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ORIGINAL PAPER

Expression of *ZmHDZ4*, a Maize Homeodomain-Leucine Zipper I Gene, Confers Tolerance to Drought Stress in Transgenic Rice

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Abstract Climate change is predicted to be a major threat to crop yield due to increasing global temperatures and periods of unpredictable rainfall. Therefore, it is important to identify some new genes that can help plants cope with drought stress. In this study, a drought-induced homeodomain-leucine zipper (HD-Zip) I gene, *ZmHDZ4*, was isolated from maize and characterized for its role in drought stress. Transient expression experiments showed that ZmHDZ4 is localized to the nucleus. Yeast one-hybrid assays demonstrated that ZmHDZ4 has trans-activation activity, and that the minimal activation domain was ZmHDZ4-1. We found that overexpression of *ZmHDZ4* in rice can enhance tolerance to drought and increase sensitivity to abscisic acid (ABA). Compared to wild-type plants, *ZmHDZ4*-expressing transgenic plants had lower relative electrolyte leakage (REL), lower malondialdehyde (MDA) levels, and increased proline contents under drought stress conditions, all of which may contribute to enhanced drought tolerance. Taken together, these results suggest that ZmHDZ4 functions as a transcriptional regulator that can positively affect plant drought tolerance. Thus, *ZmHDZ4* is an excellent candidate gene with potential applications in molecular breeding to improve crop drought tolerance.

Keywords Drought · Stress tolerance · *ZmHDZ4* · Transcription factor · ABA

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Introduction

Drought is a major abiotic stress that causes crop yield losses through its effect on plant growth and development. During the course of evolution, plants have established complex mechanisms to adapt to various adverse environments (Shinozaki and Yamaguchi-Shinozaki 2000). For example, the first response to the initial stress signals is transduction and perception, which can then affect the expression of large numbers of stress-related genes (Zhu 2002; Shinozaki et al. 2003). Among these, transcription factors (TF) are an important family of proteins that regulate the expression of their target genes by binding to gene promoters (Tran et al. 2004; Yamaguchi-Shinozaki and Shinozaki 2005). Functional investigations have shown that some stress-responsive TFs, such as *CBF1*, *OsSDIR1*, and *ABF2*, can improve stress tolerance in transgenic plants by overexpression of these genes (Kim et al. 2004; Dai et al. 2007; Gao et al. 2011).

Homeodomain-leucine zipper (HD-Zip) proteins comprise a large family of TF that appear to be unique to higher plants (Ariel et al. 2007). The HD-Zip proteins that contain a DNA-binding homeodomain (HD) and a leucine zipper (LZ) domain are unique to plants. Based on their sequence conservation, structural features, and functions, HD-ZIP proteins can be divided into four subfamilies (HD-Zip I–IV) (Ariel et al. 2007; Harris et al. 2011). Members of the different subfamilies are known to function as either transcriptional repressors or activators of gene expression (Henriksson et al. 2005; Deng et al. 2006).

Recent reports suggested that proteins from closely related HD-ZIP I families play a regulatory role in the response and adaptation of plants to environmental changes (Harris et al. 2011). For example, gene expression analyses showed that the *Arabidopsis HD-Zip I* genes *ATHB6*, *ATHB7*, and *ATHB12* were upregulated and *ATHB5* was downregulated under

water-deficit conditions and/or by externally applied abscisic acid (ABA) (Soderman et al. 1999; Lee et al. 2001). *HAHB-4*, which encodes the sunflower HD-Zip I protein, was induced by drought and ABA, and plays a central role in regulating plant responses to drought (Dezar et al. 2005; Manavella et al. 2006). Furthermore, *CpHB4* and *CpHB5* from the *Craterostigma plantagineum* HD-ZIP I family were downregulated by dehydration and were not responsive to ABA. However, the rice HD-ZIP protein OsHOX22 has been shown to affect ABA biosynthesis, and regulates drought and salt responses through the ABA signal pathway (Zhang et al. 2012). These findings imply that HD-Zip TF mediate drought responses through either ABA-dependent or ABA-independent pathways. In addition to their involvement in abiotic stress, a recent report has shown that a *Medicago truncatula* HD-Zip I protein, MtHB1, is expressed in primary and lateral root meristems and is induced by salt stress (Ariel et al. 2010).

The functions of many HD-ZIP I proteins have been well characterized in *Arabidopsis*, rice, and other plants. However, there are few reports describing the function of HD-ZIP I proteins in maize. The expression patterns of 17 *HD-Zip I* genes were analyzed following drought treatments in a previous study (Zhao et al. 2011). In this study, we identified a member of the maize HD-ZIP I family, *ZmHDZ4*, and functionally characterized its role in the response to drought and ABA treatments by expressing *ZmHDZ4* in rice. Overexpression of *ZmHDZ4* significantly improved tolerance to drought stress in rice plants. The results of our study are helpful for understanding the molecular mechanisms of this gene, and also provide a candidate gene with potential applications in molecular breeding to improve crop drought tolerance.

Materials and Methods

Plant Materials and Stress Treatments

Seeds of the maize inbred line B73 were grown in the greenhouse (14 h light/10 h dark cycle, light intensity 150 mmol s^{-1} m^{-2} *PAR* at plant height, relative humidity 35 % by day and 60 % by night, temperature 28–30 °C) for 2 weeks. Seedlings at the three-leaf stage were exposed to drought and ABA treatments. For drought stress, the seedlings were irrigated with 20 % PEG 6000 solution. For ABA treatment, seedling leaves were sprayed with 100 μM ABA solution. Leaves from the treated seedlings at the three-leaf stage were collected at 0, 1, 3, 6, 12, and 24 h after treatment. For tissue-specific gene expression analysis, the roots, stems, and leaves of seedlings at the three-leaf stage, tassels and filament at the preflowering stage, and the ears and cluster at the mature stage from a life cycle of maize were collected for RNA isolation.

Seeds of inbred line Zhonghua 11 (WT) and transgenic rice were germinated in darkness for 2 days at 30 °C and then grown in MS medium at 28 °C under a 14 h light/10 h dark cycle for 2 weeks. For the drought tolerance test, 2-week-old seedlings of WT and transgenic rice were transferred to square pots filled with a mixture of soil and sand (1:1) until they reached the five-leaf stage. At the five-leaf stage, the plants were then subjected to drought stress for 12 days by withholding water, and watering was resumed for 7 days, after which the survival rates were calculated.

Real-Time PCR Analysis

Total RNA was extracted from the different tissue samples according to the manufacturer's instructions (TianGen, Beijing, China). The complementary DNA (cDNA) synthesis reaction was performed with SuperScriptTM III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed on an ABI 7300 Real-Time system (Applied Biosystems). The primers 5'-AGGAAGAACACACACCGCTTCTA-3' (forward) and 5'-TGATCTGGCCTGTCCATGTC-3' (reverse), which are specific for *ZmHDZ4*, were used in this study. The *ACTIN1* gene was used as the internal control, and was amplified with the primers 5'-GGGATTGCCG ATCGTATGAG-3' (forward) and 5'-GAGCCACCGAT CCAGACACT-3' (reverse). The relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All experiments were carried out with two biological repeats and three technical trials.

Gene Cloning and Production of Transgenic Rice Plants

Gene-specific primers 5'-CGGGATCCATGGACAGGC CAGATCACC-3' (forward, *BamHI* site underlined), and 5'-GCTCTAGATCAGGCCACCGCATTCCACT-3' (reverse, *XbaI* site underlined) were designed to clone the 786 bp coding sequence (CDS) of *ZmHDZ4*. The PCR product was first cloned into the pEASY T1 simple cloning vector (TransGen) for sequencing and then inserted into the plant overexpression vector pCAMBIA1301. The construct was introduced into *O. sativa japonica* cv. Zhonghua11 by *Agrobacterium*-mediated transformation. Transgenic rice plants carrying the *ZmHDZ4* construct were selected by hygromycin resistance and confirmed by PCR using specific primers (HYG-F 5'-ACTCACCGCGACGTCTGT-3' and HYG-R 5'-TTTCTT TGCCCTCGGACG-3').

Subcellular Localization Assay

The full-length cDNA of *ZmHDZ4* was amplified by PCR with specific primers 5'-GCTCTAGAATGGACAGG CCAGATCACC-3' (forward, *XbalI* site underlined) and 5'-

CGGGATCCGGCCACCGCATTCCACT-3' (reverse, *BamHI* site underlined). The PCR product was inserted into pCAMBIA1305, which contains a GFP gene under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. The plasmid was then introduced into the *Agrobacterium* EHA105 by electroporation. Agrobacteria carrying the constructs were infiltrated into the leaves of 5–6-week-old *Nicotiana benthamiana* plants. Infiltrated leaves were observed 48–72 h later using a Zeiss Microsystems LSM 710.

Yeast One-Hybrid Assay

To assay the activation property of ZmHDZ4, the full-length ORF or the various truncated fragments of *ZmHDZ4* were individually fused in-frame with the yeast GAL4 DNA-binding domain in the pGBKT7 vector (Clontech). The different constructs were then transformed into yeast strain AH109. The transformed yeast cells were examined on SD/Trp^- and SD/Trp^-/His^-/Ade^-/X-α-gal medium plates for 4 days.

Measurement of RWC, REL, and MDA and Proline Contents

Following drought treatment, leaves at similar developmental stages from WT and transgenic plants were collected at predetermined times. Fresh weigh (FW), dry weight (DW), and saturated weight (SW) were used to calculate the relative water content (RWC) according to the formula: RWC = [(FW − DW)/(SW − DW)] × 100 %. Relative electrolyte leakage (REL) was measured based on a previously described method (Li et al. 2011). Malondialdehyde (MDA) content was measured by the method of Zhang et al. (2010), and the proline content was determined by the method of Bates et al. (1973).

ABA Sensitivity Assay

Plants of transgenic lines and WT were grown under the same conditions, and seeds were collected at the same time. Seeds were put on plates containing MS medium with 0, 2, 5, and 10 μM ABA solution for 7 days. Then, the germination rate, root length, and shoot length of transgenic and WT seedling were analyzed, respectively.

Results

Isolation and Sequence Analysis of *ZmHDZ4*

To elucidate the function of ZmHDZ4, the full-length cDNA of *ZmHDZ4* was cloned from maize inbred line B73 by reverse transcription PCR. Sequencing verified that *ZmHDZ4* contains an open reading frame of 786 bp. ZmHDZ4 is located on maize chromosome 2 and encodes a predicted protein of 261 amino acids (AA), with conserved HD domain (amino acids 55–111) and Zip domain (amino acids 112–156). The cDNA sequence of *ZmHDZ4* is the same as the predicted sequence in the maize B73 genome annotated database (Fig. 1).

Expression of *ZmHDZ4* Under Abiotic Stress Conditions

A previous study showed that HD-ZIP I proteins are involved in the plant stress response (Harris et al. 2011). We performed quantitative real-time PCR (qRT-PCR) to examine the expression patterns of *ZmHDZ4* in response to various stress treatments. We found that expression of *ZmHDZ4* was strongly upregulated by PEG 6000 and ABA treatments, with mRNA levels rapidly reaching their highest levels after 1 h of treatment (Fig. 2b, c). These results suggest that *ZmHDZ4* plays an important role in the response to abiotic stress and in the ABA signaling pathway. We also examined the tissue-specific expression of *ZmHDZ4* in seven representative tissues of maize B73. The results indicate that *ZmHDZ4* is ubiquitously expressed in all of the sampled tissues including root, stem, leaf, tassel, filament, ear, and cluster, with the highest level of expression detected in leaves (Fig. 2a).

The ZmHDZ4 Protein Localizes to the Nucleus

To investigate the subcellular localization of ZmHDZ4, a transient expression vector was constructed. The *ZmHDZ4* ORF without the termination codon was fused to the upstream of the GFP reporter under control of the CaMV 35S promoter. The construct was introduced into leaves of *N. benthamiana* plants and observed under a confocal microscope. Compared with the 35S::GFP construct (control), which was detected both in the nucleus and cytoplasm (date not shown), the GFP-ZmHDZ4 fusion protein was exclusively localized in the nucleus (Fig. 2d). This observation indicates that ZmHDZ4 is a putative nucleus-localized TF.

Transcription Activation Activity Analysis in Yeast

To examine whether ZmHDZ4 possesses trans-activation activity, a yeast one-hybrid system was used. The full-length *ZmHDZ4* coding region was cloned into the pGBKT7 vector downstream of the gene encoding the GAL4 DNA-binding domain (Fig. 3a). Results showed that the yeast strains carrying pGBKT7-ZmHDZ4 and the positive control not only grew well on SD/Trp^- medium, but also grew well on SD/Trp^-/His^-/Ade^- medium containing 250 μg l^{-1} aureobasidin A (Fig. 3b). To further investigate the activation site of ZmHDZ4, different truncated fragments were also inserted into pGBKT7 and separately transformed into yeast cells. We found that the lines carrying the constructs ZmHDZ4-1,

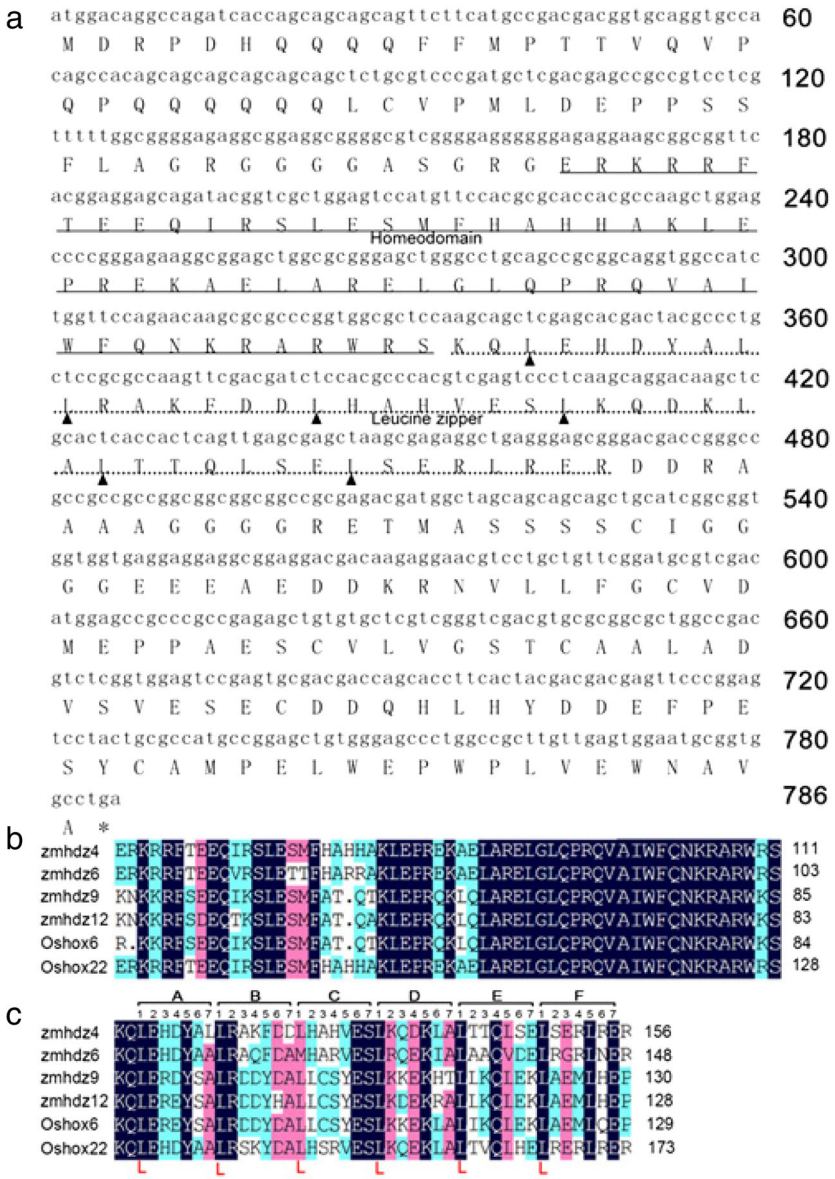

Fig. 1 Sequence analysis of maize *ZmHDZ4*. **a** ZmHDZ4 nucleotide and deduced amino acid sequences. The homeodomain (*HD*) and leucine zipper (*Zip*) motifs are indicted by *solid and dashed lines*, respectively. **b** Alignment of the homeodomains of some maize and rice HD-Zip I protein family members. **c** Alignment of the leucine zipper motifs. The six heptad repeats with Leu residues at position L in each heptad are indicated. The locus names of the corresponding genes are as follows: *ZmHDZ4* (GRMZM2G351330), *ZmHDZ6* (GRMZM2G117164), *ZmHDZ9* (GRMZM2G041462), *ZmHDZ12* (GRMZM2G034113), *OsHOX6* (Os09g35910), and *OsHOX22* (Os04g45810)

ZmHDZ4-2, ZmHDZ4-3, and ZmHDZ4-4 grew well on SD/Trp^{-}/His^{-}/Ade^{-} medium containing 250 μg l^{-1} aureobasidin A (Fig. 3b). Therefore, in yeast, ZmHDZ4 has trans-activation activity, and the minimal active region is defined by ZmHDZ4-1 (amino acids 1–54) (Fig. 3a, b).

Overexpression of *ZmHDZ4* Enhances Tolerance to Drought Stress in Rice

To examine the effect of ZmHDZ4 on improving stress tolerance, a total of 16 independent transgenic plants were generated. Two independent transgenic lines (L1 and L2) with single-copy of transgene confirmed by Southern blotting (data not shown), which represent different expression levels of *ZmHDZ4*, were selected for further stress tolerance experimentations. Both WT and plants overexpressing *ZmHDZ4* were subjected to drought stress for 12 days, after which watering was resumed for 7 days. As shown in Fig. 4a, WT plants displayed leaf rolling and wilting, but the transgenic plants exhibited the phenotype to only a slight extent following drought stress. The relative water contents (RWC) of the plants were measured, and we found that the *ZmHDZ4* transgenic plants had higher RWCs than did the WT plants after different days stress (Fig. 4b). For example, about 50 % RWCs in transgenic plants after continuously 12 days withholding water. By contrast, only approximately 30 % RWCs in WT plants can be detected. After recovery for 7 days, the survival rate was determined, and only 25 % of the WT plants survived. In contrast, more than 70 % of *ZmHDZ4* transgenic plants survived (Fig. 4c).

Previous studies showed that plants subjected to abiotic stress often showed an increase in malondialdehyde (MDA)

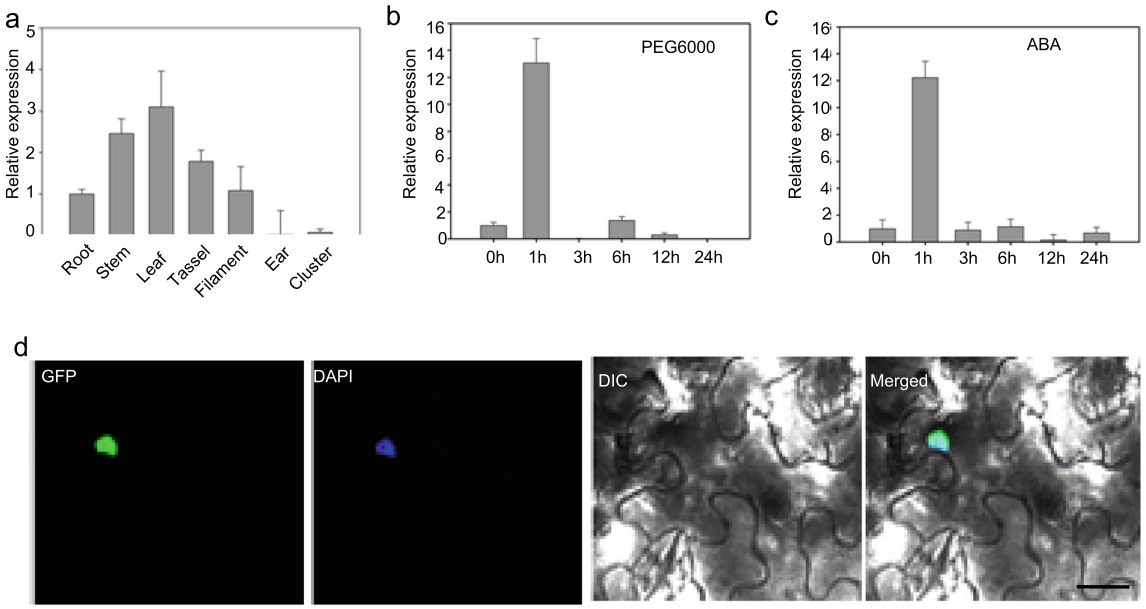

Fig. 2 Gene expression patterns and subcellular localization of ZmHDZ4 in maize. **a** Tissue-specific expression pattern of *ZmHDZ4*. **b**, **c** qRT-PCR analysis of *ZmHDZ4* expression in response to PEG 6000 and ABA treatments at six time-points from 0 to 24 h. *Bars* express the relative fold-change on a linear scale. Maize *ACTIN* was used as reference gene, and ratios were normalized against the 0 h sample. *Error bars* represent the SD of four biological replicates. **d** Subcellular localization of the ZmHDZ4 protein. *Bar* = 20 μm

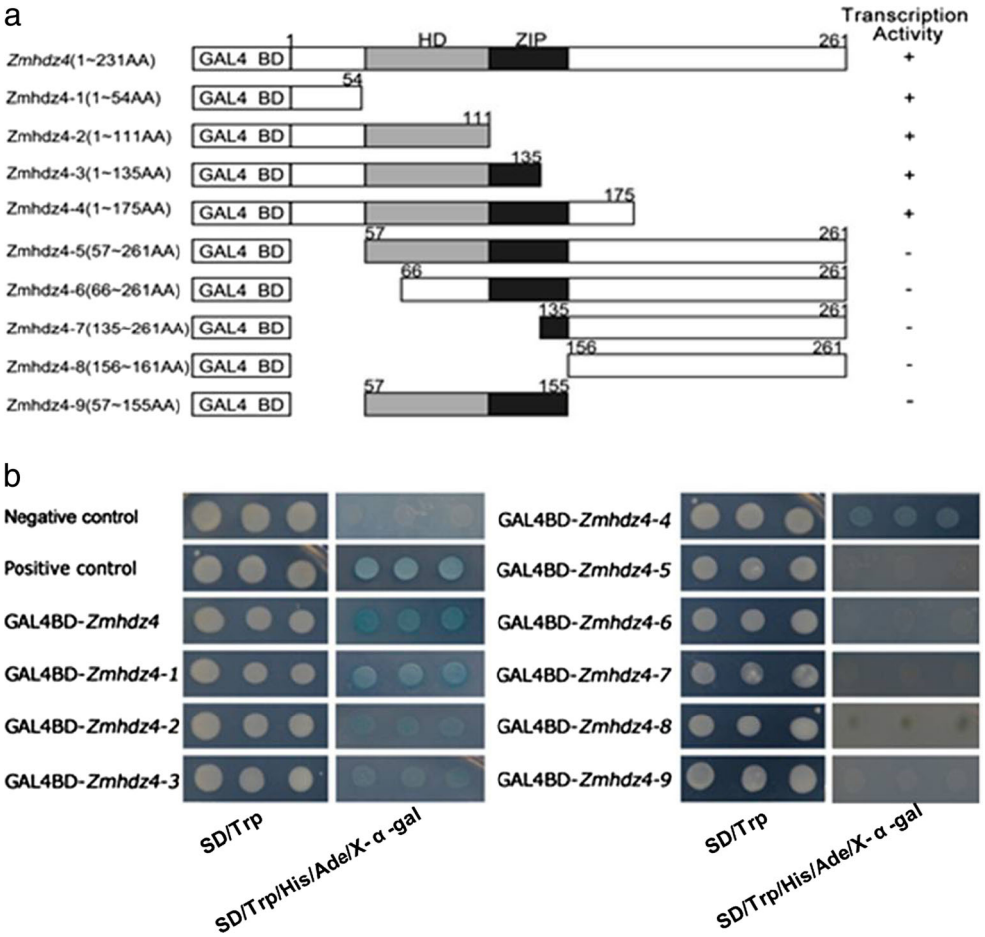

Fig. 3 Trans-activation activity assay of the ZmHDZ4 protein. **a** Schematic representation of each *ZmHDZ4* gene fragment used in the assays showing length and relative position. *HD* homeodomain (*gray*), *Zip* leucine zipper (*black*). **b** Trans-activation assay in yeast with truncated ZmHDZ4 proteins. The different deletion fragments in the pGBKT7-ZmHDZ4 constructs were transformed into yeast strain AH109, and tested on SD/Trp$^-$ and SD/Trp$^-$/His$^-$/Ade$^-$/X-a-gal plates

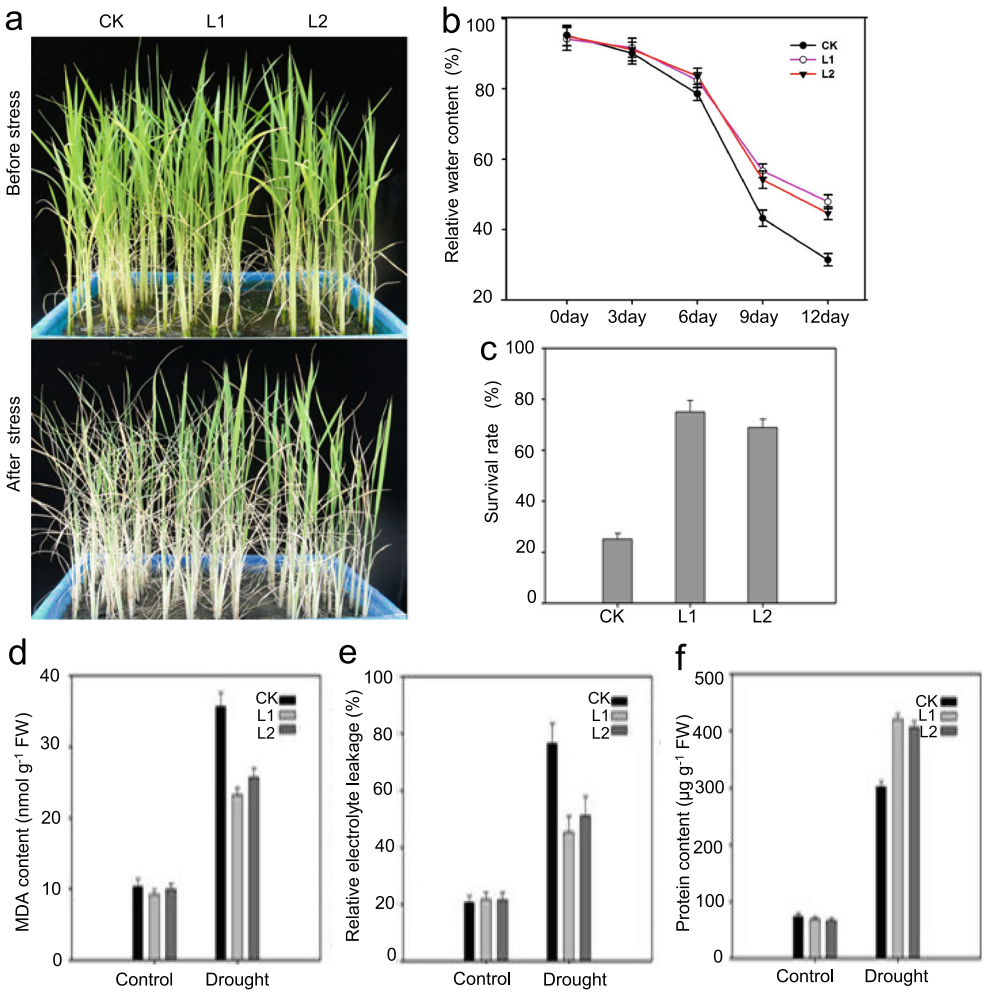

Fig. 4 Enhanced drought tolerance of transgenic rice lines overexpressing maize *ZmHDZ4*. **a** Phenotype of WT and transgenic plants before and after drought treatment for 14 days at the five-leaf stage followed 7 days of recovery. **b** RWC of leaves during drought stress. **c** Relative survival of the WT control (*CK*) and transgenic plants after recovery for 7 days. **d**, **e**, and **f** The MDA contents, REL, and proline contents, respectively, of WT and transgenic plants before and after drought stress

levels (Kong et al. 2011). Following drought stress, the MDA contents increased in both WT and transgenic plants, but the WT plants (more than 35 %) had higher levels than did the *ZmHDZ4* transgenic plants (25 %) (Fig. 4d). There were no obvious differences under normal conditions between the WT and *ZmHDZ4* transgenic plants; however, the WT plants (close to 80 %) exhibited a higher relative electrolyte leakage (REL) than did the transgenic plants (less than 50 %) after exposure to drought stress (Fig. 4e). The proline content was also examined under both normal and drought stress conditions. Again, there was no significant difference between the WT and transgenic plants under normal conditions, but the transgenic plants had higher proline contents after drought stress (Fig. 4f). Therefore, these results indicate that overexpression of *ZmHDZ4* in rice plants can enhance tolerance to drought stress.

Increased ABA Sensitivity in *ZmHDZ4* Transgenic Plants

The fact that ABA treatment induced the expression of *ZmHDZ4*, and that the plants overexpressing *ZmHDZ4* also showed enhanced drought tolerance, urged us to examine whether *ZmHDZ4* can affect ABA signaling. To analyze the sensitivity of the *ZmHDZ4*-overexpressing plants, seeds were germinated on solid MS medium containing either 0, 2, 5, or 10 μM ABA. As shown in Fig. 5, there was no obvious difference in germination rate, root length, and shoot height under normal conditions between WT and *ZmHDZ4* transgenic plants. Seed germination was severely inhibited by all concentrations of ABA in both WT and *ZmHDZ4* transgenic plans, but the inhibition was much stronger in transgenic plans than in WT plants (Fig. 5b). For example, approximately 45 % of WT seeds germinated compared with only 30 % of transgenic seeds in culture medium containing 10 μM ABA. The root length and shoot height of both WT and *ZmHDZ4* transgenic plants were also measured when grown in the presence of different concentrations of ABA. The growth of rice seedlings overexpressing *ZmHDZ4* was inhibited more severely than in WT plants (Fig. 5c, d). These results show that overexpression of *ZmHDZ4* in rice increases sensitivity to ABA.

Discussion

In maize, 55 members of the HD-ZIP family have been identified and classified into four subfamilies (Zhao et al. 2011). A

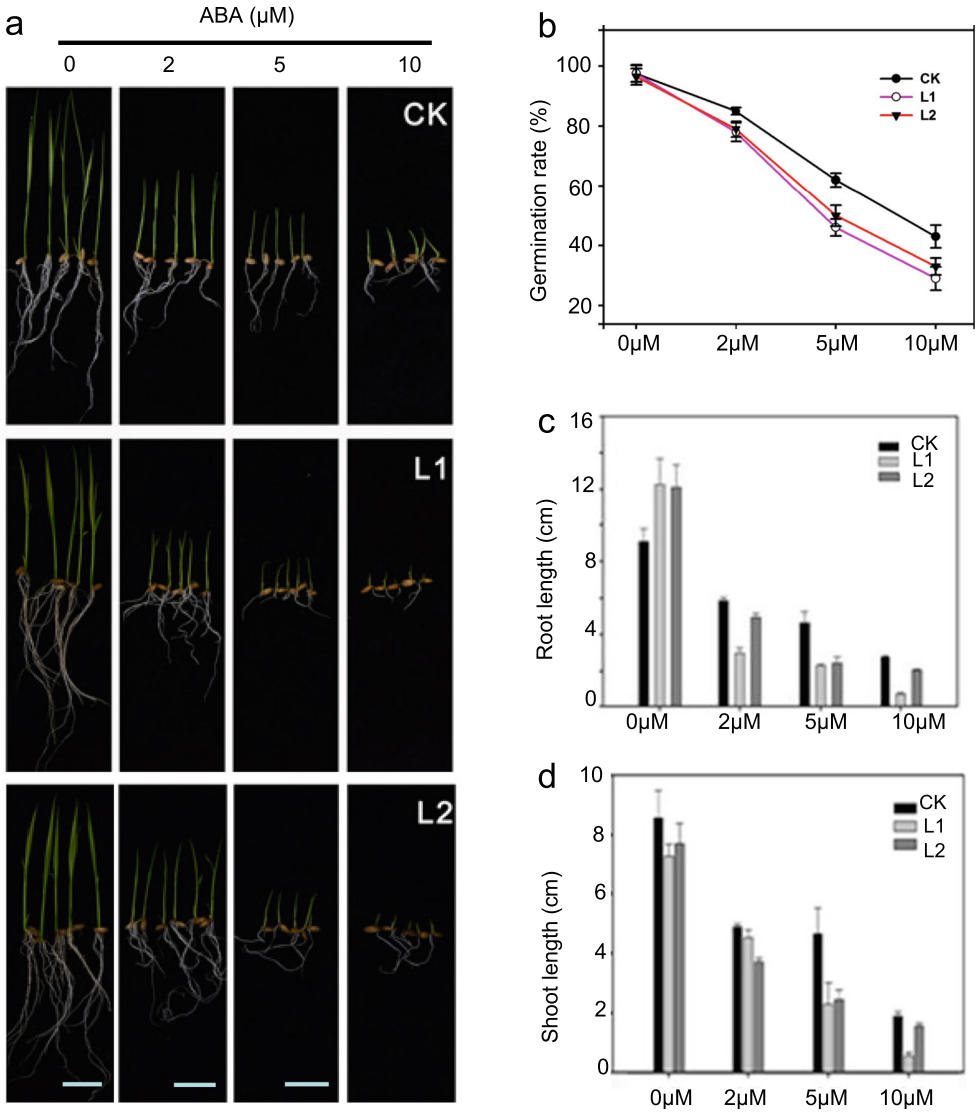

Fig. 5 Analysis of ABA sensitivity in transgenic rice plants expressing maize *ZmHDZ4*. **a** Phenotypes of WT and transgenic plants grown in the presence of different concentrations of ABA for 14 days. **b** Germination of WT and *ZmHDZ4* transgenic rice seeds in the presence of different concentrations of ABA for 7 days. **c** Root lengths and **d** shoot heights of WT and *ZmHDZ4* transgenic rice seedlings grown in the presence of different concentrations of ABA for 10 days following germination. The *error bars* indicate the SD of three independent experiments. *Bars* = 2 cm

gene in the HD-ZIP I subfamily, *ZmHDZ4*, was shown to be induced by drought treatment (Zhao et al. 2011). However, the function of *ZmHDZ4* in the environmental stress response has not been fully investigated. In this study, we isolated the *ZmHDZ4* gene from maize and functionally characterized its role in drought stress tolerance in transgenic rice.

Based on the high degree of similarity between the ZmHDZ4 protein and other reported HD-ZIP I subfamily proteins from rice, we confirmed that the gene we isolated from maize is an HD-ZIP I subfamily member with a conserved HD domain (amino acids 55–111) and a Zip domain (amino acids 112–156). In addition, we used a GFP-tagged protein fusion construct to demonstrate that ZmHDZ4 localizes to the nucleus in tobacco leaf cells, which is consistent with the function of a TF. The activation properties of ZmHDZ4 were analyzed in yeast one-hybrid experiments, showing the activation of reporter gene expression without the requirement for exogenous activation domains, which is consistent with previous findings (Zhao et al. 2014). To further define the activation domain, several truncated fragments of ZmHDZ4 were transformed into yeast to assay their trans-activation capabilities. Results of this experiment showed that the HD and Zip domains are not necessary for the trans-activation activity of ZmHDZ4. This result is contrary to a previous study, in which both the HD and Zip domains were shown to be required for the trans-activation of OsHOX22 (Zhang et al. 2012). Further investigations are required to understand the reasons for the differences in the two studies.

Previous research has shown that the overexpression of HD-ZIP I TF genes can improve abiotic stress tolerance (Ariel et al. 2010; Zhang et al. 2012). To our knowledge, only one HD-ZIP I gene, *ZmHDZ10*, has been studied in maize. Overexpression of *ZmHDZ10* was shown to confer enhanced drought stress tolerance in transgenic plants (Zhao et al. 2014). Here, in order to evaluate the effects of constitutive expression of *ZmHDZ4* in the response to abiotic stresses,

we generated transgenic rice plants overexpressing *ZmHDZ4*. We found that overexpression of *ZmHDZ4* in rice enhanced drought stress tolerance, and this was mainly verified by the higher RWC, higher survival rates, lower MDA contents and REL, and the higher proline content of the transgenic plants under conditions of drought stress. Abiotic stress can cause lipid peroxidation, resulting in MDA accumulation (Kong et al. 2011). MDA production in plants reflects the degree of oxidization of cell membrane lipids (Mittler 2002). REL is an important indicator of membrane damage. The lower MDA levels and REL content in transgenic plants suggests that the degree of cell membrane damage caused by abiotic stress was less than in WT plants. In addition, the higher proline content in transgenic plants compared to WT plants could account for higher osmolality, resulting in lower water potential, thus making the plants more effective at imbibing moisture (Song et al. 2012). These results strongly imply that the ZmHDZ4 protein can as a key positive regulator to enhance drought tolerance in plants.

ABA signaling is crucial for triggering plant responses to abiotic stresses, including drought, high salinity, and low and high temperatures, by regulating the expression of stress/ABA-responsive genes (Xiong et al. 2002; Xiong et al. 2006). Some HD-Zip I proteins regulate abiotic stress responses through either ABA-dependent or ABA-independent pathways (Gago et al. 2002; Deng et al. 2006; Agalou et al. 2008; Shan et al. 2011). Similar to these HD-Zip I proteins, we found that expression of *ZmHDZ4* was also induced by ABA treatment, and overexpression of *ZmHDZ4* in Zhonghua 11 led to an increase in ABA sensitivity, suggesting that *ZmHDZ4* may participate in ABA signal transduction pathways either directly or indirectly.

In conclusion, a drought-induced HD-ZIP I gene was isolated from maize and characterized for its role in drought stress responses. Transgenic rice plants expressing *ZmHDZ4* showed enhanced drought tolerance mediated by changes in REL and MDA and proline levels compared to the WT plants. These results suggest that *ZmHDZ4* is a candidate gene with potential applications in molecular breeding to improve crop drought tolerance.

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