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RESEARCH ARTICLE

Genome-wide identification of HD-ZIP transcription factors in maize and their regulatory roles in promoting drought tolerance

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Abstract Drought is the main limiting factor of maize productivity, therefore improving drought tolerance in maize has potential practical importance. Cloning and functional verification of drought–tolerant genes is of great importance to understand molecular mechanisms under drought stress. Here, we employed a bioinformatic pipeline to identify 42 *ZmHDZ* drought responsive genes using previously reported maize transcriptomic datasets. The coding sequences, exon–intron structure and domain organization of all the 42 genes were identified. Phylogenetic analysis revealed evolutionary conservation of members of the *ZmHDZ* genes in maize. Several regulatory elements associated with drought tolerance were identified in the promoter regions of *ZmHDZ* genes, indicating the implication of these genes in plant response to drought stress. 42 *ZmHDZ* genes were distributed unevenly on 10 chromosomes, and 24 pairs of gene duplications were the segmental duplication. The expression of several *ZmHDZ* genes was upregulated under drought stress, and *ZmHDZ9* overexpressing transgenic plants exhibited higher SOD and POD activities and higher accumulation of soluble proteins under drought stress which resulted in enhanced developed phenotype and improved resistance. The present study provides evidence for the evolutionary conservation of HD-ZIP transcription factors homologs in maize. The results further provide a comprehensive insight into the roles of *ZmHDZ* genes in regulating drought stress tolerance in maize.

Keywords Maize · HD-ZIP · Drought stress · Overexpression · Genetic transformation

Introduction

Abiotic stresses such as salinity, drought and heat are major factors that influence the growth, development and yield of crop plants (Abou-Elwafa and Shehzad 2021). To cope with these adverse stresses, plants adopt complex responses at the physiological, metabolic, and molecular levels (Tan et al. 2017), accordingly, numerous defense responsive genes are transcriptionally activated or inhibited during this process (Sekhwal et al. 2015). The expression of stress-responsive genes is mainly controlled by specific transcription factors (TFs) (Hu et al. 2008; Rabara et al. 2014). TFs are proteins that bind to a specific DNA sequence to control the transcription rate of a gene. Based on the characteristics of the DNA-binding domain, TFs are classified into different families, including v-avian myeloblastosis viral oncogene homolog (MYB), NAC, WRKY, APETALA2/Ethylene Responsive Factor (AP2/ERF) and homeodomain-leucine zipper (HD-ZIP) (Du et al. 2014; Li et al. 2016, 2019b; Wang et al. 2020; Zhang et al. 2020; Zhao et al. 2018). *HD-ZIP*, which is exclusively found in plants, is a transcription factor composed of conserved HD and LZ motifs and plays pivotal roles in regulating plant development and in response to abiotic stresses (Ariel et al. 2007). Since the first plant HD-Zip

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gene *KNOTTED1* was cloned from maize, HD-ZIP genes were identified and characterized in Arabidopsis (Ciarbelli et al. 2008), rice (*Oryza sativa*) (Meijer et al. 2000), tobacco (*Nicotiana tabacum*) (Li et al. 2019a), sunflower (*Helianthus annuus*) (Dezar et al. 2010), wheat (*Triticum aestivum*) (Yue et al. 2018) and soybean (*Glycine max*) (Belamkar et al. 2014; Valdés et al. 2012).

According to the encoded protein structure, physiological function and conserved domain, HD-Zip family members were classified into four categories: I–IV (Ariel et al. 2007). The HD-ZIP I genes are mostly implicated in regulating plant response to abiotic, environmental and biological stresses, optical signal reaction and organ development. In Arabidopsis, *HOMEOBOX7* (*AtHB7*) and *HOMEOBOX12* (*AtHB12*) play crucial roles in the primary response to drought by mediating the negative feedback effect on ABA signaling in plant response to drought (Ré et al. 2014). The overexpression of *ZmHDZ1* in rice reduces plant tolerance to salinity stress, suggesting that *ZmHDZ1* adversely regulates plant tolerance to salinity via ABA-mediated signal transduction pathways (Wang et al. 2017). The expression of the HD-ZIP II gene was regulated by optical signals and involved in organ development, hormone response and shade avoidance. Similar to *A. thaliana* and several other dicots, numerous rice drought-responsive genes including *HOX6*, *HOX22* and *HOX24*, have been assigned to HD-ZIP groups I and II, and the expression of these genes was upregulated under drought conditions (Agalou et al. 2008). The overexpression of *Hahb-4* in Arabidopsis resulted in shortened stems and internodes, tight catkins and improved drought tolerance (Dezar et al. 2005; Manavella et al. 2006). The genes belonging to HD-ZIP group III mainly control the formation of vascular tissue, polar transport and embryo development. Five HD-ZIP III genes were identified in Arabidopsis, among them, *IFL1*, *AtHB9* and *AtHB14* are related to the development of the apical meristem, vascular bundle and lateral tissues, which affect the development of root tip during the embryonic stage and control the formation of the root tip (Byrne 2006). The HD-ZIP genes of group IV are mostly implicated in root development and epithelial cell differentiation. The Arabidopsis *HOMEOBOX10* (*AtHB10*) manipulates the transcription of specific genes in the epidermal cells and mostly functions in the hairy root, epidermal hair and seed coat (Dezar et al. 2010). Taken together, the overexpression of exogenous HD-ZIP genes could promote abiotic stress tolerance of transgenic plants.

Maize is an important food and industrial crop worldwide, and water shortage is a key factor in reducing maize yield, therefore improving drought tolerance in maize has potential practical significance (Su et al. 2021). Cloning and functional verification of drought–tolerance genes is of great importance to understand molecular mechanisms under drought stress. In this study, 42 maize *HD-ZIP* (*ZmHDZ*) genes associated with drought tolerance were identified from previous transcriptomic datasets (accession number PRJNA477643) generated under drought-stressed and rewatering treatments (Cao et al. 2019). The full-length genomic and coding sequences, exon–intron structure and domain organization of the 42 genes were identified. Moreover, phylogenetic and expression profile analyses of these genes were performed. A representative *HD-ZIP* gene, i.e., *ZmHDZ9*, was functionally characterized by overexpression in the *A. thaliana* Col-0 wild type (WT). *ZmHDZ9* transgenic plants exhibited a higher drought–tolerant phenotype compared to the WT plants, indicating that *ZmHDZ9* enhances drought tolerance in transgenic plants.

Materials and methods

Identification and bioinformatics analysis of *HD-ZIP* genes in maize

The Hidden Markov Model (HMM) profile of the HD domain (PF00046) obtained from Pfam database (http://pfam.sanger.ac.uk/) was employed as a query to identify all HD-containing sequences in maize by searching HD domain sequence against the maize genome database using BlastP program (p value = 0.001). The PFAM and SMART (http://smart.embl-heidelberg.de/) were implemented to identify homologs of the HD-ZIP domains-containing (PF02183) transcription factors in maize (*ZmHDZs*). The genomic sequence, molecular weight (MW), isoelectric point (PI) and localization information of *ZmHDZ* genes were redeemed from the EnsemblPlants database (http://plants.ensembl.org/index.html). The Gene Structure Display Server 2.0 was employed for the annotation of exon–intron structures of *ZmHDZ* genes. The unrooted phylogenetic tree was generated using the Neighbor-Joining algorithm and the Dayhoff PAM matrix as in the MEGA6 software (http://www.megasoftware.net/). PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used for annotation of the promoter regions of *ZmHDZ* genes (2 kb upstream of the transcription start site (TSS)) for the identification of *cis*-elements. Seven *cis*-regulatory elements including the ABA-responsive element (ABRE), MYB binding site (MBS), SA-responsive elements (TCA), TC-rich, MeJA-responsive elements (CGTCA-motif), LTR and TATC-box were chosen for further analysis. A coexpression network map of genes was generated by the online software Cytoscape version 3.7.1 (https://cytoscape.org). The chromosomal location and duplication information of the identified 42

ZmHDZ genes were analyzed using TBtools software (Chen et al. 2020a).

RNA extraction and RT-qPCR

Trizol reagent (TaKaRa, Dalian, China) was used to extract total RNA from leaves according to the manufacturer's procedure. After DNase treatment, the integrity and purity of isolated RNA were analyzed using spectrophotometry and electrophoresis on1% agarose gel. cDNA was synthesized using about 2 µg of RNA using the First Strand cDNA Synthesis SuperMixKit (YEASEN) for RT-qPCR assay.

RT-qPCR analysis was conducted according to the procedure of TB Green *PremixExTaq*^{TM} II (TaKaRa, Dalian, China). The 18S RNA was chosen as a reference gene. The $2^{-\Delta\Delta CT}$ method was employed to estimate the relative expression (Livak and Schmittgen 2001). RT-qPCR experiments were conducted using three biological and three technical replicates.

Subcellular localization

The full-length cDNA sequence of *ZmHDZ9* was ligated into the pSAT1-cCFP-C and pSAT1-nVenus-C vectors (Biovector NTCC Inc., Beijing, China) to produced ZmNF-YC2-GFP, ZmAP2-GFP and ZMM4-GFP fusion proteins. The recombinant plasmids were introduced into the maize protoplast following the procedure described by Gomez-Cano et al. (2019). After overnight incubation at 24 °C in the dark, a laser scanning confocal microscope (excited at 488 nm for GFP) was employed for the detection of GFP signals in the protoplast (Carl Zeiss LSM710, Jena, Germany).

The complete coding region of *ZmHDZ9* was amplified and ligated into the pMDC83-GFP vector to generate a fusion protein. The recombinant vector was transferred by electroporation into *Agrobacterium tumefaciens* EHA105 competent cells and transiently expressed in 4-weeks old *N. Benthamiana* leaves following the infiltration procedure described by Yang et al. (2000). The laser scanning fluorescence microscope (Carl Zeiss LSM710) was employed to assay the GFP-associated fluorescence of fusion constructs after 48 h.

Vector construction and generation of transgenic plants

The complete *ZmHDZ9* cDNA sequence was amplified. After restriction enzyme digestion, the PCR product was ligated into the corresponding restriction sites of the binary vector pFGC5941 derived by the CaMV 35S promoter. The intactness of the binary vectors to *ZmHDZ9* cDNA sequence was verified by enzymatic restriction digestion and PCR assay, followed by sequencing. The construct (pFGC5941-*ZmHDZ9*) was transferred by electroporation into *Agrobacterium tumefaciens* LBA 4404 competent cells and transformed into *A. thaliana*Col-0 wild-type plants using the floral dip approach (Clough and Bent 1998). The integration of the transgene in the genomes of T1 plants was verified by PCR assay (Abou-Elwafa et al. 2010). Two independent homozygous lines, OE1 and OE2, were chosen for further analysis.

Drought stress assays of *ZmHDZ9* transgenic Arabidopsis plants

After surface sterilization, seeds of OE1 and OE2 lines were stored for 15 d at 4 °C. Sterilized seeds were cultivated on 1/2 MS medium and grown in a climate chamber (light intensity of 100 µmol m^{-2} s^{-1} of photosynthetically active radiation for 14 h at 22 °C). Four-leaf old seedlings were transplanted into a 3:1 soil and vermiculite mixture. Drought stress was applied to 9–10 leaves-old seedlings by withholding water for 12 days.

Measurement of physiological and biochemical parameters

Twelve days after water withholding, leaves were sampled for measuring physiological parameters. Leaves from four Arabidopsis seedlings were randomly sampled from each treatment. Chlorophyll and soluble protein contents in the leaves were measured referencing the guidance of physiological experiments (Zhang 1992). Chlorophyll content was analyzed using the HPLC procedure (Montefiori et al. 2009). The activity of superoxide dismutase (SOD) and peroxidase (POD) was assayed using analytical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the procedure described by Zhang et al. (2012).

Coexpression and statistical analysis

According to the provided target gene expression in each sample, Pearson correlation analysis between the 42 *ZmHDZ* genes based on their expression levels measured as Fragments per kilobase of transcript per million mapped fragments (FPKM) was performed to obtain correlation coefficients and p value significance between genes. Finally, the filtered network into Cytoscape version 3.7.1 (https://cytoscape.org) was employed to create the coexpression network map. Student's t-test was employed to statistically analyze the differences in experimental data between groups. p values of ≤ 0.05 or 0.01 indicate significant or highly significant respectively, differences

Table 1 Molecular information of 42 *ZmHDZ* genes identified in the maize genome

Gene name	ID gene identifier	Chromosome	Protein size (aa)	Molecular weight (kDa)	Theoretical IP
ZmHDZ1	GRMZM2G021339	4	339	37	4.36
ZmHDZ2	GRMZM2G122076	4	272	17.82	6.55
ZmHDZ3	GRMZM2G003304	1	270	29.99	4.75
ZmHDZ4	GRMZM2G351330	2	261	29.38	4.76
ZmHDZ5	GRMZM2G178741	9	344	37.57	6.5
ZmHDZ6	GRMZM2G117164	5	235	26.41	4.77
ZmHDZ7	GRMZM2G002915	2	283	31.22	5.12
ZmHDZ8	GRMZM2G056600	7	261	28.46	4.5
ZmHDZ9	GRMZM2G041462	7	239	26.24	5.22
ZmHDZ10	GRMZM2G041127	2	305	33.04	4.53
ZmHDZ11	GRMZM2G139963	1	344	37.6	6.75
ZmHDZ12	GRMZM2G034113	2	244	26.79	5.22
ZmHDZ13	GRMZM2G132367	1	326	35.53	4.85
ZmHDZ14	GRMZM2G134260	9	293	31.1	9.65
ZmHDZ15	GRMZM2G366130	2	325	34.52	7.59
ZmHDZ16	GRMZM2G068672	6	261	27.37	9.73
ZmHDZ17	GRMZM2G307397	5	247	26.54	10.15
ZmHDZ18	GRMZM2G148074	1	321	34.83	8.62
ZmHDZ19	GRMZM2G047715	4	331	35.54	5.33
ZmHDZ20	GRMZM2G477415	5	221	23.8	9.3
ZmHDZ21	GRMZM2G106276	9	272	28.03	9.7
ZmHDZ22	GRMZM2G105834	9	296	31.56	9.38
ZmHDZ23	GRMZM2G131476	1	262	28.24	8.14
ZmHDZ24	GRMZM2G127537	7	333	36.02	7.28
ZmHDZ25	GRMZM2G044752	2	277	25.05	8.92
ZmHDZ26	GRMZM2G126808	1	292	30.96	8.63
ZmHDZ27	GRMZM2G003509	1	894	96.87	7.21
ZmHDZ28	GRMZM2G178102	3	865	93.94	6.98
ZmHDZ29	GRMZM2G469551	1	872	96.35	6.08
ZmHDZ30	GRMZM2G001289	2	794	85.19	5.55
ZmHDZ31	GRMZM2G004957	10	797	85.65	6.38
ZmHDZ32	GRMZM2G118063	10	719	76.66	4.78
ZmHDZ33	GRMZM2G122897	10	844	91.06	5.91
ZmHDZ34	GRMZM2G126646	4	698	76.17	6.62
ZmHDZ35	GRMZM2G145690	5	692	75.81	6.58
ZmHDZ36	GRMZM2G396527	10	268	29.29	5.22
ZmHDZ37	GRMZM2G008286	8	176	19.6	7.48
ZmHDZ38	GRMZM2G004334	6	687	74.91	6.04
ZmHDZ39	GRMZM5G803812	5	348	37.53	6.45
ZmHDZ40	GRMZM2G023291	1	856	93.17	6.58
ZmHDZ41	GRMZM2G119999	1	305	33.34	4.57
ZmHDZ42	AC233899.1_FG004	9	370	39.87	7.99

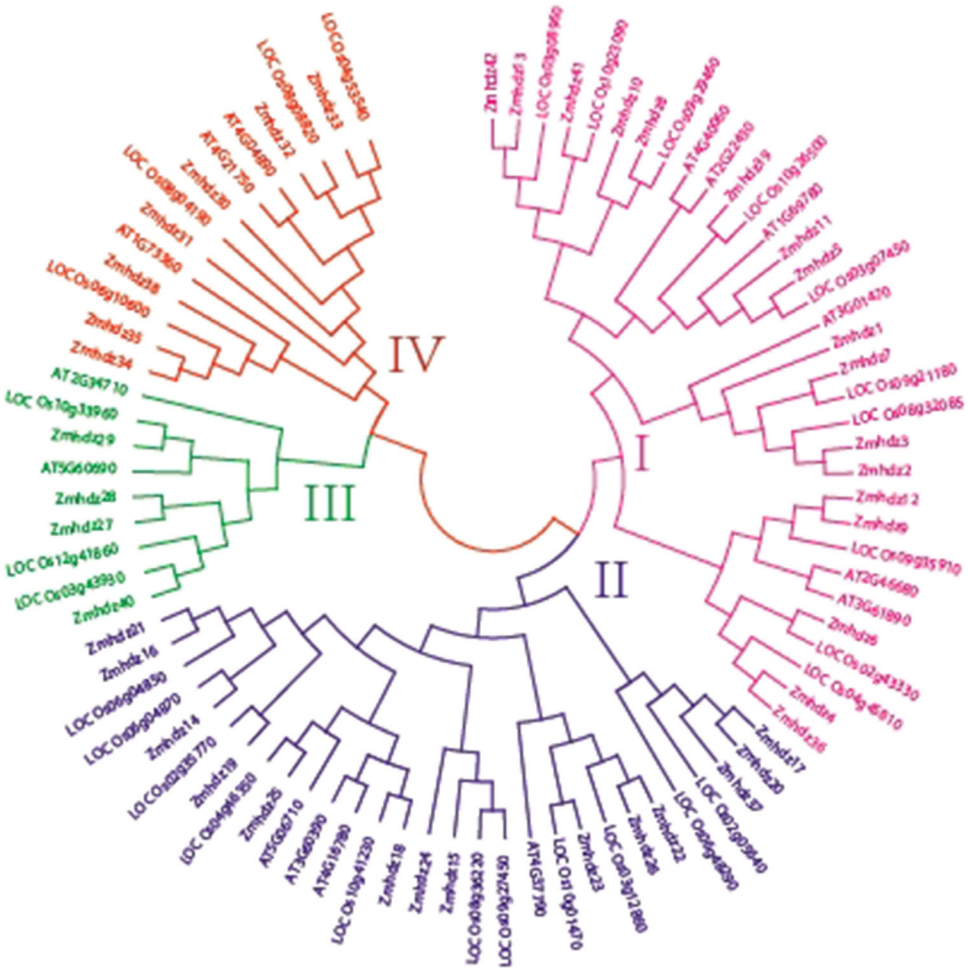

Fig. 1 Phylogenetic analysis of *HD-ZIP* genes in maize, Arabidopsis and rice. An unrooted phylogenetic tree was generated using the Neighbor-Joining algorithm and the Dayhoff PAM matrix employed in the MEGA6 software with 1000 bootstrap replicates. Different colors represent different subgroups

between sample groups. All experimental data were presented as averages of three replicates.

Results

Identification of *ZmHDZ* genes under drought-stressed and rewatering conditions

Forty-two HD-ZIP genes, designated as *ZmHDZ1* to *ZmHDZ42*, implicated in drought tolerance regulation redeemed from the previously generated transcriptomic datasets (Cao et al. 2019), were screened using the online tools Pfam and SMART (Table 1). The 42 *ZmHDZ* genes encode polypeptides with 176 to 894 amino acids. The estimated molecular weight of ZmHDZ proteins ranged from 17.82 (ZmHDZ2) and 96.87 (ZmHDZ27) kDa, and the isoelectric point (PI) ranged from 4.36 (ZmHDZ1) to 10.15 (ZmHDZ17).

The evolutionary relationship of HD-ZIP genes in maize, Arabidopsis and rice was analyzed by phylogenetic analysis. A phylogenetic tree comprising 42 maize, 15 Arabidopsis and 28 rice HD-ZIP protein sequences was constructed (Fig. 1). Phylogenetic analysis classified ZmHDZ proteins into four groups, each of which contains Arabidopsis and rice members, indicating that HD-ZIP genes in maize, Arabidopsis and rice have a parallel evolution relationship. Seventeen *ZmHDZ* genes were assigned to group I, 14 *ZmHDZ* genes were assigned to group II, 7 *ZmHDZ* genes were assigned to group III, and 4 *ZmHDZ* genes were assigned to group IV. Furthermore, most of the *ZmHDZ* genes in the same evolutionary subgroup shared a similar exon–intron structure in terms of intron/exon number and position. The number of exons exhibits substantial variations among the 42 *ZmHDZ* genes, with most of the *ZmHDZ* genes having 2–4 exons, whereas only four of them have 17–19 exons (Fig. 2).

Stress-related *Cis*-elements analysis in the promoters of *ZmHDZ* genes

To further reveal the structural characteristics and potential regulatory mechanisms of *ZmHDZ* genes, the MEME SUITE online tool was employed to annotate the promoter

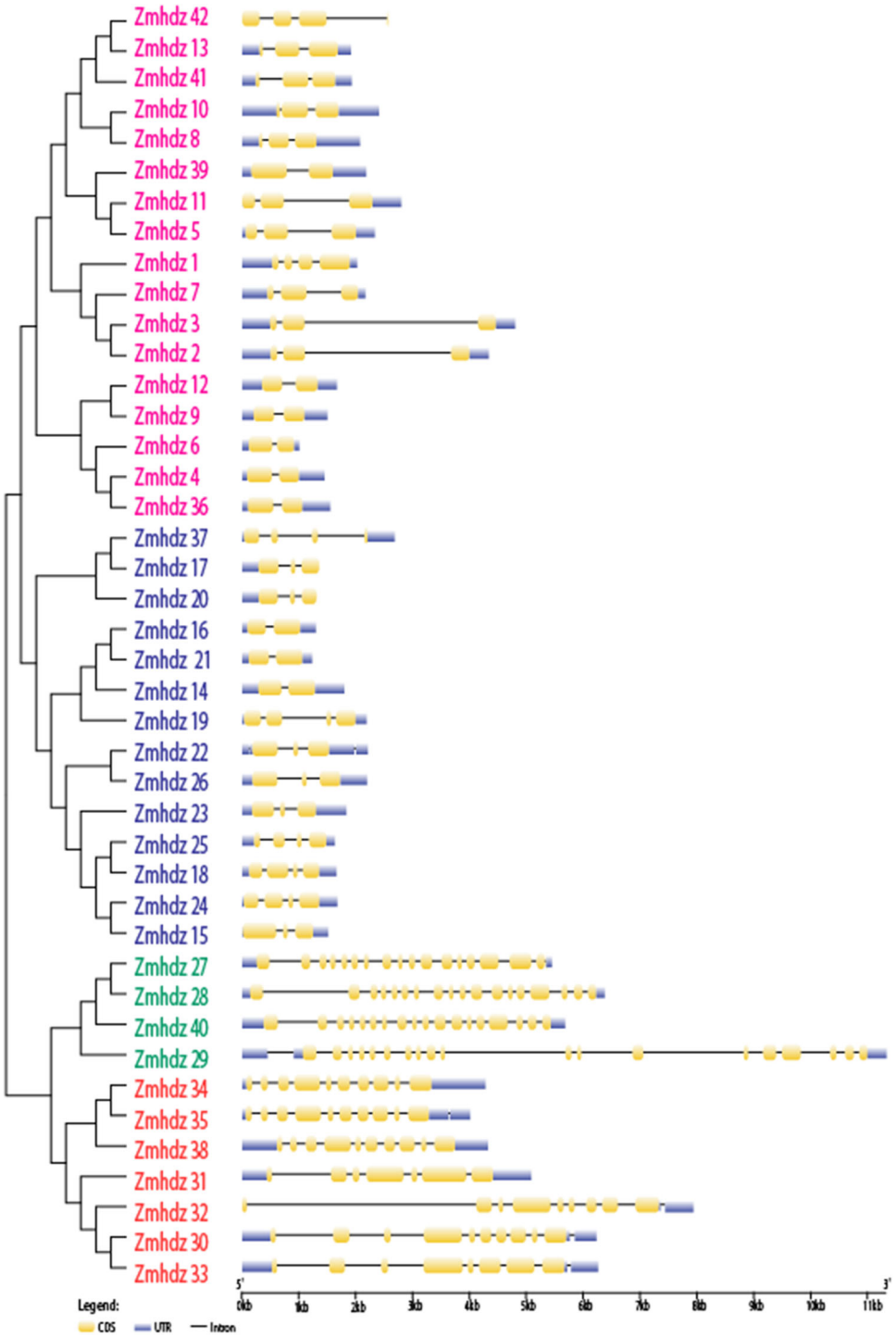

Fig. 2 Structural analysis of 42 *ZmHDZ* genes. Yellow boxes indicate exons, blue boxes indicate the UTRs. Exon and intron sizes can be determined using the horizontal line at the bottom

region of *ZmHDZ* (2 kb upstream TSS) to identify the stress-related *cis*-elements, such as ABRE, MBS, TCA, TC-rich, CGTCA, LTR and TATC-box. The annotation revealed the clustering of the ABRE, CGTCA, MBS, TC-rich and LTR *cis*-regulatory elements in the promoter regions of most of the *ZmHDZ* genes (Fig. 3). For instance, the ABRE was identified in 36 *ZmHDZ* genes; among them, *ZmHDZ36* contained 14 ABRE, followed by eight in each of *ZmHDZ4*, *ZmHDZ17*and *ZmHDZ6*, and six ABREs in *ZmHDZ9*. MBS, which is involved in drought stress response, was identified in 20 *ZmHDZ* genes. Three MBS elements were identified in each of *ZmHDZ24* and *ZmHDZ26*, two were detected in each of *ZmHDZ12*, *ZmHDZ21*, *ZmHDZ37* and *ZmHDZ42*. The CGTCA-motif, which is a MeJA-responsive element involved in the methyl jasmonate inducibility, was found in 38 *ZmHDZ*

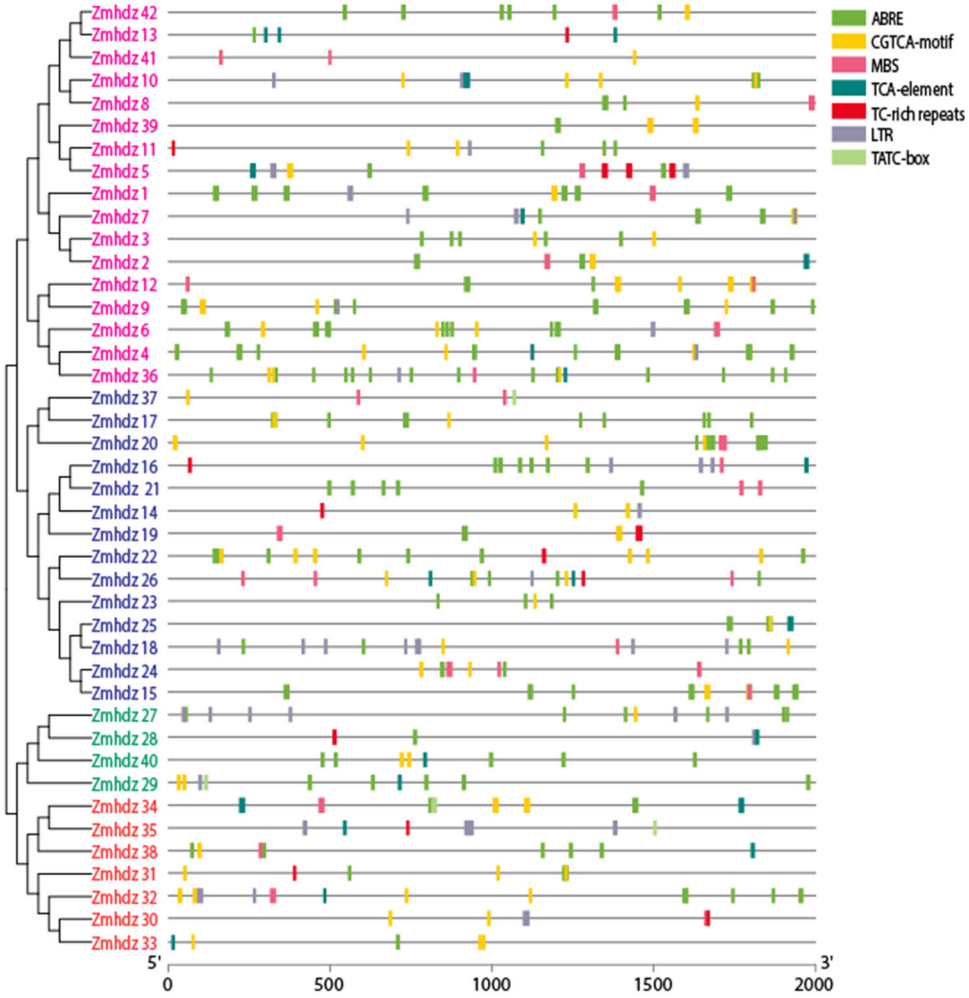

Fig. 3 Distribution of the major identified stress associated *cis*-elements in the promoter region (− 2 kb upstream TSS) of *ZmHDZ* genes. ABRE, CGTCA-motif, TCA-element, and TATC-box: hormone-responsive element; MBS: dehydration-responsive element; TC-rich repeats: stress-responsive element, LTR: low-temperature response. Different colors represent different *cis*-regulatory elements

gene promoter regions, such as *ZmHDZ9*, *ZmHDZ32*, *ZmHDZ36* and*ZmHDZ6*. Six CGTCA elements were enriched in *ZmHDZ22*, indicating the implication of most of *ZmHDZ* genes in regulating tolerance to abiotic stresses.

Chromosomal distribution and gene duplication of *ZmHDZ* genes

The identified 42 *ZmHDZ* genes were unevenly distributed on the 10 maize chromosomes. The highest number of *ZmHDZ* genes (10 genes) was mapped on chromosome 1, whereas only a single gene was mapped on each of chromosomes 3 and 8. A total of 24 pairs of duplicated genes were identified, all of which were fragment repeats, such as *ZmHDZ12/ZmHDZ9*, *ZmHDZ13/ZmHDZ42*, and *ZmHDZ16/ZmHDZ14*. Eleven of the 42 *ZmHDZ* genes participated in gene replication and promoted the diversity of the *ZmHDZ* gene family, suggesting that some *ZmHDZ* genes might promote the evolution of the *ZmHDZ* gene family through segmental duplication (Fig. 4).

Expression patterns of *ZmHDZ* genes under drought-stressed and rewatering conditions

To explore the potential role of *ZmHDZ* genes, the expression profiles of the 42 *ZmHDZ* genes were analyzed (Fig. 5). The results showed that the 42 genes exhibited different expression profiles. The expression levels of 13 *ZmHDZ* genes, such as *ZmHDZ4*, *ZmHDZ6* and *ZmHDZ9*, *ZmHDZ13* and *ZmHDZ29*, were upregulated at 60 and 96 h under drought stress induction and downregulated after rewatering, indicating that they were strongly induced by drought and may play pivotal roles in regulating plant tolerance to drought stress. The expression of *ZmHDZ21*, *ZmHDZ34* and *ZmHDZ41* was downregulated at 60 and 96 h under drought stress induction but was upregulated after rewatering. The expression of the remaining 26 genes, such as *ZmHDZ38, ZmHDZ3*, *ZmHDZ17*, exhibited irregular expression patterns.

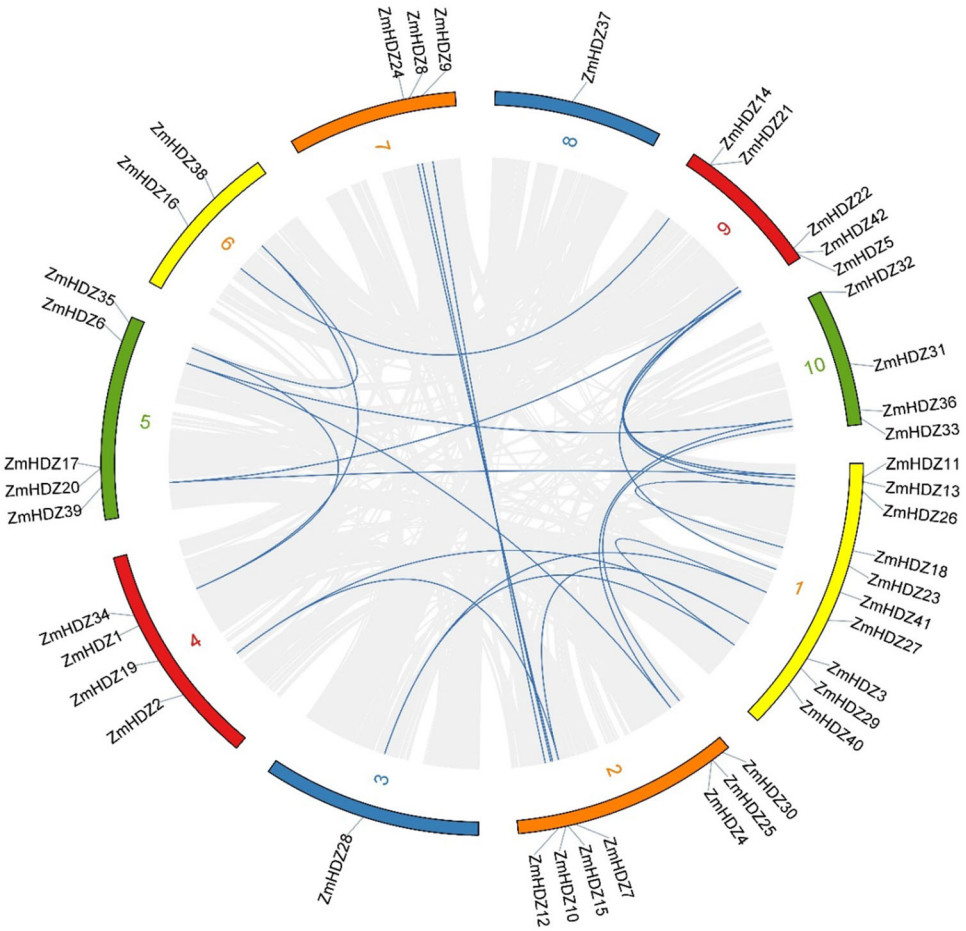

Fig. 4 Schematic representations for chromosomal allocation and *ZmHDZ* genes relationships. Grey lines reveal collinearity between maize genomes, and the blue lines represent repeated *ZmHDZ* gene pairs

Coexpression network analysis of *ZmHDZ* genes

A coexpression map based on gene expression similarity was constructed to identify the biological function and networks between the 42 *ZmHDZ* genes. In many cases, gene coexpression may imply the presence of a functional linkage between genes. As shown in Fig. 6, the nodes represent genes, and genes with similar profiles connected and formed a network. *ZmHDZ4*, − 6, − 9, − 14, − 27, − 32, and − 40 exhibited more connection with other genes, indicating that they are key regulatory genes.

Subcellular localization of *ZmHDZ9*

ZmHDZ9, a core gene upregulated under drought stress, was selected as a candidate gene for further functional analysis. Subcellular localization of ZmHDZ9 protein was detected. The recombinant vectors pMDC83-ZmHDZ9-GFP and pMDC83-GFP were constructed and transferred into tobacco (*N. benthamian*) leaves. The GFP control was evenly observed either in the nucleus and the cytoplasm, while pMDC83-ZmHDZ9-GFP fusion protein was exclusively observed in the nucleus, indicating that ZmHDZ9 protein is localized in the nucleus (Fig. 7).

Overexpression of *ZmHDZ9* improves drought tolerance in transgenic Arabidopsis plants

Overexpression analysis of *ZmHDZ9* in *A. thaliana* wild-type (WT) plants was employed to dissect the function of *ZmHDZ9* in regulating drought tolerance. Two independent homozygous transgenic lines, OE1 and OE2, were chosen for analysis. There were no visible morphological variations between the transgenic and WT plants. However, 12 days after water withholding, the leaves of the WT plants appeared yellow and wilted, whereas transgenic plants exhibited vigorous, even though the tip section of the leaves became yellow, indicating that *ZmHDZ9* transgenic plants exhibited drought stress-tolerant phenotype (Fig. 8A).

The related physiological and biochemical parameters were also assayed. No significant differences were observed in the leaf contents of chlorophyll and soluble protein, as well as the SOD and POD activities between the WT and transgenic plants under non-stressed conditions.

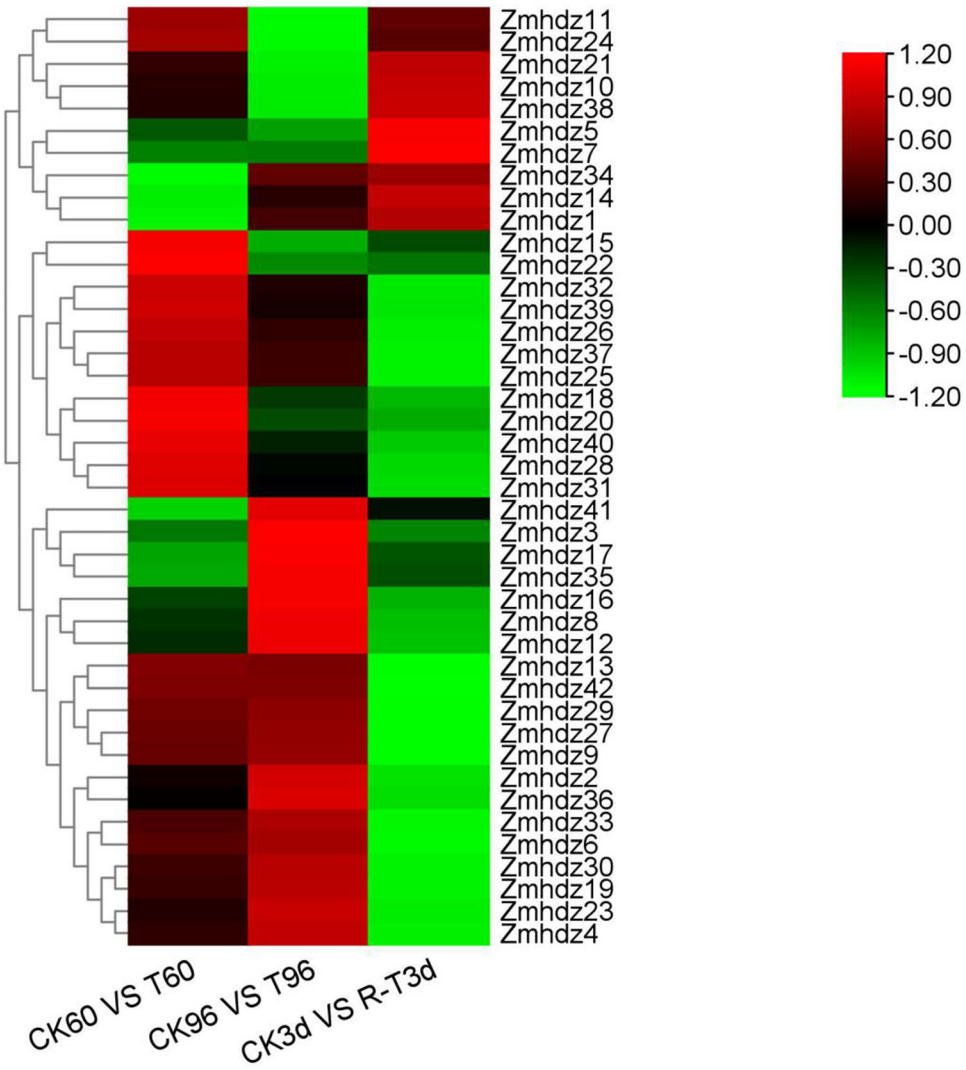

Fig. 5 Expression profiles of the 42 *ZmHDZ* genes under drought and rewatering conditions. Drought stress is induced at three-leaf stage for 60 h (T60) and 96 h (T96) by 20% PEG6000 and rewatered after 3d denoted as T3d. CK60, CK96, and CK3d indicate the control non-transgenic plants exposed to water deficit for 60 and 96 h, and 3 d after rewatering, respectively. The ratio between the expression levels of CK60 and the T60 is expressed as $\log_2$(FC) of CK60 versus T60. Similarly, $\log_2$(FC) of CK96 versus the T96 and $\log_2$(FC) of CK3d versus the T3d were calculated

After 12 days of water withholding, leaf chlorophyll and soluble protein contents and the SOD and POD activities of the two transgenic lines overexpressing *ZmHDZ9* were significantly ($p < 0.01$) higher than those of the WT plants, indicating that the overexpression of *ZmHDZ9* could withstand drought stress (Fig. 8B–E).

Discussion

HD-ZIP transcription factors play pivotal roles in regulating plant growth, development and response to various abiotic and biotic stresses. However, comprehensive studies to dissect the roles of these TFs have less been reported in maize. In this study, employing the online tools Pfam and SMART, 42 *ZmHDZ* drought-responsive genes were identified from the previously reported maize transcriptomic datasets, and further bioinformatics and expression profile analysis was conducted. Phylogenetic analysis showed that the 42 *ZmHDZ* genes were allocated into four groups, which was consistent with the classification of HD-Zip genes in rice and Arabidopsis (Fig. 1), and genes in the same subgroup exhibited similar gene exon–intron structure. Analyzing the type and number of *cis*-regulatory elements in the gene promoter region is helpful to explore its function and regulation mechanism (Lechner et al. 2011). In this study, several *cis*-regulatory elements including, ABRE, MBS, TCA, TC-rich, CGTCA, LTR and TATC-boxes were observed in the promoter regions of the 42 *ZmHDZ* genes, among which, ABRE, CGTCA, MBS, TC-rich and LTR were observed in most of the *ZmHDZ* genes. In addition, several *ZmHDZ* genes contain both ABRE and MBS regulatory elements. ABRE is an ABA-responsive regulatory element with a conserved, 8-bp long (PyACGTGG/TC) *cis*-element and an ACGT core sequence. However, a single ABRE copy is not sufficient for ABA-responsive gene expression induction. To function as an active *cis*-regulatory element, ABRE requires

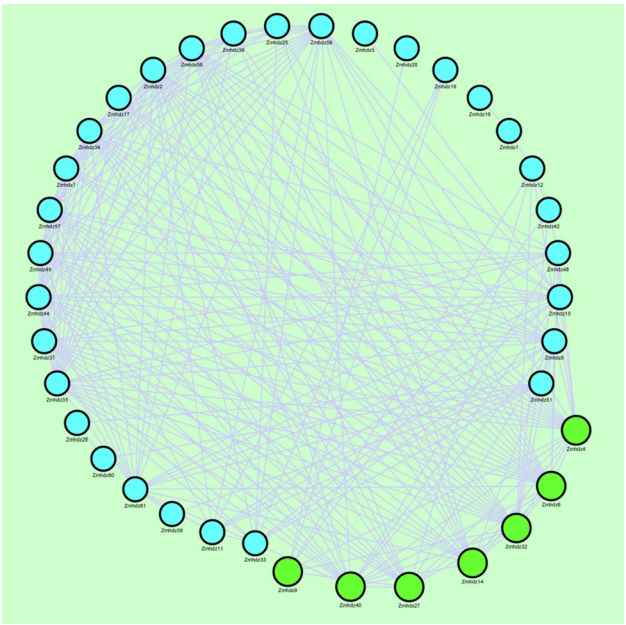

Fig. 6 Coexpression network map of *ZmHDZ* genes. The straight line represents the regulatory relationship of gene existence. The larger the green circle, the stronger association of the gene with other genes

other ABRE copies or another specific *cis*-regulatory element in proximity, which is designated as the coupling element (Fujita et al. 2011; Nakashima et al. 2009). MBS is an MYB binding site required for MYB transcriptional activation of drought inducible genes (Kaur et al. 2017). Furthermore, ABRE and TCA elements have been reported as key *cis*-regulatory elements that play crucial roles in regulating plant response to various stresses (Li et al. 2020), indicating that *ZmHDZ* is a positive regulator of drought stress in maize. The 42 *ZmHDZ* genes were unevenly distributed on the 10 chromosomes of maize. Collinearity analysis of the 42 *ZmHDZ* genes showed that 31 *ZmHDZ* genes (73.8%) were in a replication relationship, for a total of 24 gene pairs, all of which were segmental repeats. Tandem and segmental duplications are the driving forces for gene family expansion and evolution. The protein sequence and gene structure of two genes in one gene pair were highly similar, which could ensure the stability of maize evolution.

Under drought and recovery treatments, 42 *ZmHDZ* genes exhibited different expression profiles. The expression profiles of several *ZmHDZ* genes, such as *ZmHDZ4*, *ZmHDZ6*, *ZmHDZ9* and *ZmHDZ13*, were upregulated under drought stress and downregulated after rewatering, whereas other genes exhibited irregular expression profiles, indicating that different genes are involved in different regulatory pathways and have different functions. Coexpression analysis is a powerful approach to deduce the transcriptional regulation of genes. *ZmHDZ* 4, − 6, − 9, − 14, − 27, − 32 and − 40 exhibited more connection with other *ZmHDZ* genes, indicating that those genes are likely to be more implicated in drought tolerance in maize.

Transgenic expression of HD-ZIP genes was found to promote plant ability to tolerate abiotic stresses (Zhu et al. 2018). The ectopic overexpression of *AtHDG11* significantly promoted drought and salt tolerance in tall fescue

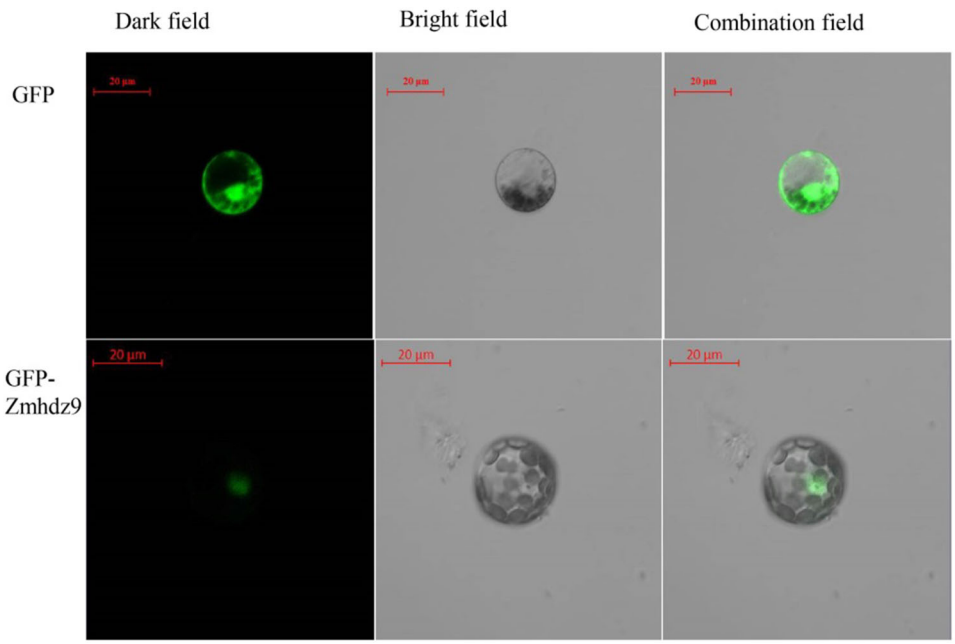

Fig. 7 *ZmHDZ9* subcellular localization analysis. Fusion proteins were transiently expressed under the control of the CaMV35S promoter in tobacco leaves and detected using a laser scanning confocal microscope. The green color indicates GFP signals. Scale Bars = 20 μm

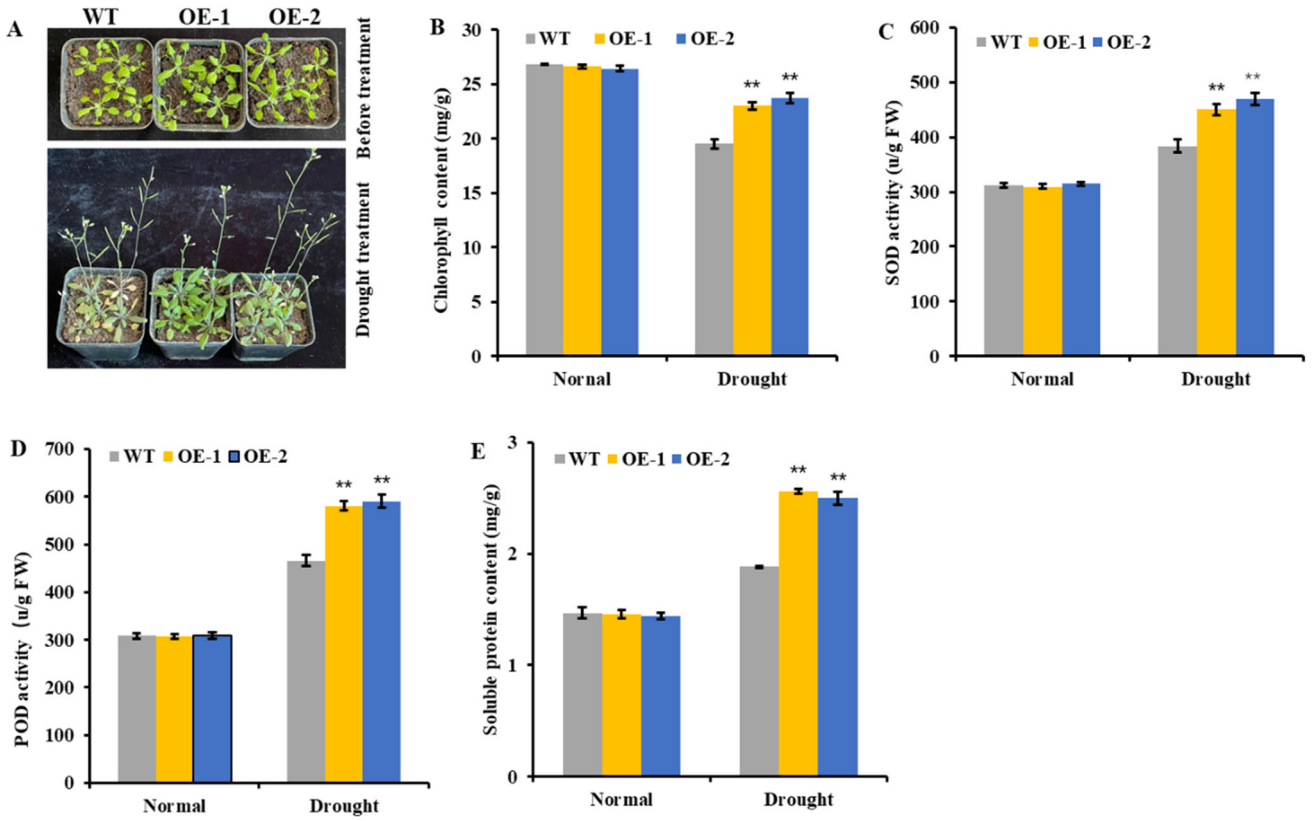

Fig. 8 Overexpression of *ZmHDZ9* enhanced drought tolerances in transgenic Arabidopsis plants. **A** Phenotypes of WT and *ZmHDZ9*-OE seedlings after 12 days water withholding **B** chlorophyll content, **C** SOD activity, **D** POD activity, **E** Soluble protein content. * and ** indicate $P \leq 0.05$ or 0.01, respectively. Values are presented as averages of three biological replicates ± SD

(*Festuca arundinacea* Schreb.) (Cao et al. 2009). Moreover, the contents of proline, abscisic acid (ABA), and soluble sugar, as well as POD and SOD activities, were highly elevated in transgenic rice plants overexpressing *AtEDT1/HDG11*under drought stress (Yu et al. 2013). In rice, the overexpression of *ZmHDZ4* improves drought tolerance and enhances plant sensitivity to induced abscisic acid (Wu et al. 2016). To further dissect the molecular function of *ZmHDZ* genes, *ZmHDZ9*, which was positively upregulated under drought stress, was selected for functional analysis by overexpression in the *A. thaliana* Col-0 wild-type strain. *ZmHDZ9* transgenic Arabidopsis plants showed much better tolerance than Col-0 WT plants under drought stress. Thus, it is suggested that *ZmHDZ9* has a similar biological function with its Arabidopsis homolog, *AtHB7*, which was reported to be implicated in plant response to drought stress (Lee and Chun 1998), and the overexpression of *AtHB7* enhances the development and regeneration of transgenic tomato plants (Pehlivan 2019). Chlorophyll is an essential component of the photosynthetic system in plants (Abou-Elwafa and Amein 2016; Chen et al. 2020b). In the study, the results showed that chlorophyll content was drastically reduced in both the *ZmHDZ9* transgenic and Arabidopsis WT plants in response to drought stress. However, the reduction was much higher in the WT plants. Thus, it is conceivable that *ZmHDZ9* transgenic plants exhibited an enhanced developed phenotype and improved tolerance to drought conditions. Drought exacerbates the accumulation of reactive oxygen species (ROS) in plants, causing oxidative stress. POD and SOD are both important antioxidant enzymes in plants that maintain the balance of free radicals by eliminating harmful free radicals in plants (Lobo et al. 2010; Sharma et al. 2012). The observed higher SOD and POD activities in *ZmHDZ9* transgenic plants compared to the WT plants indicate that the overexpression of *ZmHDZ9* could protect cells from oxidative damage induced by drought stress. Soluble proteins are important osmotic regulatory substances and nutrients. The accumulation of soluble proteins in plant cells can improve cells' ability to retain water and protect cell membranes; thus, they are frequently used as indicators of tolerance ability (Jiang et al. 2020; Zhang et al. 2021). The higher abundance of soluble protein in *ZmHDZ9* transgenic plants compared to the WT plants under water deficit conditions indicates that the overexpression of *ZmHDZ9* improves drought tolerance in transgenic Arabidopsis plants.

In conclusion, a bioinformatic pipeline was efficiently implemented to identify 42 *ZmHDZ* drought-responsive genes using previously generated maize transcriptomic datasets. Phylogenetic analysis revealed the first evidence for evolutionary conservation of components of the HD-ZIP TFs in maize. Several regulatory elements associated with drought-inducible genes were identified in the promoter regions of *ZmHDZ* genes, indicating the implication of these genes in regulating drought tolerance in plants. The expression of several *ZmHDZ* genes was elevated under drought conditions. Transgenic plants overexpressing *ZmHDZ9* exhibited higher activities of SOD and POD and the accumulation of more soluble proteins in the plant cells under drought stress, which resulted in an enhanced developed phenotype and improved resistance. The present survey reveals the first evidence for evolutionary conservation of HD-ZIP transcription factor homologs in maize. In addition, our results provide a comprehensive insight into the roles of *ZmHDZ* genes in regulating drought–tolerance in maize.

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Declarations

Conflict of interest The authors declare that they have no conflict of interests.

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