yeast cells transformed with pGBKT7-53 and pGADT7-T; Negative control, yeast cells transformed with pGBKT7-Lam and pGADT7-T. Experiments were performed three times with similar results.

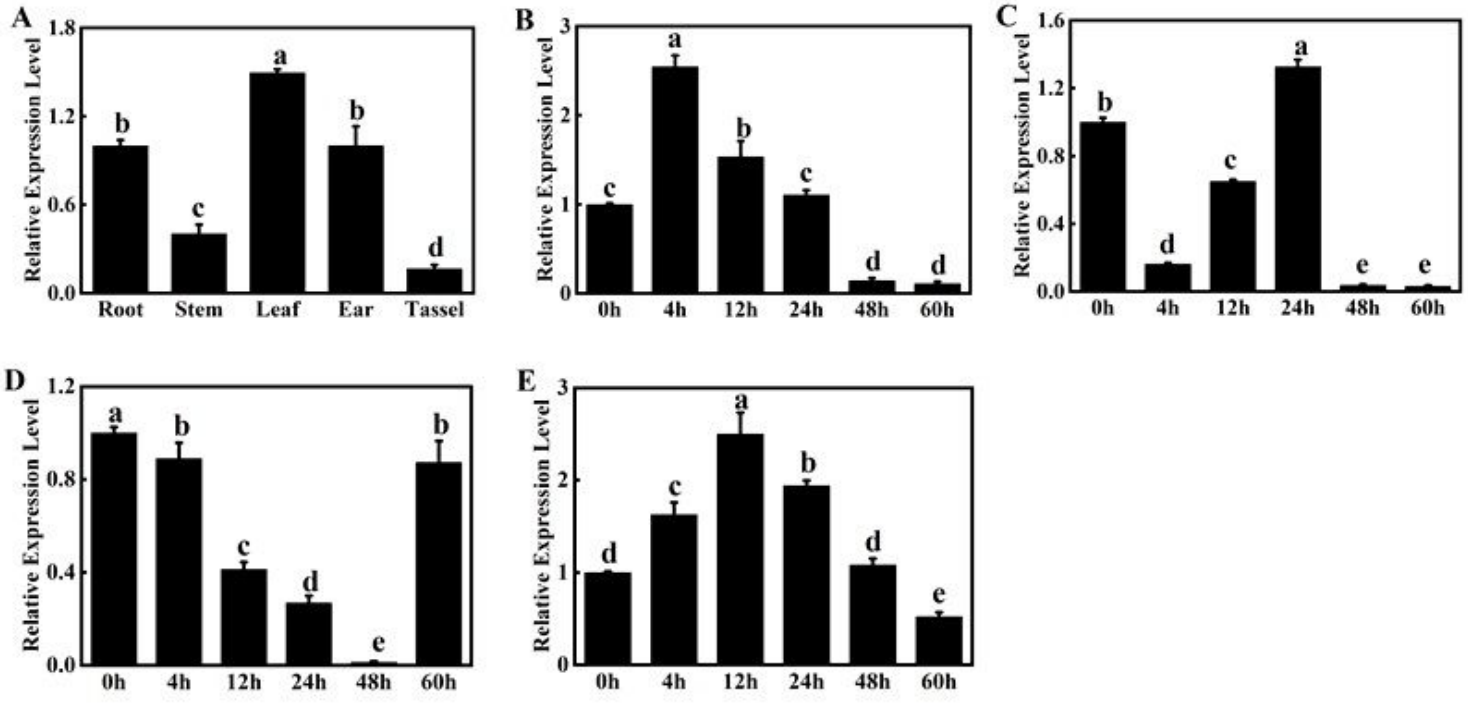

Figure 4

The ZmZHD9 expression in different tissues and response to abiotic stress. (A) Tissue-specific expression patterns of ZmZHD9 in maize. The expression level in root was set as 1. (B-E) The expression profiles of ZmZHD9 under various stress treatments, including (B) 20% PEG6000 (drought); (C) 300mM NaCL (high salinity); (D) 37°C (heat) and (E) 5μM abscisic acid (ABA) treatment. The maize 18S (small subunit ribosomal RNA gene) was used as an internal control. The expression at 0h is set as 1. Data represent the mean value ± standard deviation (SD) of three biological replicates. Different lowercases letters denote statistically significant differences from the control treatment at $p \leq 0.05$ by t-test.

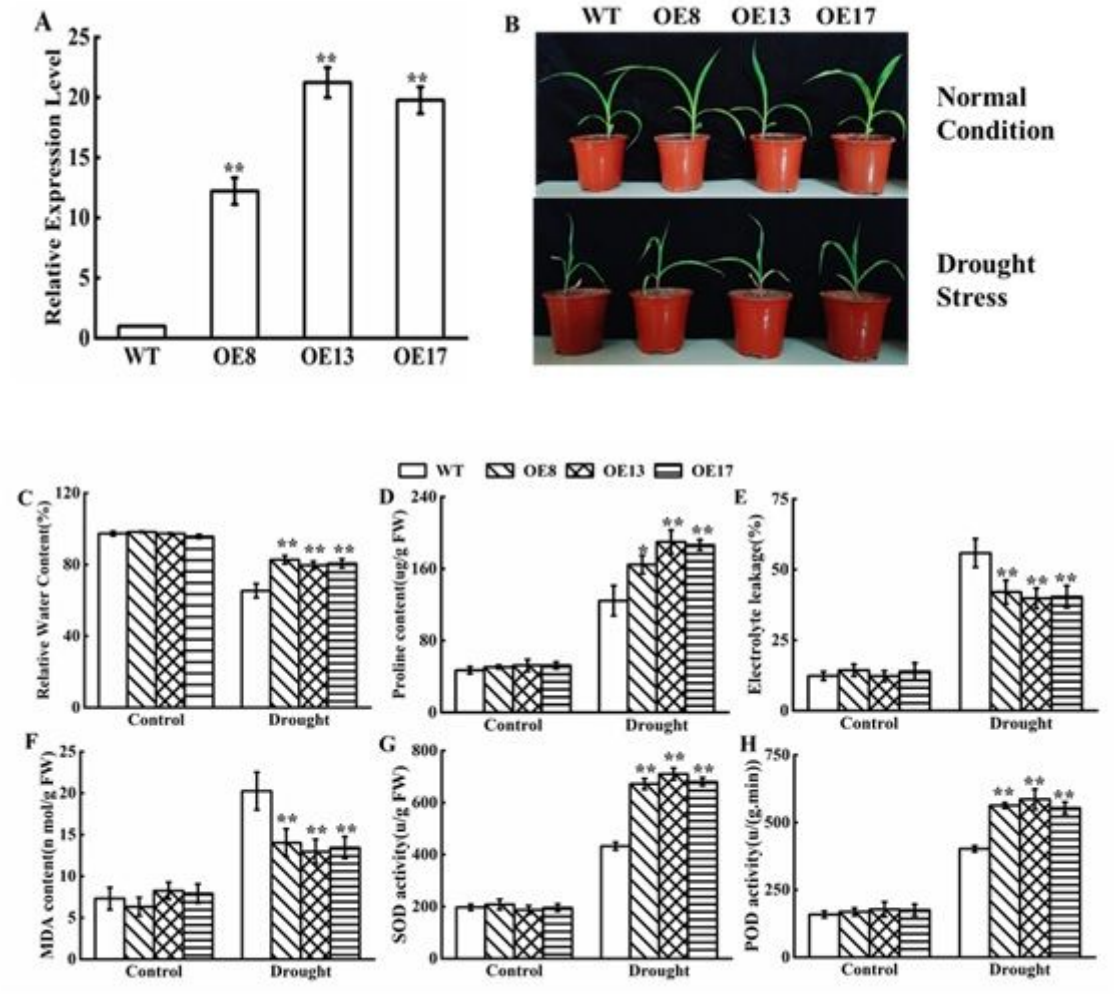

Figure 5

ZmZHD9 overexpression in maize enhances drought tolerance. (A) The relative expression levels of ZmZHD9 in transgenic lines and WT. (B) The visual phenotype of the transgenic maize plants during drought treatment. ZmZHD9-OE transgenic plants and wild-type plants under normal condition and drought stress for 10 days. (C) Relative water content (RWC); (D) Proline content; (E) Relative electrolyte leakage (REL); (F) MDA content; (G) SOD activity; (H) POD activity. Each data point is the mean (± SE) of three experiments. Significant differences from the WT control are indicated by asterisks (Student's t-teat, *p≤0.05;**p≤0.01)

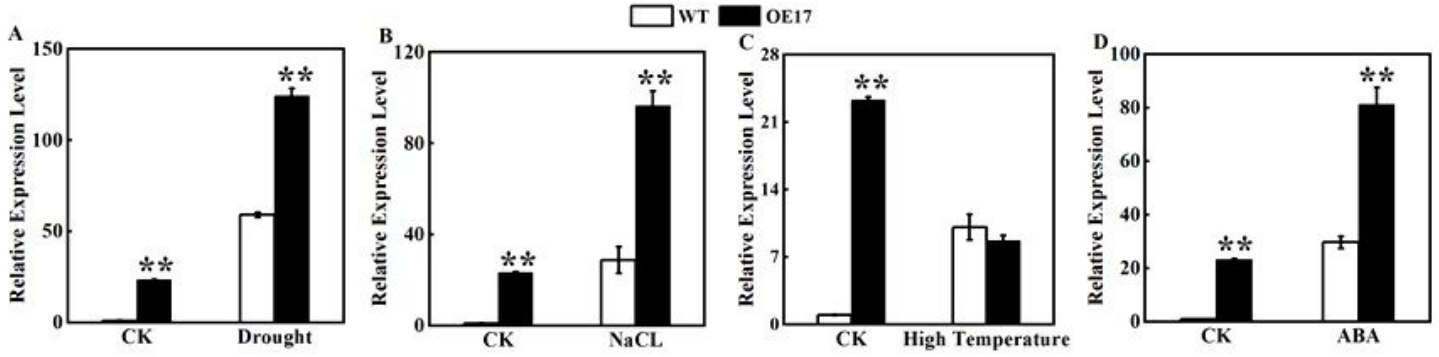

Figure 6

The expression profile of ZmZHD9 in transgenic line (OE17) and wild-type (WT) under different abiotic stress. (A) 20% PEG (drought stress) (B) NaCL (high salinity) (C) 37°C (heat treatment) (D) abscisic acid (ABA). Leaves of maize plants were harvested at the 24h after treatment for gene expression analysis. The experiment included three biological replicates. Data represent the mean value ± standard deviation (SD) (n=3). Significant differences from the WT control are indicated by asterisks (Student's t-teat, *p≤0.05;**p≤0.01)

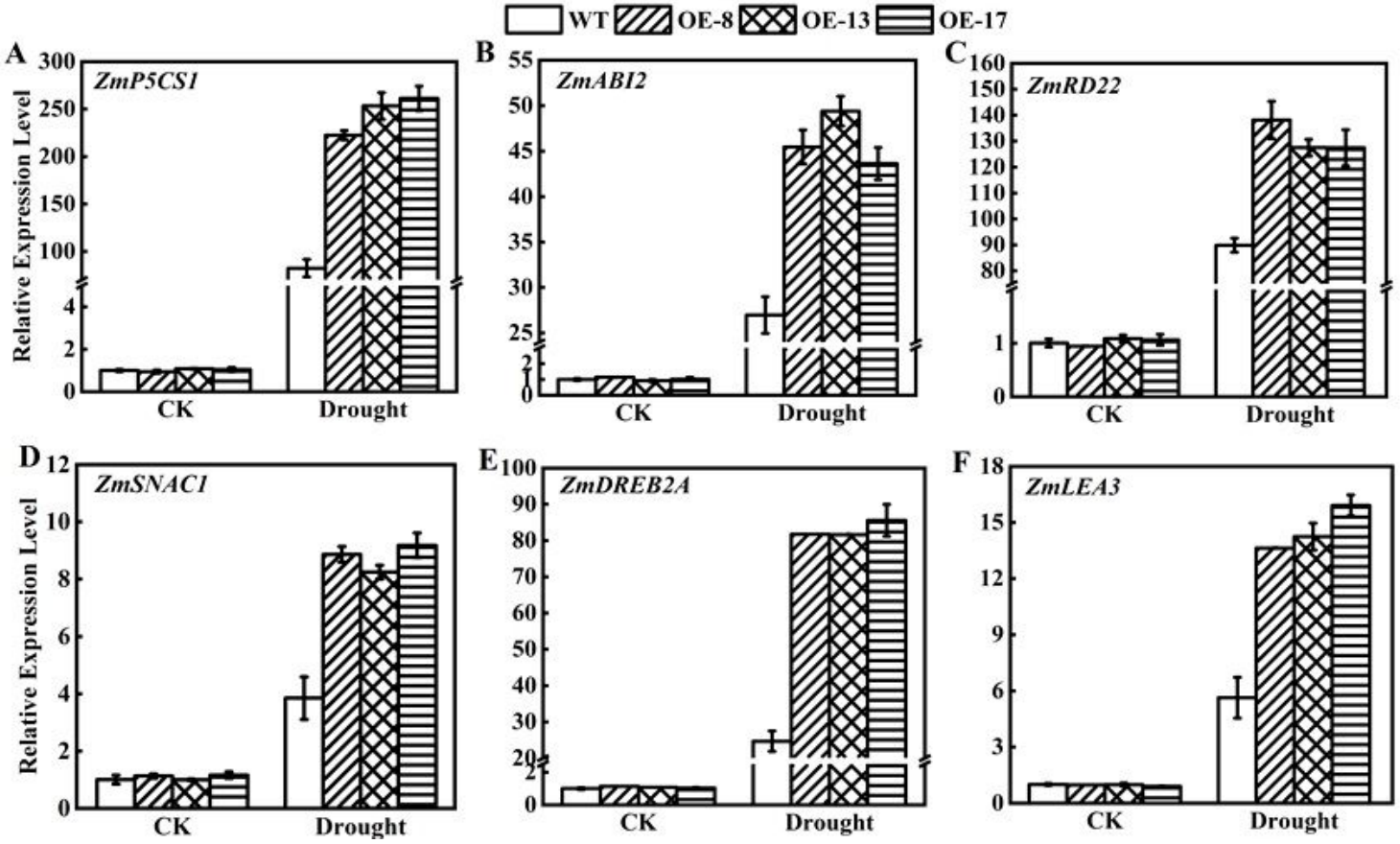

Figure 7

The expression of stress-responsive genes in the WT and transgenic plants under normal (CK) and drought stress (Drought) condition. The experiment included three biological replicates. Data represent

the mean value ± standard deviation (SD) (n=3). Significant differences from the WT control are indicated by asterisks (Student's t-teat, *p≤0.05;**p≤0.01)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS11.xlsx