Heterologous Overexpression of ZmHDZIV13 Enhanced Drought and Salt Tolerance in Arabidopsis and Tobacco

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Research Article

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Abstract

The homeodomain leucine zipper (HD-Zip) IV transcription factor is indispensable in the response of plants to abiotic stress. Systematic studies have been carried out in *Arabidopsis*, rice and other species from which a series of stress resistance-related genes have been isolated. However, the function of the HD-Zip-IV protein in maize is not clear. In this study, we cloned the HD-Zip-IV gene *ZmHDZIV13* and identified its function in the stress response. Our phylogenetic analysis showed that *ZmHDZIV13* and *AtHDG11* had high homology and might have similar functions. The heterologous overexpression of *ZmHDZIV13* in *Arabidopsis* resulted in sensitivity to abscisic acid (ABA), salt tolerance during germination and drought tolerance in seedlings. Under drought stress, the transgenic *Arabidopsis* showed stronger drought resistance than the wild-type showed (control). The malondialdehyde content of *ZmHDZIV13* transgenic plants was lower than that of the control, and the relative water content and proline content were significantly higher than those of the control. After the drought was relieved, the expression of *P5CS1*, *RD22*, *RD29B*, *RD29A*, *NCED3* and *ERD1* were upregulated in transgenic *Arabidopsis*. Also, modified tobacco plants (*35S::ZmHDZIV13*) exhibited proper stomatal changes in response to drought conditions. These results show that *ZmHDZIV13*, as a stress-responsive transcription factor, plays a role in the positive regulation of abiotic stress tolerance and can regulate an ABA-dependent signaling pathway to regulate drought response in plants.

Introduction

Plant growth and development is a complex and diverse biological process. This development process is often affected by various biotic and abiotic stress factors, such as pathogens, low temperature, drought and salinity. In response to these stresses, plants induce various regulatory mechanisms occurring at cellular, molecular, physiological and biochemical levels[1]. The earliest abiotic stress signals that plants perceive and transmit are through molecular signaling pathways affecting the expression levels of a series of associated genes[2]. Among them, transcription factors play an important regulatory role in plant stress response. A transcription factor, also known as a transacting factor, is a protein molecule with a special structure that regulates gene expression. Some transcription factors are related to plant resistance. When plants are subjected to abiotic stress, a series of signal transmissions stimulate the expression of transcription factors. The stimulated transcription factors combine with corresponding cis-acting elements to inhibit or enhance gene expression and its downstream effects. In recent years, hundreds of transcription factors related to plant stress resistance (e.g., drought, high-salt, low-temperature), growth and development have been discovered one after another[3].

The homeodomain leucine zipper (HD-zip) protein is an important transcription factor unique to plants. It has been reported in *Arabidopsis*, sunflower, rice, tomato, alfalfa, wheat, barley and potato [4-11]. The HD-ZIP protein includes two important functional domains, the HD domain for DNA binding and a closely connected ZIP domain that is related to protein dimerization. There are 48 HD-ZIP family proteins in the *Arabidopsis* genome, and they are divided into four subfamilies (HD-ZIP I-IV) according to DNA binding specificity, physical and chemical properties and other functional domains[12]. Related studies have

shown that HD-Zip IV participates in root development and epithelial cell differentiation during plant growth and development while regulating the accumulation of anthocyanins and the formation of trichomes[13]. In addition, the HD-Zip IV family has a START domain, which plays a very important regulatory role in response to drought and other abiotic stresses[14]. At present, multiple HD-Zip IV genes have been cloned from *Arabidopsis thaliana*, *Oryza sativa*, and *Zea mays* L. [15-17]. For example, the *AtHDG11* (*HOMEODOMAIN GLABROUS11/ENHANCED DROUGHT TOLERANCE1*) gene from *A. thaliana* is an important member of the HD-START (HD-Zip IV) transcription factor family. The expression of this gene in plants can effectively promote root elongation and stomatal closure, thereby significantly increasing plant resistance to drought[14]. Cao *et al.* [18] transferred *At HDG11* into tall fescue (*Festuca elata*) and the overexpression of the gene enhanced drought and salt tolerances in transgenic plants. Wei *et al.* [19] constructed and transformed an *Arabidopsis At HDG11*-overexpression vector into ryegrass (*Lolium perenne*) and found that compared with wild-type (WT) plants, the transgenic plants had lower levels of malondialdehyde (MDA) under drought stress and maintained high levels of superoxide dismutase (SOD) and catalase (CAT) activity. Yu *et al.* [20]also found that the overexpression of *At HDG11* in cotton (*Gossypium* spp.) and *Populus tomentosa* produced strong drought and salt tolerance, and the transgenic plants had more developed root systems. Li *et al.* [21]found that the overexpression of *AtHDG11* enhanced drought resistance in wheat (T*riticum aestivum* L.), and the transgenic plants had low stomatal density, low water-loss rate, high proline content, and greater accumulation and activity of catalase and superoxide dismutase compared with those of WT plants. Zhu *et al.* [22]found that the overexpression of *AtEDT1/HDG11* in Chinese kale (*Brassica oleracea* var. *alboglabra*) enhances drought and osmotic stress tolerance, the content of proline and the activity of active oxygen scavenging enzymes in transgenic Chinese kale leaves were significantly higher than those in wild Chinese kale. Banavath *et al.* [23]found that overexpression of *At HDG11* improved drought resistance and salt tolerance in transgenic peanut plants (*Arachis hypogaea* L.) by increasing free proline content, water-use efficiency (WUE), chlorophyll content and photosynthetic rate and decreasing stomatal density. Guo *et al.* [24]showed that *AtEDT1/HDG11* enhanced drought resistance by regulating stomatal density and WUE of *A. thaliana*.

Initially, we conducted a BLAST search for homologous protein sequences in a corn database (MaizeGDB) and found that *AtHDG11* (GenBank No. NM_105996) in *Arabidopsi*s and *ZmHDZIV13* (GenBank No. BK008038) in maize are in the same clade with a degree of similarity of 53.51%, indicating high homology. We speculated that *ZmHDZIV13* has a similar function to the function of the *AtHDG11* gene. At present, no research has been reported on the *ZmHDZIV13* gene in maize, and thus its biological function remains unclear. In this study, we cloned the maize HD-Zip-IV gene *ZmHDZIV13* (BK008038) and the function was identified. We found that stress induced by drought, salt (NaCl), ABA and mannitol treatments increased the expression of *ZmHDZIV13*. Overexpression of *ZmHDZIV13* caused ABA hypersensitivity and osmotic stress during the germination period of *Arabidopsis*. Moreover, compared to that of the WT, overexpression increased tolerance to drought stress. These results show that *ZmHDZIV13*, as a stress-responsive transcription factor, plays a role in the positive regulation of abiotic

stress tolerance and is of great significance for improving maize stress resistance and enriching resources based on maize resistance genes.

Materials And Methods

Plant Materials

The experiment was carried out in the Gansu Key Laboratory of Arid Land Crop Science, Gansu Agricultural University. WT *Arabidopsis* seeds were obtained from this laboratory, tobacco (*Nicotiana tobaccum*, T12) seeds were provided by the Gansu Academy of Agricultural Sciences, and maize inbred lines (*Z. mays*, Zheng 58) were provided by Pingliang Breeding Station in Gansu Province.

Bacterial strain, plasmid and transformation

Total RNA was extracted from the immature tassels of the maize inbred line Zheng 58, reverse transcribed into cDNA as a template, and PCR amplified to obtain the full-length cDNA fragment (2372 bp) of *ZmHDZIV13*. The fragment was recovered and ligated into the pUCm-T-Easy vector. The complete sequence obtained by sequencing verification is stored in GenBank under the accession number BK008038.

The primers were designed to amplify the target gene, *ZmHDZIV13*, fragment. We introduced an *Xba* I restriction site at the 5' end of the corresponding target gene fragment and a *Sma* I restriction site at the 3' end to connect with the pUCm-T cloning vector. Then the target gene fragment was obtained by double digestion of *Xba* I and *Sma* I and connected with a 9580-bp fragment of pCAMBIA3300-Ubi-PRO II MCS-*bar* to obtain the pCAMBIA3300-35S-*ZmHDZIV13-bar* plant expression vector. The constructed plant expression vector pCAMBIA3300-35S-*ZmHDZIV13-bar* was transformed into *Agrobacterium tumefaciens* LBA4404, a single colony of bacteria was selected and the *Agrobacterium* plasmids were extracted for PCR identification. The plant expression vector was identified by double enzyme digestion of *Sma* I and *Xba* I. Finally, *A. tumefaciens* LBA4404 containing this vector was screened for and introduced into wild *A. thaliana* and wild tobacco by the flower-soaking method [45]. Homozygous transgenic lines from the T3 generation were used for further assays.

Measurement of germination rate and root traits and evaluation of drought resistance

Thirty *Arabidopsis* T3 seeds were selected from each WT and transgenic lines, surface disinfected, and treated with different concentrations of ABA (0, 0.2, 0.4, 0.6, and 0.8 μM), salt (0, 50, 100, 150, and 200 mM) and mannitol (0, 50, 100, 150, and 200 mM) in their MS growth medium. The seeds were cultivated at 4°C for three days and then transferred to growing conditions of 22°C and a 16-h light/8-h dark cycle. After seven days, the germination rate was measured and the green cotyledons were scored. The seedlings continued to grow vertically for 20 days inside an artificial climate room (16h light/8h dark, 22°C), and the root length, number of lateral roots and root dry weight were recorded. Each experiment was performed with three replicates for each treatment.

A pot (5 cm × 5 cm) experiment was used to identify the drought resistance of *Arabidopsis* seedlings. Peat/forest soil and vermiculite (1:1v/v) were mixed and used as the substrate. Before drought stress treatment, the seedlings were supplemented with water every three days and Hogland nutrient solution every two weeks. After the seeds germinated, the seedlings were not watered for two weeks to simulate drought conditions. After the two weeks of drought, the seedlings were rewatered, and the survival rate and relative leaf water content (RWC) of the seedlings were measured after seven days[46].

Measurement of leaf stomatal density, photosynthesis rate, transpiration rate, and water-use efficiency

A leaf-surface imprinting method[14] was used to compare sampled leaves of the same age and relative position from WT and transgenic tobacco seedlings. We prepared leaf samples by applying a drop of nail polish on the paraxial surface of the leaf, removing the drying layer with adhesive tape, putting that layer on a glass slide, and putting a drop of water on the imprint on the slide. We observed prepared samples under a Leica CTR6000 electron microscope. Five visual fields were observed and photographed under a Leica CTR6000 (Leica, Germany) electron microscope. Stomatal lengths and widths were measured by Image J software (National Institutes of Health). Photosynthetic (*P*) and transpiration (*T*) rates were measured by a portable photosynthesis system (LI-COR, Li-6400, LI-COR Environmental, Lincoln, NE, USA). Each measurement was sampled three times. We calculated WUE by *P/T*.

Measurement of MDA and proline contents

The content of malondialdehyde was determined according to the method of Chen (2002). We sampled 1.0 g transgenic or WT plant tissue after completing the drought stress treatment on plants. Then 2 ml of 10% trichloroacetic acid (TCA) and a small amount of quartz sand were added to grind samples. These coarse homogenates received further grinding after adding 8 ml TCA before centrifuging each homogenate at 4000 R/min for 10 minutes. We collected 2 ml of centrifuged supernatant (2 ml of distilled water was used as a control) and added 2 ml of 0.6% thiobarbituric acid solution. The mixture was then placed into a boiling water bath for 15 min, removed, and quickly cooled before centrifuging the mixture. We measured the absorbances of the supernatant at 532, 600 and 450 nm. MDA content was calculated according to the following steps. First, we calculated the content of MDA in the extract: MDA content in the extract (umol/L) = 6.45 (OD_{532}-OD_{600}) - 0.56OD_{450}. Second, we calculated the content of MDA in the fresh tissue sample: MDA content in fresh plant tissue (μmol/g FW) = MDA content in the extract (μmol/ml) × total extract (ml) / fresh weight of plant tissue (g).

The content of proline was determined by the acid ninhydrin method describd by Chen *et al.* (2002). We ground 0.5-g samples of transgenic or WT plant tissue into a homogenate with 5 ml of 3% sulfosalicylic acid before transferring the homogenate into a centrifuge tube to be placed into a boiling water bath for 10 min. Sample tubes were then removed to cool before filtering each solution; the filtrate was collected to continue extracting the proline. From the filtrate, 2 ml were taken to mix with 2 ml glacial acetic acid and 4 ml of 2.5% acid ninhydrin reagent. The mixture was heated in a boiling water bath for 30 min, after which the solution will turn red. After cooling, we added 4 ml toluene, shook the mixture for 30 s, allowed

any precipitates to settle to the bottom of the tube and transferred the supernatant into a 10 ml centrifuge tube to spin at 3000 R/min for 5 min. The red toluene mixture of proline in the upper layer of each tube was gently transferred into a cuvette, and the toluene solution was used as the blank control. The absorbance values of samples were determined at 520 nm and compared to a standard curve to determine the contents of proline (X) in each sample. The formula used to calculate the contents is: proline content (μg/g) = {(X × total amount of extract (ml)) / (fresh weight of sample (g) × amount of extract used in content determination (ml))}.

Gene expression analysis

Semi-quantitative RT-PCR and quantitative real-time PCR were used to analyze the overexpression of the *ZmHDZIV13* gene. Total RNA was extracted from the leaves of transgenic and wild *A. thaliana* strains using the RNA Extraction Kit (TIANGEN, China). The concentration and quality of RNA were determined by NanoDrop-2000 (Thermo, U.S.A). The first strand of cDNA was synthesized by a Reverse Transcription Kit (TIANGEN, China). The real-time fluorescence quantitative PCR was carried out following a two-step method according to SYBR Premix Ex TaqTM (Takara, Japan) kit's manual. PCR reaction conditions were as follows: pre-denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s and annealing at 56°C for 30 s. The relative expression of genes was calculated and analyzed by the $2^{-\Delta\Delta}$CT method. *His2A* was used as the internal standard to adjust the relative expression levels of transgenic and WT plants. Each treatment was repeated three times.

Results

Informatics analysis of *ZmHDZIV13*

The full-length cDNA sequence of *ZmHDZIV13* was cloned from the inbred line Zheng 58 by RT-PCR, resulting in a length of 2097 bp. The full-length DNA sequence of the gene (GenBank accession number: BK008038) was obtained by searching the National Center for Biotechnology Information database with the cDNA sequence as a probe. Sequence alignment showed that the gene contained 10 exons and 9 introns. The DNA sequence of *ZmHDZIV13* was used as a probe to search through a maize genome database (https://maizegdb.org/). We found that the *ZmHDZIV13* gene was located near the telomere on chromosome 4. Protparam analysis showed that the *ZmHDZIV13* gene, with a predicted molecular weight of 7.62 kDa and an isoelectric point of 6.19, encoded 698 amino acids. CD-Search was used to analyze the conserved domain of the *ZmHDZIV13* protein. The results showed that the *ZmHDZIV13* protein had two conserved domains, HD and START, which was consistent with that of the HDG protein (Fig. 1).

Transformation of *ZmHDZIV13* into *Arabidopsis*

The results of the transformation of *Agrobacterium* showed that the plant expression vector pCAMBIA3300-35S-*ZmHDZIV13*-bar was successfully introduced into the strain LBA4404. The transgenic line *35S::ZmHDZIV13* was screened by adding 300 μg/L basta in the medium. We obtained 53 homozygous lines of *ZmHDZIV13* in the T3 generation. Expression analysis of *ZmHDZIV13* resulted in 39

resistant plants with specific bands amplified near the 2097-bp size, while no corresponding bands were found in the untransformed plants. The PCR-positive transformation rate of resistant plants was 81.25%, indicating that the exogenous gene was successfully integrated into the *Arabidopsis* genome. The amplification results are shown in Figure 2 (homozygous lines L3, L7, L25).

Morphological and physiological characteristics in response to drought stress

After seven days of drought treatment, the survival rate and leaf RWC of the T3 generation of overexpression and WT plants were determined. The results showed that the survival rates of transgenic (L3, L7, L25) and WT plants were 100% when given sufficient water. In contrast, the survival rates of WT, L3, L7 and L25 grown in the drought stress condition decreased by 82.0%, 57.63%, 64.41% and 68.38% of that of the WT control, respectively. The survival rates of L3, L7 and L25 were 135.39%, 97.72% and 75.67% higher than those of the WT under stress, respectively. Where water availability was sufficient, the RWCs of WT, L3, L7 and L25 remained above 91%, and the differences among these groups were not obvious. With the temporary drought, the RWCs of leaves of L3, L7 and L25 were reduced. Moreover, RWC of the WT reached the lowest amount, 59.09%, while the RWCs of the *ZmHDZIV13*-transgenic plants basically remained above 50% and were 70.89% (L3), 66.88% (L7) and 30.40% (L25) higher than that of the WT (Fig. 3). The results showed that the water holding capacity of the T3 generation of plants that overexpressed *ZmHDZIV13* was stronger than that of WT plants subjected to the temporary drought.

The contents of MDA and free proline are important indexes used to reflect drought resistance of crops. Proline is an osmoprotectant that reduces the osmotic potential of cells in response to drought stress. MDA is a product of plant cell membrane lipid peroxidation. Drought stress causes plant cells to lose water and eventually damages the membrane system observed in the ruptures of the vacuolar membrane and damages to the lipid membrane structure. In our experiment, the contents of MDA and proline were determined from WT, L3, L7 and L25 plants exposed to the drought stress. The results showed that the contents of MDA and proline in the non-transformed and transformed lines were similar to the corresponding contents in the regularly irrigated group. After seven days of water deprivation, the MDA contents of L3, L7 and L25 decreased significantly by 13.67%, 15.63% and 13.60%, respectively, and the proline contents of L3, L7 and L25 increased significantly by 10.14%, 9.74% and 4.99%, respectively, from that of their non-deprived groups (Fig. 3). The results showed that the physiological indexes related to drought resistance observed from the transformed plant leaves were improved in response to the temporary drought treatment.

Morphological characteristics in response to different ABA levels

ABA signaling stimulates plant responses to various stress factors by regulating the expression of stress- and ABA-responsive genes and the closure of leaf stomata [25]. In order to determine whether the *ZmHDZIV13* gene improves drought resistance in transgenic *Arabidopsis* through the ABA signaling pathway, the phenotypes of WT and transgenic *A. thaliana* seedlings grown on MS medium containing 0.6 μM ABA were observed and compared. There were no significant differences in seedling and root lengths between WT and the transgenic L3, L7 and L25 plants grown in MS medium without ABA (Fig. 4).

In the medium containing 0.6 μM ABA, the growth of WT and transgenic *Arabidopsis* seedlings was significantly inhibited, and the degree of inhibition was significantly greater in transgenic than in WT plants. These results indicate that overexpression of *ZmHDZIV13* can significantly increase the sensitivity of transgenic plants to ABA.

Morphological characteristics in response to NaCl treatments and osmotic stress in *Arabidopsis*

The WT *Arabidopsis* and *35S::ZmHDZIV13* transgenic lines (L3, L7, and L25) were subjected to osmotic stress tests on a medium supplemented with NaCl and mannitol to determine potential interactive effects of genetics and environmental conditions. The results showed that the germination rate of transgenic plants increased with the increase of NaCl concentrations, but the germination rate of transgenic plants was lower than that of WT plants (Fig. 5). These results indicate that *ZmHDZIV13* can improve germination and cotyledon-emergence rates of transgenic plants under osmotic and salt stresses.

Root length, number of lateral roots and root dry weight of *35S::ZmHDZIV13* and WT *Arabidopsis* seedlings under NaCl and mannitol stresses were measured or counted. The number of lateral roots from *ZmHDZIV13* was higher than that from WT (Figs. 5 and 6). Transgenic *35S::ZmHDZIV13* seedlings showed strong osmotic resistance under high (150 mM) NaCl/mannitol stress. With the addition of 150 mM NaCl, the average root length and root dry weight of transgenic *Arabidopsis* seedlings decreased by 57.59% and 4.59%, respectively, while the number of lateral roots increased by 10.34%. The root length, root dry weight and number of lateral roots of WT decreased by 80.95%, 35.23% and 67.47%, respectively. Due to treatment with 150 mM mannitol, the average root length and root dry weight of transgenic *Arabidopsis* seedlings decreased by 11.97% and 11.36%, respectively, while the number of lateral roots increased by 88.50%. The root length, root dry weight and number of lateral roots of WT decreased by 11.94%, 54.55% and 72.29%, respectively (Fig. 6). The results showed that *35S::ZmHDZIV13* seedlings exhibited stronger tolerance to the drought stress, and it might be due to the increase of number of lateral roots.

Relative expression levels of stress response genes under drought stress

In order to elucidate the molecular mechanism of drought tolerance of the *ZmHDZIV13* gene, the transcription levels of six drought-related genes were compared by quantitative PCR. The results showed that ABA and drought-stress-inducible genes *(P5CS1*, *RD22*, *RD29B*, *RD29A*, *ERD1*, and *NCED3*) were highly expressed in dehydrated plants, and the expression levels of drought genes in transgenic plants were significantly higher than that in WT plants (Fig. 7). For example, the relative expression levels of *RD29B* in *ZmHDZIV13* transgenic lines L3, L7 and L25 were respectively 127.70, 126.41 and 153.05 at the 6th day of dehydration. In addition, we found that drought could induce the expression of *ERD1*, but there was no significant difference in expression between transgenic and WT *Arabidopsis*. Before the drought treatment, with the exception of *RD29B*, the expression levels of all other genes in transgenic plants were lower than those in WT.

Leaf density and water-use efficiency in transgenic tobacco

Heterologous reproduction experiments were carried out with transgenic tobacco, the genetic structure pCAMBIA3300-*35S-ZmHDZIV13-bar* was transferred into tobacco and further studied using three independent over expression lines T2 homozygous lines (L7, L10 and L17) (Fig. 8). The results showed that stomatal density of transgenic *ZmHDZIV13*-overexpression lines in L10 and L17, but not in L7, were lower than that in WT lines (Fig. 8A and E), while the size of a single stomate of each transgenic line was greater than that of WT lines (Fig. 8F). The lower stomatal density may be related to the greater size of epidermal cells (Fig. 8B), and the decrease of stomatal density may help to reduce water loss from the leaves of transgenic plants. The photosynthetic rates were significantly greater, transpiration rates were significantly lower (Fig. 8B and C), and WUEs were significantly higher in transgenic than in WT lines (Fig. 8D).

Discussion

Current studies have shown the induced expression of transcription factors, such as *Os MYB3R-2*, *CBF1*, *Os SDIR1*, *ABF2*, and *SlHZ24*, in response to stress[26–29], can improve the resistance of plants to stress, and more and more evidence shows that HD-ZIP family proteins participate in plant response to stress[30]. The HD-Zip IV subfamily of transcription factors are mostly and specifically expressed in the epidermal tissues of plant organs, and they are mainly involved in accumulation of anthocyanins, epidermal differentiation and trichome formation on plant organs[8, 31]. There are 16 members of the IV subfamily in *Arabidopsis* [31]. Studies have found that the *ATHB10* gene mainly functions in the root hair, epidermal hair and seed coat[32]. Vernoud *et al.* [33] found that the *OCL4* gene of maize is mainly expressed in the leaf epidermis. Overexpression of this gene will inhibit the development of transgenic *Arabidopsis* leaf epidermal trichomes, while silencing the expression of this gene will cause abnormal phenotypes in epidermal hair differentiation in plants. Research by Li *et al.* [34] showed that overexpression of *HGD3* causes anthers to remain closed and leads to male sterility. In our study, we cloned the full-length cDNA of *ZmHDZIV13* (an HD-Zip IV gene), analyzed the effects of endogenous ABA, NaCl, and mannitol on *ZmHDZIV13*-transgenic *Arabidopsis* and tobacco lines, and evaluated how *ZmHDZIV13* regulates downstream genes in transgenic *Arabidopsis*.

ABA signaling stimulates plant responses to various stress factors by regulating the expression of stress and ABA-responsive genes and the closure of leaf stomata[25]. Studies on a similar HD-Zip I gene, *Hahb-4*, was expressed in sunflower (*Helianthus annuus* L.) at the early stage of development and could be induced by abiotic stress from water deficiency or by ABA [35]. Studies on the HD-Zip I gene in rice showed that overexpression of *ZMHDZ10* enhanced drought and salt tolerance, but increased sensitivity to ABA[36]. In addition, previous studies have found that overexpression of *AtEDT1/HDG11* can lead to ABA hypersensitivity, thus inhibiting germination and inducing stomatal closure[22]. In this study, we found that overexpression of *ZmHDZIV13* can significantly improve the sensitivity of transgenic *Arabidopsis* to exogenous ABA, which indicates that *ZmHDZIV13* may play a positive role in regulating plant stress response through an ABA-dependent signal transduction pathway. *Oshox22*, *Athb7*, *Athb12*, and *Zmhdz10* are members of the HD-Zip I family[37], and they have been reported to regulate plant responses to various stress factors by participating in the ABA signaling pathway. Similar to the genes of

HD-Zip I, *ZmHDZIV13* was not only induced by drought, but also induced by exogenous ABA, indicating that *ZmHDZIV13* may be directly or indirectly involved in the ABA signaling pathway. More direct evidence was observed where the overexpression of *ZmHDZIV13* enhanced plant sensitivity to exogenous ABA, lending further support of the role of *ZmHDZIV13* in the ABA signal transduction pathway. The results also showed that the expression of ABA responsive genes in transgenic plants was activated due to stress induction. In conclusion, these results indicate that *ZmHDZIV13* can regulate plant acclimation to drought through the ABA signaling pathway.

In the process of plant evolution, in order to adapt to the changing external environment and to maintain their own growth, plants have formed a set of complex and rigorous stress response mechanisms. When plants are stimulated by external stress, the content of reactive oxygen species within the plants accumulates, resulting in lipid peroxidation and peroxidation stress, which is not conducive to the normal growth of plants. Drought tolerance in transgenic maize containing the *ZmHDZIV13* gene was investigated. After 7 days of drought, the untransformed plants exhibited serious damage, for example, their leaves showed different degrees of wilting and curling. In contrast, drought tolerance in transformed maize plants was significantly enhanced and these plants maintained normal growth and produced healthy-looking phenotypes. Malondialdehyde is commonly used as an indicator of the degree of lipid peroxidation[38]. In response to the drought stress, MDA contents in transgenic plants overexpressing *ZmHDZIV13* were significantly lower than that in the control group, indicating that overexpression of *ZmHDZIV13* can reduce the degree of membrane lipid peroxidation in transgenic plants and reduce the degree of cell membrane damage. Free proline in plants has important functions. In plants under stress, free proline can stabilize subcellular structure, maintain intercellular permeability and reduce cell damage by accumulating proline[39]. Our results showed that the free proline content in transgenic plants was significantly higher than that in WT group subjected to drought. Meanwhile, the accumulation of proline reduced the water potential in *Arabidopsis* and inhibited the loss of water from cells, which is consistent with the RWCs observed in leaves under drought stress. The water content of leaves in the transgenic group was significantly higher than that in WT. The results also showed that cell membrane damage in transgenic plants under stress was less than the damage in the WT. Moreover, resistance to membrane lipid peroxidation was significantly enhanced, which improved the growth and development of individuals and further protected these transgenic *Arabidopsis* plants from the abiotic stress. These results indicate that *ZmHDZIV13* is overexpressed in maize inbred lines, which helps maintain plasma membrane stability and improve antioxidant capacity.

Reportedly, overexpression of stress-inducible transcription factors usually improves the resistance of plants to various stresses by regulating the expression of downstream stress-response genes[40], thus it is necessary to identify the target genes downstream of *ZmHDZIV13*. Overexpression of *ZmHDZIV13* in *A. thaliana* can significantly improve plant tolerance to drought and high salinity, indicating that the stress-response pathway regulated by *ZmHDZIV13* may be conserved in plants[36]. In this study, we found through homologous alignment, the genes that play a key role in the drought signaling pathway in *A. thaliana* and detected the expression of stress-related genes (*ERD1*, *RD29A*, *P5CS1*, *RD22*, *NCED3* and *RD29B*) in transgenic *A. thaliana* by fluorescence quantitative PCR. The results showed that the

expression levels of all genes, except of *RD29B*, in transgenic plants were lower than those in WT plants when given sufficient water. In response to the drought-stress treatment, the expression levels of all genes were up-regulated to varying degrees, and the expression levels of all genes in transgenic seedlings were significantly higher than those in WT seedlings. These results indicate that overexpression of the *ZmHDZIV13* gene plays an important role in increasing the expression of six marker genes in transgenic plants, and they also indicate that *ZmHDZIV13* may participate in an ABA-dependent signal transduction pathway. The *RD29B*, *RD22* and *RD29A* genes have been shown to be primarily involved in drought stress and ABA response[41] and can improve plant tolerance to stress, such as dehydration, and ABA induction[2, 36, 42]. Therefore, *RD29B*, *RD22* and *RD29A* may be indirectly regulated by ABA induced by drought stress. Zhu *et al.* [22]also showed that *AtEDT1/HDG11* enhanced abiotic stress resistance in Chinese kale through auxin and ABA-mediated signal transduction. In addition, *RD29B*, *RD22* and *RD29A* may participate in ABA signal transduction in maize. *NCED3* improves drought resistance and salt tolerance by regulating ABA synthesis in plants [2]. Previous studies have shown that *OsNCED3* is a drought-stress responsive gene in rice that regulates ABA levels and stress resistance when grown under drought stress[43] *ERD1* is an early drought-inducible gene. After drought stress, the expression of *ERD1* was significantly induced in both transgenic and WT *Arabidopsis*, which further supports the conclusion that *ZmHDZIV13* may be involved in the ABA signal transduction pathway. Previous studies have shown that some HD-Zip transcription factors are involved in ABA-independent pathways regulating *ERD1* expression [41, 36]. Also playing an important role in plant stress response is *P5CS1* that encodes proline synthase [44]. The induced expression of *P5CS1* can lead to the accumulation of proline, which in the *ZmHDZIV13*-overexpressing transgenic plants, proline accumulation results in strong stress resistance. Therefore, the activation of these stress resistant genes plays a crucial role in improving the stress resistance of *ZmHDZIV13* transgenic plants. In conclusion, *ZmHDZIV13* may be an important upstream gene regulating ABA biosynthesis and signal transduction.

Conclusion

In this study, we isolated *ZmHDZIV13* from maize HD-Zip-IV transcription factor family, and characterized its role in drought and salt stress and its sensitivity to ABA. The results showed that *ZmHDZIV13* mediated signal transduction, thus regulating genes related to abiotic stress response and realizing cell protection. This is similar to our previous study on *ZmHDZIV14*(Fang *et al.*, 2020), These results also provide valuable information for further research and application of *ZmHDZIV13* gene in drought resistance improvement of maize and other crops.

Declarations

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Author contributions YP designed the experiments. PF and HY performed the experiments. YP, PF and FW analyzed the data. YP, PF and FW wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interests The authors declare that they have no conflicts of interest.

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Figures

Figure 1

Homology analysis of ZmHDZIV13 and At HDG11 amino acid sequences

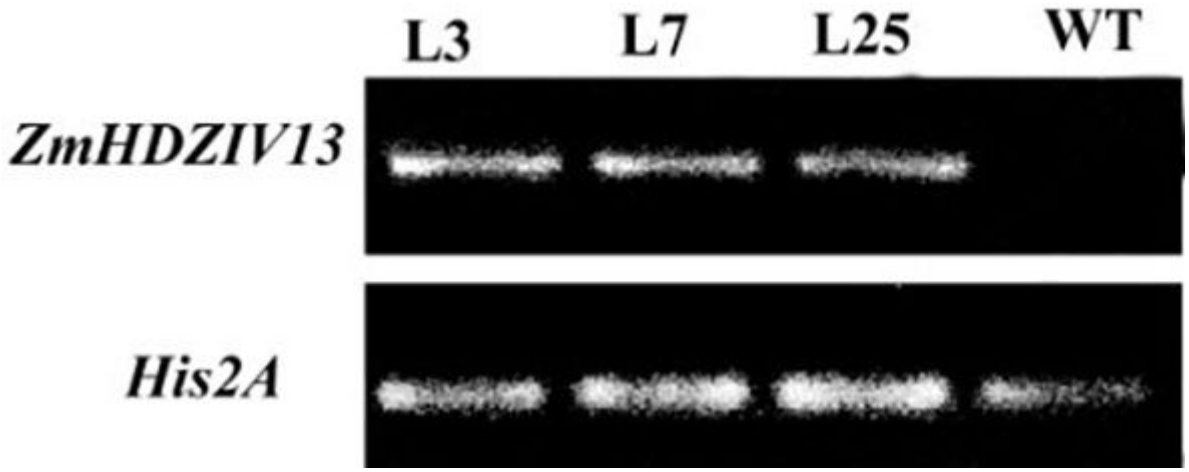

Figure 2

RT-PCR of the 35S::ZmHDZIV13 transgenic and WT Arabidopsis lines. His2A served as the internal standard for adjustment of relative expression levels.

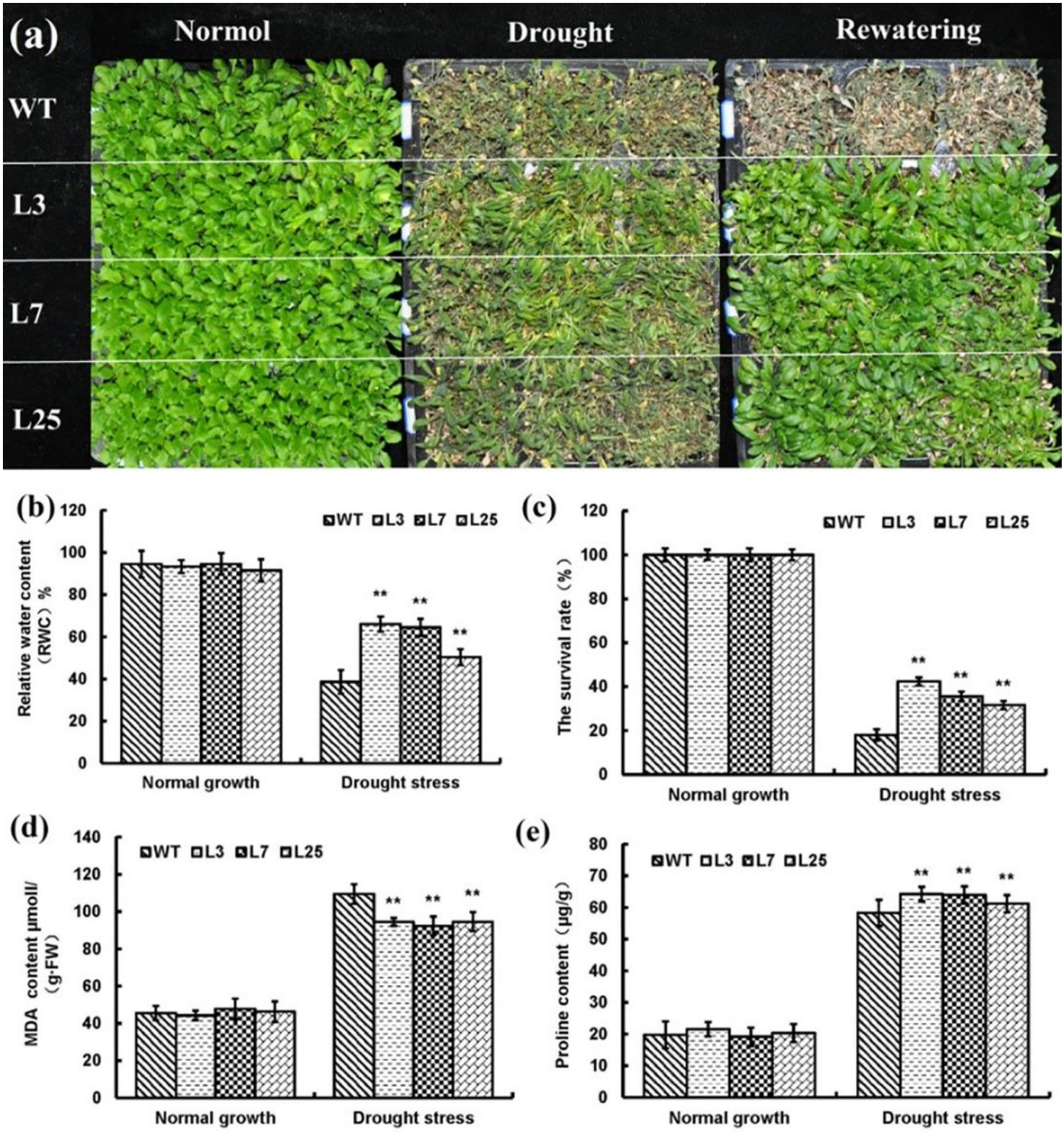

Figure 3

The morphological and physiological characteristics of transgenic lines (L3, L7 and L25) and wild type Arabidopsis thaliana under drought stress were studied. a. Morphology and development under normal water condition, drought condition and after the restoration of watering of the plant were studied. b~e. RWC, survival rate, MDA content and proline content were measured under normal water and drought stress.

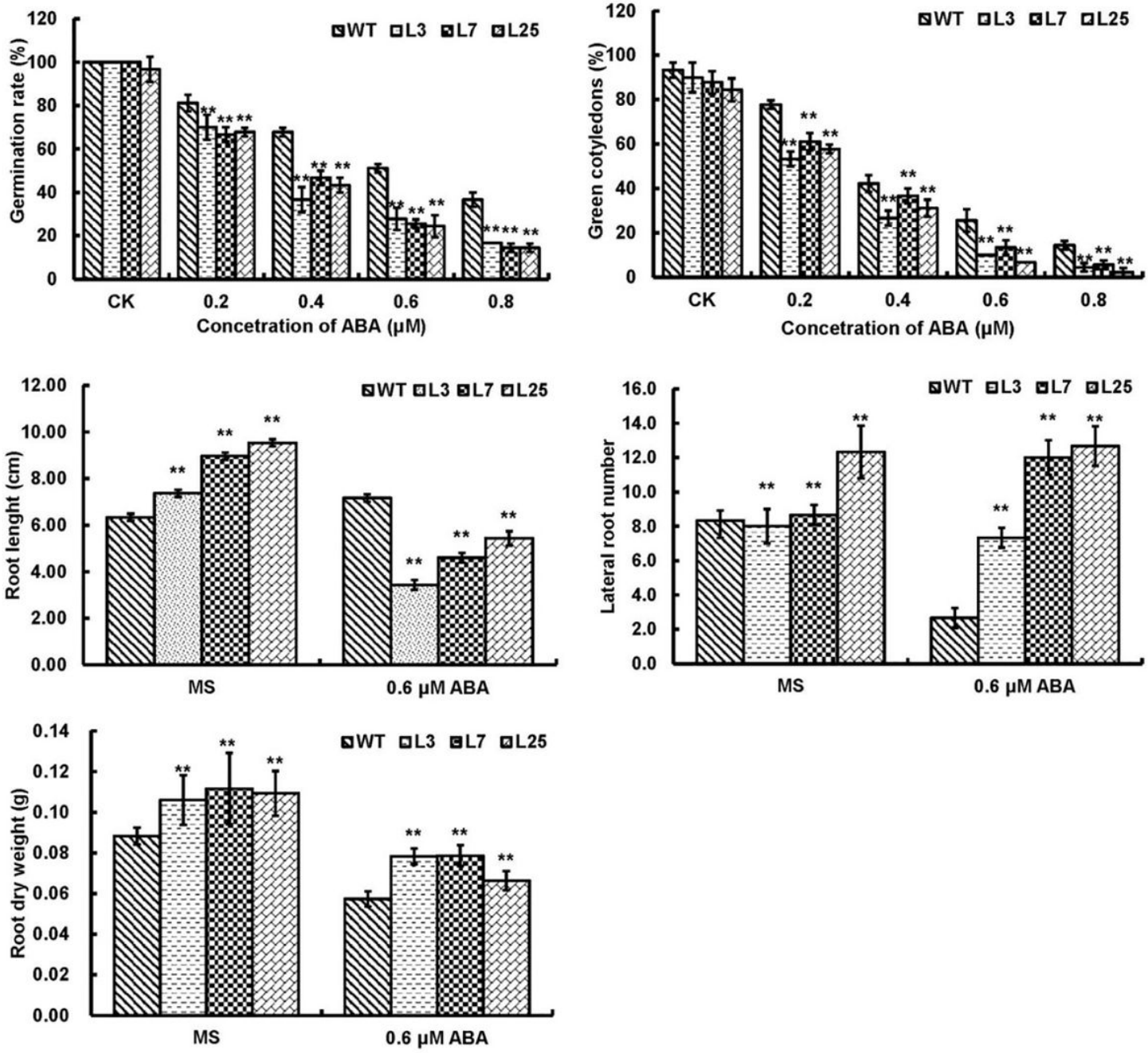

Figure 4

Morphological characteristics of transgenic (L3, L7 and L25) and WT lines at different ABA levels (CK: 0.2, 0.4, 0.6 and 0.8 μ m ABA).a. Germination rate(%).b. Green cotyledons (%).c. The root length was measured at 20 th days. d. The number of lateral roots was measured at 20th days. e. Root dry weight measured on the 20th day s(g).

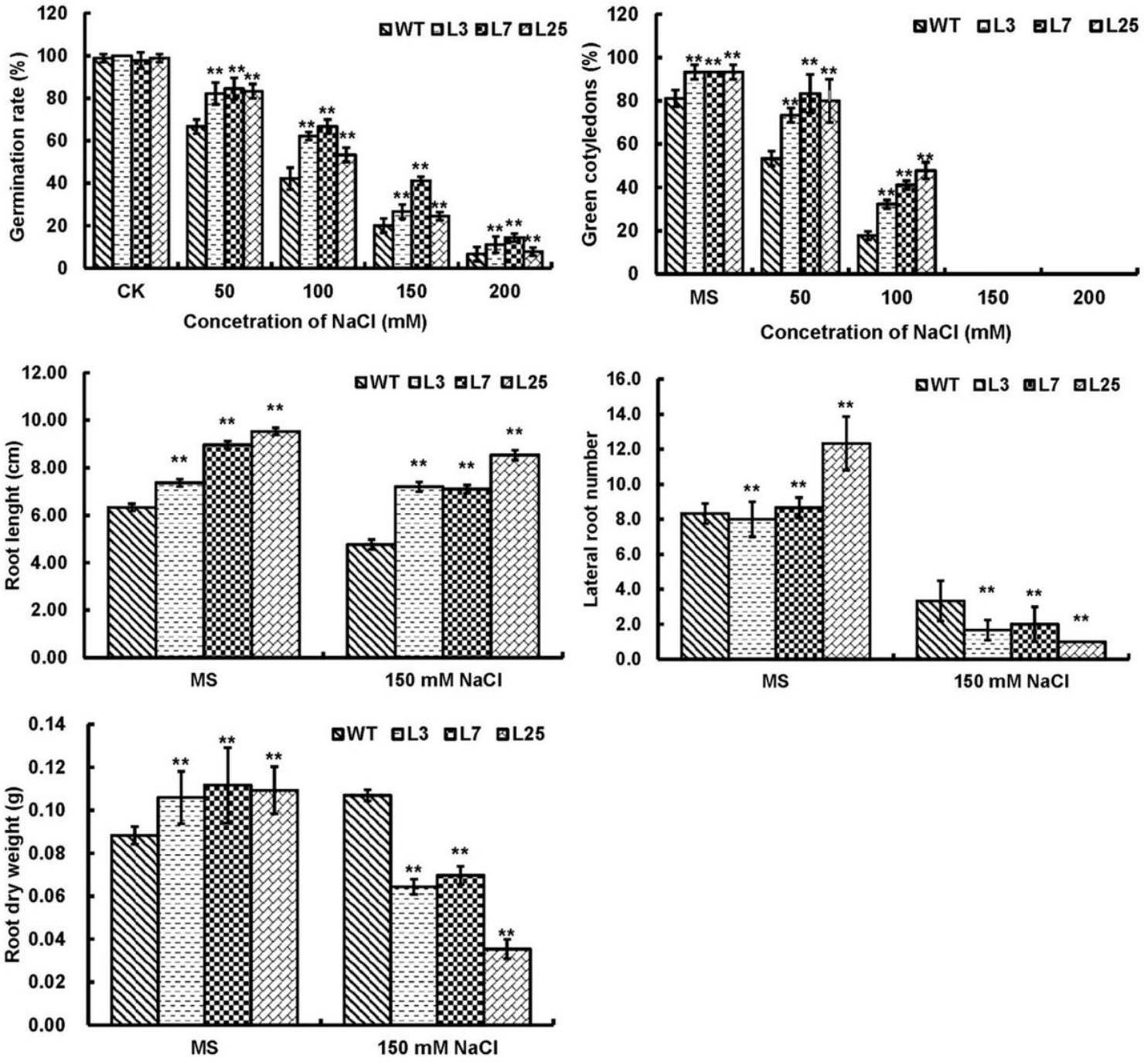

Figure 5

The morphological characteristics of transgenic Arabidopsis thaliana were different under the NaCl treatments (CK, 50,100,150 and 200 mM). a. Germination rate(%).b. Green cotyledons (%).c. The root length was measured at 20 th days.d.The number of lateral roots was measured at 20th days. e. Root dry weight measured on the 20th days(g).

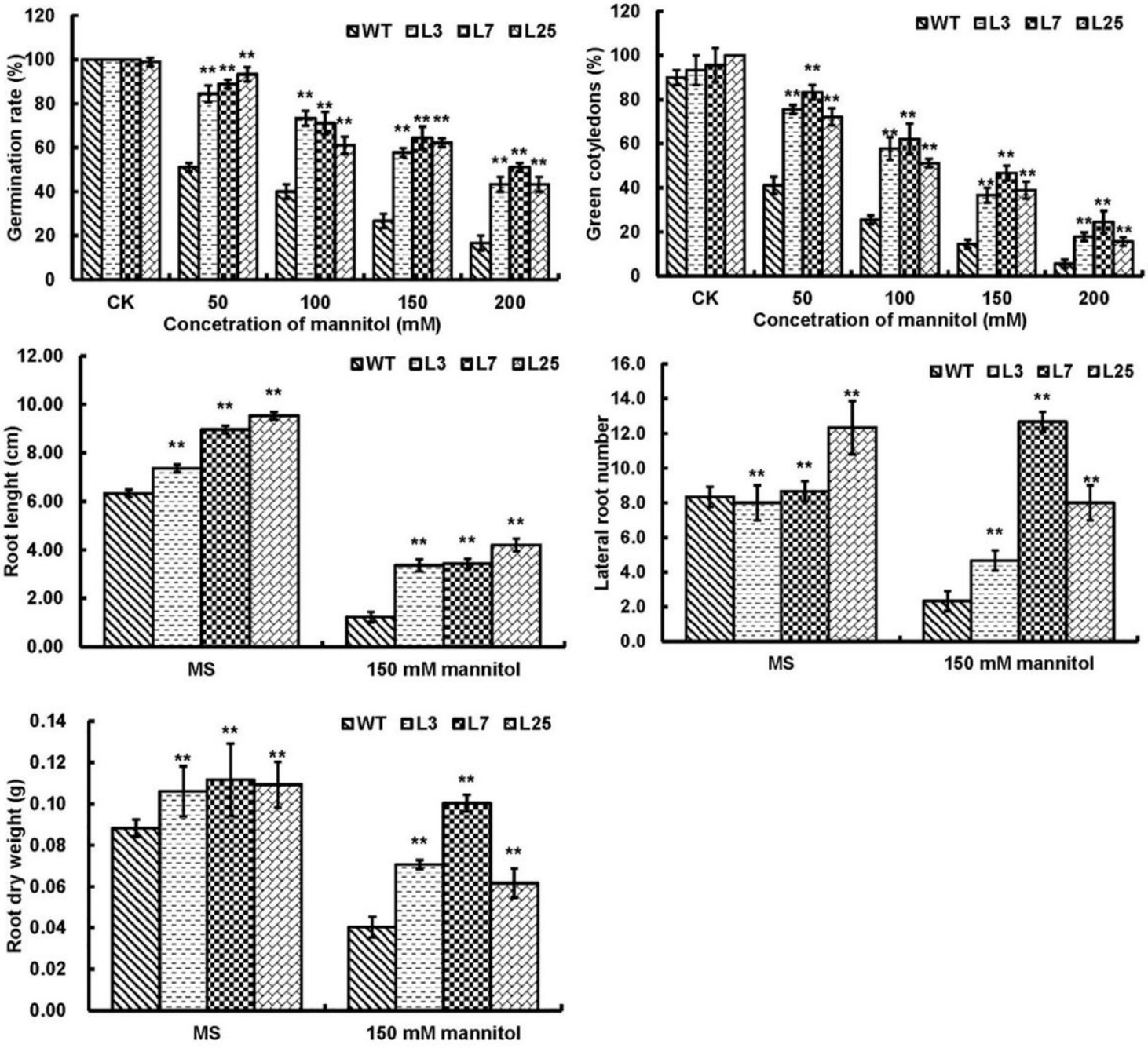

Figure 6

The morphological characteristics of transgenic Arabidopsis thaliana were different under the mannitol treatments (CK, 50,100,150 and 200 mM). a. Germination rate(%). b. Green cotyledons (%). c. The root length was measured at 20 th days. d. The number of lateral roots was measured at 20th days. e. Root dry weight measured on the 20th days(g).

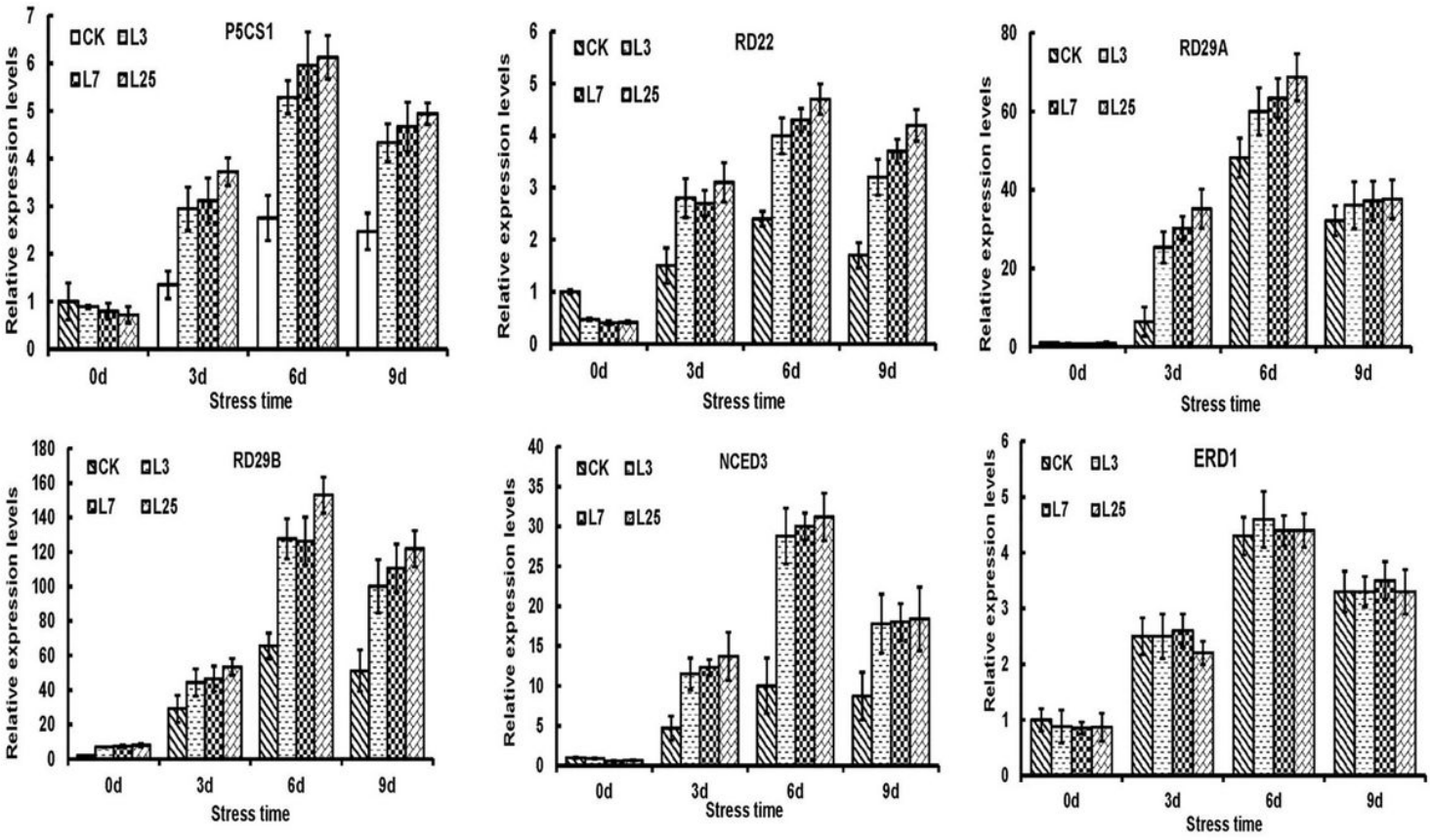

Figure 7

Relative expression levels of six stress response genes (P5CS1, RD22, RD29B, RAB18, NCED3 and ERD1) in Arabidopsis lines (L6, L19 and L28) at 0, 3, 6 and 9 days after drought stress.

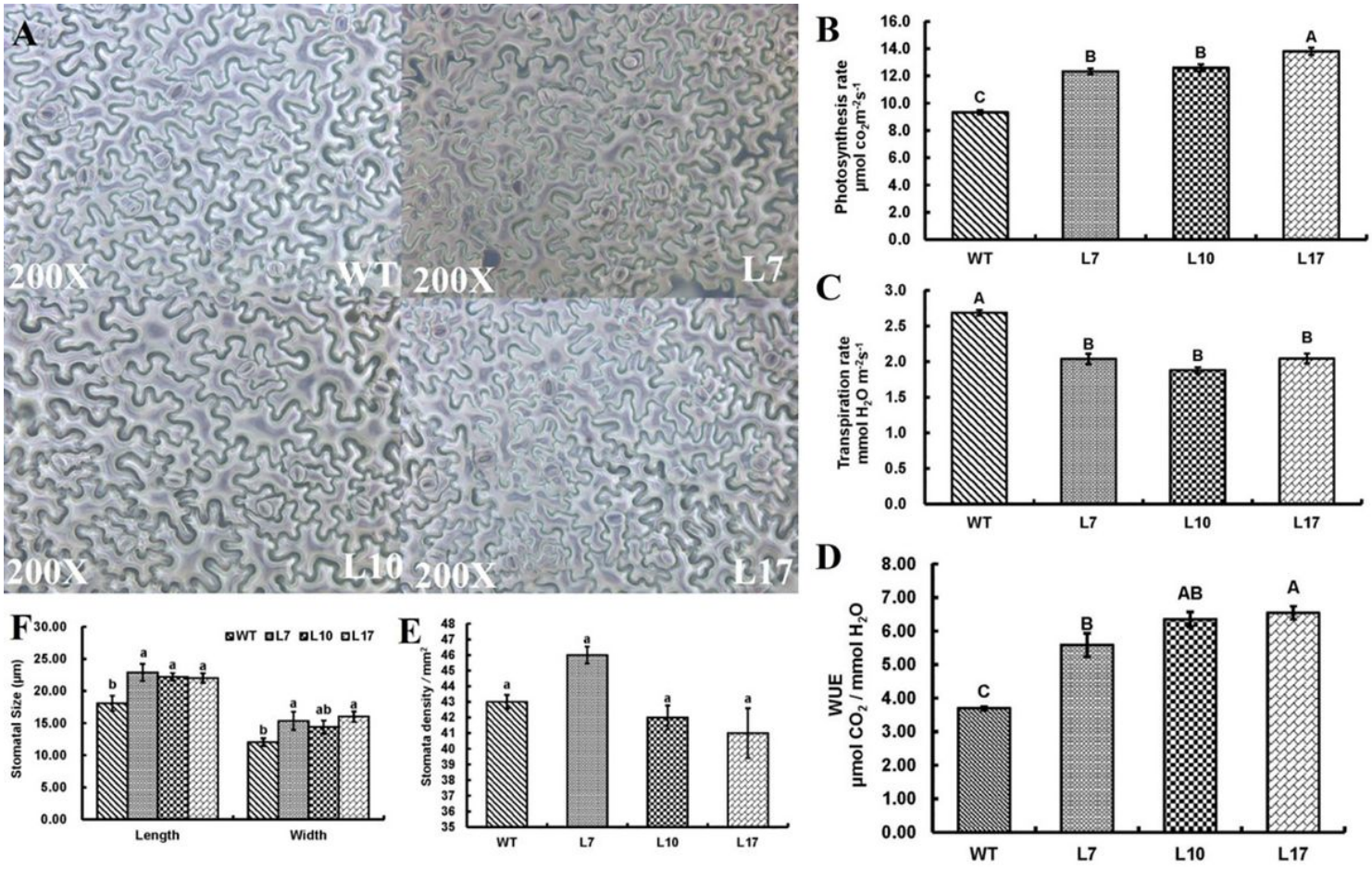

Figure 8

Stomatal density (A, E), stomatal size (F), photosynthesis rate (B), ranspiration rate (C), and water use efficiency (WUE) (D) in transgenic (L7, L10, and L17) and WT tobacco lines.