Plant Mol Biol Rep (2016) 34:353–364
DOI 10.1007/s11105-015-0926-2

ORIGINAL PAPER

ZmDBF3, a Novel Transcription Factor from Maize (*Zea mays* L.), Is Involved in Multiple Abiotic Stress Tolerance

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Published online: 14 August 2015

Abstract The dehydration-responsive element-binding (DREB) transcription factors play important roles in regulation of plant responses to abiotic stresses. In the present study, *ZmDBF3*, a novel DREB transcription factor gene from maize (*Zea mays* L.), was cloned and characterized. Sequence analyses revealed that *ZmDBF3* is classified into A-4 group. It was demonstrated that *ZmDBF3* was induced in by salt, drought, cold, and high temperature, as well as by signaling molecules abscisic acid (ABA), but no significant changes were observed under salicylic acid (SA) and methyl jasmonate (MeJA) conditions. The results of transient expression assays and transcriptional activity analysis revealed that ZmDBF3 is a nuclear protein with transcriptional activity. Overexpression of *ZmDBF3* in yeast (*Saccharomyces cerevisiae*) exhibited increased survival rate under NaCl, KCl, Na_2CO_3, $NaHCO_3$, PEG6000, freezing, and sorbitol treatment, compared with the control. Furthermore, ectopic expression of *ZmDBF3* in *Arabidopsis* significantly enhanced tolerance to salt, drought, and freezing tolerance. Taken together, the findings indicated that the ZmDBF3 is a novel member of DREB transcription factor which may act as a regulatory factor involved in multiple stress response pathways.

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Keywords ZmDBF3 · Maize · Abiotic stress · Yeast expression · *Arabidopsis*

Introduction

Abiotic stresses, especially drought and high salinity, are major limiting factors that retard plant growth and severely decrease crop productivity. To survive, plants have to evolve complex mechanisms to adapt to ever-changing environmental conditions. Drought stress induces a range of physiological and biochemical responses in plants, including stomatal closure, inhibition of photosynthesis, and respiration activation (Shinozaki and Yamaguchi-Shinozaki 2007). Additionally, plants respond to abiotic stresses at the cellular and molecular levels, including stress perception, signal transduction to cellular components, gene expression, and metabolic changes (Agarwal et al. 2006; Shinozaki and Yamaguchi-Shinozaki 2007).

Transcription factors have been proved to mediate the function of multiple stress response genes in a coordinated manner, which are considered as attractive targets for application through genetic engineering. The dehydration-responsive element-binding (DREB) proteins are the major transcription factors that induce a set of abiotic stress-inducible gene expression in the ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki 2006). DREBs are parts of APETALA2/ethylene-responsive factor (AP2/ERF) superfamily, specially bind the *cis*-element of dehydration responsive element (DRE, A/GCCGAC), in the promoter region of many stress-inducible genes (Yamaguchi-Shinozaki and Shinozaki 1994; Jiang et al. 1996). Two groups of *DREB* genes were discovered in the *Arabidopsis*, *DREB1s* and *DREB2s* (Agarwal et al. 2006). Expression of the *Arabidopsis DREB1* genes is induced by cold, but not by

dehydration or high-salinity. By contrast, the *DREB2* genes can be induced by dehydration, high-salinity, and heat stresses, but not by cold stress (Lata and Prasad 2011). Hence, DREB1s and DREB2s are involved in two separate signal transduction pathways under low temperature and dehydration, respectively. However, *DREB1D*, *DREB1E*, and *DREB1F* are also induced by osmotic stress, suggesting the existence of cross talk between the DREB1 and the DREB2 pathways (Haake et al. 2002; Nakashima et al. 2009).

Numeral studies demonstrated that overexpression of special *DREB* genes in transgenic plants using constitutive or inducible promoters resulted in plants more tolerant to drought, salt, heat, and freezing stresses (Lata and Prasad 2011). For example, transgenic *Arabidopsis* plants overexpressing *DREB1B* under control of the cauliflower mosaic virus (CaMV) 35S promoter showed strong tolerance to freezing stress (Jaglo-Ottosen et al. 1998). Similarly, transgenic *Brassica napus* or tobacco plants overexpressing the *Arabidopsis* DREB1/CBF genes increased the freezing tolerance and induced the expression of cold-related genes (Jaglo-Ottosen et al. 2001; Kasuga et al. 2004). The overexpression of *AtDREB1A* driven by the stress-inducible *rd29A* promoter in transgenic wheat also showed improved tolerance to drought stress (Pellegrineschi et al. 2004). Besides in *Arabidopsis*, DREB transcription factors have been identified in other various plant species, such as rice (*Oryza sativa*), soybean (*Glycine max*), sorghum (*Sorghum bicolor*), hot pepper (*Capsicum annum*), tobacco (*Nicotiana tabacum*), and chickpea (*Cicer arietinum*) (Lata and Prasad 2011). These observations suggested that the DREB regulation can be used to improve the tolerance of various kinds of agriculturally important crops to multiple abiotic stresses by gene transfer.

Maize (*Zea mays* L.), one of the most important crops in the world, is frequently impacted by drought, high salinity, and low temperature. Therefore, how to improve abiotic stress tolerance in maize is a priority target in many breeding programs. Previously, two *DREB* genes (*ZmDREB1A* and *ZmDREB2A*) belonging to the DREB1 and DREB2 subgroups, respectively, were identified and characterized (Qin et al. 2004; Qin et al. 2007). *ZmDREB1A* was accumulated in response to cold stress, while transcripts of *ZmDREB2A* were increased by cold, dehydration, salt, and heat stresses in maize seedlings (Qin et al. 2007; Qin et al. 2004). Moreover, constitutive or stress-inducible expression of *ZmDREB2A* resulted in improved drought stress tolerance in plants (Qin et al. 2007). However, until now, much less is known about functions of the other DREB transcription factors in maize. In the present study, we isolated *ZmDBF3* gene from maize and examined its expression pattern in response to various abiotic stresses and signal molecules. Furthermore, the role of *ZmDBF3* in tolerance to abiotic stresses was examined by ectopic expression in both yeast cells and *Arabidopsis*.

Materials and Methods

Plant Material, Growth Conditions and Treatments

Maize (*Z. mays* L. cv. B73) was employed to isolate the *ZmDBF3* gene and to evaluate its expression patterns under various treatments. Seeds were germinated in pots containing vermiculite and cultured in growth chambers (16-h light/8-h dark cycle with a light intensity of 200 μmol m^{-2} s^{-1} at 25 °C). Similar sized seedlings at the same stage (8 days old) were selected and subjected to the following treatments. For salt, drought, abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate (MeJA) treatments, the roots of the seedlings were immersed in Hoagland's solutions containing 250 mM NaCl, 20 % polyethylene glycol 6000 (PEG 6000) and 100 μM ABA, 2 mM SA, and 100 μM MeJA, respectively. Cold treatment was conducted by exposing the seedlings to 4 °C in a chamber with a 16-h light/8-h dark regime for 24 h. For high-temperature stress, seedlings were kept in a chamber at 40 °C for 0.5, 1, 3, 4, 5, and 6 h. Leaves from plants in each treatment were collected at special time points, immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

Amplification and Sequence Analysis of *ZmDBF3*

Genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method. The full-length coding sequence of *ZmDBF3* was amplified with Pfu DNA polymerase (TransGen, Beijing, China) from the DNA by gene-specific primers (forward ATGAGCTTGCTTGTGACTG; reverse TCTTGCTCGTCAGTGCTCC) based on the sequence from NCBI database (NM_001157828). The products were purified and cloned into pMD18-T vector (Takara, Dalian, China) for sequencing. The isoelectric point (p*I*) and molecular weight (MW) of ZmDBF3 were predicted with ExPASy (http://expasy.org/tools/pi_tool.html) for computation. Multiple sequence alignment was performed by DNAMAN software. Phylogenetic analysis was generated by neighbor-joining method and displayed using MEGA4.0 based on the similarity of amino acid sequences (Tamura et al. 2007).

Subcellular Localization of ZmDBF3 Protein

Primers (ZmDBF3-F: CGAGCTCATGAGCTTGCTTGTGACTG, the underlined indicates *Sac* I restriction site, ZmDBF3-R: CACCATGGTGGCGACCGGTGGGTGCTCCCAGAGCAGCAAC) and primers eGFP-F: AGCACCCACCGGTCGCCACCATGGTGAGCAAGGGCGAG, eGFP-R: 5′-TCCCCCGGGTTACTTGTACAGCTCGTC, the underlined indicates *Sma* I site) were used to amplify ORF of *ZmDBF3* without the termination codon and eGFP, respectively. The products were linked by

overlapping PCR method, and the recombinant fragment was digested by *Sac* I/*Sma* I and inserted into the corresponding sites of pCHF-1301 vector (Li et al. 2014) and verified by sequencing. Then, the resultant DNA was transformed into *Agrobacterium* EHA105. Transient expression of the *ZmDBF3::eGFP* fusion construct and *eGFP* control was performed by introducing the resultant DNAs into onion epidermal cells using *Agrobacterium*-mediated genetic transformation method. Transformed cells were cultured on Murashige and Skoog (MS) medium for 16–48 h and observed under a confocal microscope (Olympus, Japan).

Transcriptional Activation Assay

The complete coding sequence of *ZmDBF3* was amplified by PCR with primers (ZmDBF3-F: CCGGAATTC TCTTGCTCGTCAGTGCTCC (*Eco*R I), ZmDBF3-R: CGCGGATCCTCTTGCTCGTCAGTGCTCC (*Bam*H I)) and cloned into the pGBKT7 vector. The pGBKT7-ZmDBF3 construct, pGAL4 plasmid (as s positive control), and the negative control (pGBKT7 vector) were transformed into yeast strain AH109. Transformed cells were confirmed by PCR and then plated on SD/-Trp or 10 mM 3-AT of SD/-Trp-His-Ade medium. Transcription activation was evaluated according to the growth status of yeast cells after incubating plates at 30 °C for 3 days with 5-bromo-4-chloro-3-indolyl-b-D-galacto- pyranoside (X-gal).

Abiotic Stress Analysis of *ZmDBF3* in Yeast Cells

The full-length sequence of *ZmDBF3* was amplified and directionally cloned into the yeast expression vector pYES2 (named pYES2-ZmDBF3), which contained the Ura3 selection marker driven by a GAL1 promoter. The pYES2-ZmDBF3 construct and pYES2 (the negative control) were transformed to the yeast strain *INVSc1* (His-, Leu-, Trp-, and Ura-) according to the pYES2 vector kit instructions (Invitrogen). The positive recombinant *INVSc1* strains harboring pYES2-DREB1B and pYES2 were cultured in SC-U liquid medium containing 2 % glucose at 30 °C for 12–16 h until the OD600 reached 0.4. Stress tolerance assays were performed according to the methods described previously (Gao et al. 2011).

Plasmid Construction and *Arabidopsis* Transformation

The full-length coding sequence of *ZmDBF3* was amplified by PCR using primers CGAGCTCATGAGCTTGCTT GTGACTG (*Sac* I) and TCCCCCGGGTCTTGCTC GTCAGTGCTCC (*Sma* I). The PCR products were cloned into the pCHF1301 vector driven by the CaMV 35S promoter (Li et al. 2014). The recombinant plasmid was introduced into the EHA105. *Arabidopsis* plants transformation was performed by the floral dip method (Clough and Bent 1998). Transgenic *Arabidopsis* was selected on MS medium containing 30 μg/l hygromycin and confirmed by PCR analysis. T3 generation plants were used for stress tolerance analysis in this study.

Analysis of Drought, Cold, and Salt Tolerance in Transgenic *Arabidopsis*

In all experiments, *Arabidopsis* seeds were surface-sterilized with 10 % NaOCl for 3–6 min and germinated on MS agar medium with 1 % (*w*/*v*) sucrose and 0.6 % (*w*/*v*) agar. The growth conditions of *ZmDBF3* transgenic plants (T3 generation) and the control (Col-0) were the same as described above. For salt and drought stress on plates, the *Arabidopsis* seeds were germinated on MS agar for 5 days and then transferred to fresh MS medium containing different concentrations of NaCl (100, 200, and 300 mM) and mannitol (200, 300, and 400 mM), respectively. The plates were positioned vertically on shelves to assist the evaluation of root growth rates. The survival rates were recorded for another 5 days after transferring. For the salt tolerance test at the adult stage, 3-week-old plants were irrigated with 350 mM NaCl solution. When the soil was completely saturated with salt solution, free NaCl solution was removed and the plants were cultured under normal conditions. Photos were taken on the fifth and tenth day after salt treatment. For drought tolerance analysis, 3-week-old plants were cultured without watering for 2 weeks. For freezing stress treatment, 3-week-old *ZmDBF3* transgenic and control plants were exposed −6 °C for 3 h and then cultured under normal conditions described as above. All abiotic stress tolerance experiments were conducted in triplicate.

qRT-PCR

Total RNAs from maize leaves were isolated using RNAiso Plus reagent (Takara) according to the manufacturer's instructions. Genomic DNA digestion in total RNA and reverse transcription were carried out using PrimeScript® RT reagent Kit With gDNA Eraser kit (Takara). qPCR was performed in a total volume of 20 μl, 1 μl of diluted complementary DNA (cDNA), 200 nM for each primer, 0.4 μl of ROX Reference Dye II (50×), and 10 μl of 2× SYBR Green PCR Master Mix (Takara) on an ABI 7500 Real Time System (PE Applied Biosystems, USA). The qPCR program included a preliminary step of 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 58 °C for 40 s. *ZmGAPDH* (GenBank accession number NM_001112119) was used as internal control for normalization of different cDNA samples, respectively. Relative gene expression was calculated according to a $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The primer sequences are as follows: *ZmGAPDH*-L CCGTGTTCCTACTGTGGATG,

ZmGAPDH-R TGTCACCAAGGAAGTCGGTA, *ZmDBF3*-L GGCTGCGATGGAGATGGAGA, and *ZmDBF3*-R GGGCATTGTCGGCGTTGTT.

Results

Isolation and Structure Analysis of ZmDBF3

Using a gene-specific primer pair designed based on *ZmDREB1B* (accession number NM_001157828) nucleotide sequence, a 783-bp fragment containing full length of the gene was successfully obtained by PCR method from B73 maize DNA samples since there is no intron predicted in this gene. Sequence analysis revealed that *ZmDREB1B* consisted of 783-bp open reading frame (ORF) encoding 260 amino acids. The deduced amino acids sequence of ZmDREB1B had a calculated molecular weight of 28 kDa with a *p*I of 5.11 (http://web.expasy.org/compute_pi/). To analyze the phylogenetic relationship of ZmDREB1B with the group DREB/CBF proteins from other plants, a phylogenetic tree was constructed based on the amino acid sequences of these proteins. As shown in Fig. 1a, ZmDBF2, StDREB1B, AtTINY, and AtTINY2 were in the same group, which belongs to subgroup A-4. Moreover, ZmDREB1B showed high homolog to ZmDBF2; therefore, this gene was named as *ZmDBF3* instead. Comparison of amino acids sequences of these proteins indicated that the ZmDBF3 protein contains a conserved DNA-binding domain (AP2/ERF domain) which is composed of 64 amino acid residues (73–136), with conserved valine (V) and glutamic acid (E) residues at the 14th and 19th positions (Fig. 1b).

Nuclear Localization and Transcriptional Activity of ZmDBF3 Protein

To investigate the subcellular localization of ZmDBF3 in plant cells, we conducted transient expression assays with *35S::eGFP* and a chimeric *35S::ZmDBF3-eGFP* construct (Fig. 2a) in onion epidermal cells. As shown in Fig. 2b, GFP fluorescence distributed evenly in the nucleus and the cytoplasm with the control plasmid 35S-*eGFP*, while the CaMV *35S*::*ZmDBF3-eGFP* fusion protein was targeted to the nucleus. These results clearly indicate that ZmDBF3 is a nuclear localized protein.

To test whether ZmDBF3 has transcriptional activity, the entire coding region was fused to the GAL4 DNA-binding domain in the vector pGBKT7, and the construct was transformed into yeast. Colony-lift filter assays showed that the reporter gene *Lac*Z was expressed in the yeast cells transformed with the pGAL4 or pGBKT7-ZmDBF3 constructs (Fig. 2c), indicating that the ZmDBF3 has transcriptional activity in yeast.

Expression Pattern of *ZmDBF3* Under Abiotic Stresses and Signaling Molecules

The transcript profiling of *ZmDBF3* in response to abiotic stresses and signaling molecules was investigated by qRT-PCR analysis. As shown in Fig. 3a, the expression of *ZmDBF3* was enhanced about 22-fold at 1 h and then declined following PEG treatment. Salt stress led to a significant increase of *ZmDBF3* transcript level within 1 h and reached a maximum at 6 h and then decreased at 24 h (Fig. 3b). After ABA treatment, the transcript level of *ZmDBF3* was strongly induced at 4 h time-point and continuously accumulated up to 24 h of treatment (Fig. 3c). Similar to cold treatment, *ZmDBF3* mRNA accumulated quickly in response to high temperature and reached maximum at 5 h of stress treatment (Fig. 3d, e). However, no significant changes of *ZmDBF3* were detected at each tested time-point as compared with non-treated control, when subjected to MeJA or SA (Fig. 3f, g). Above results indicated that *ZmDBF3* may play important roles in abiotic stress to help plants adapting to adverse environmental conditions.

Overexpression of *ZmDBF3* in *S. cerevisiae INVSc1* Cells Improved Tolerance to Multiple Abiotic Stresses

ZmDBF3 was introduced into yeast cells to analyze the possible function of this protein under different abiotic stresses. The growth performance of yeast cells transformed with pYES2 (control) or pYES2-ZmDBF3 was tested under various abiotic stress conditions. As shown in Fig. 4a, the growth of *ZmDBF3* transgenic yeast cells was inhibited relative to control under stress-free conditions, when diluted to 10^{-5}, indicating that *ZmDBF3* expression retards the growth of yeast cells. However, *ZmDBF3*-overexpressing yeast cells exhibited increased survival rate compared to the control, when subjected to NaCl, KCl, 6 % Na_2CO_3, and $NaHCO_3$ treatment (Fig. 4b–d, f, g). No significant difference in growth levels between the *ZmDBF3* transgenic and control yeast cells under 8 % Na_2CO_3, 40 % PEG6000, sorbitol, and freezing (Fig. 4e, h–j). These results indicated that *ZmDBF3* might be involved in the yeast multiple abiotic stress tolerance.

Ectopic Expression of *ZmDBF3* in *Arabidopsis* Resulted in Enhanced Salt and Drought Tolerance

To investigate the function of *ZmDBF3* in plants, transgenic *Arabidopsis* plants expressing the *ZmDBF3* under the control of the CaMV 35S promoter were obtained. Five-day-old Col-0 and transgenic seedlings were transferred to the MS plates containing different concentrations of NaCl and mannitol after germination on MS medium. As shown in Fig. 5a, b, no significant difference between the transgenic plants and control was observed on MS medium containing 100 mM NaCl or

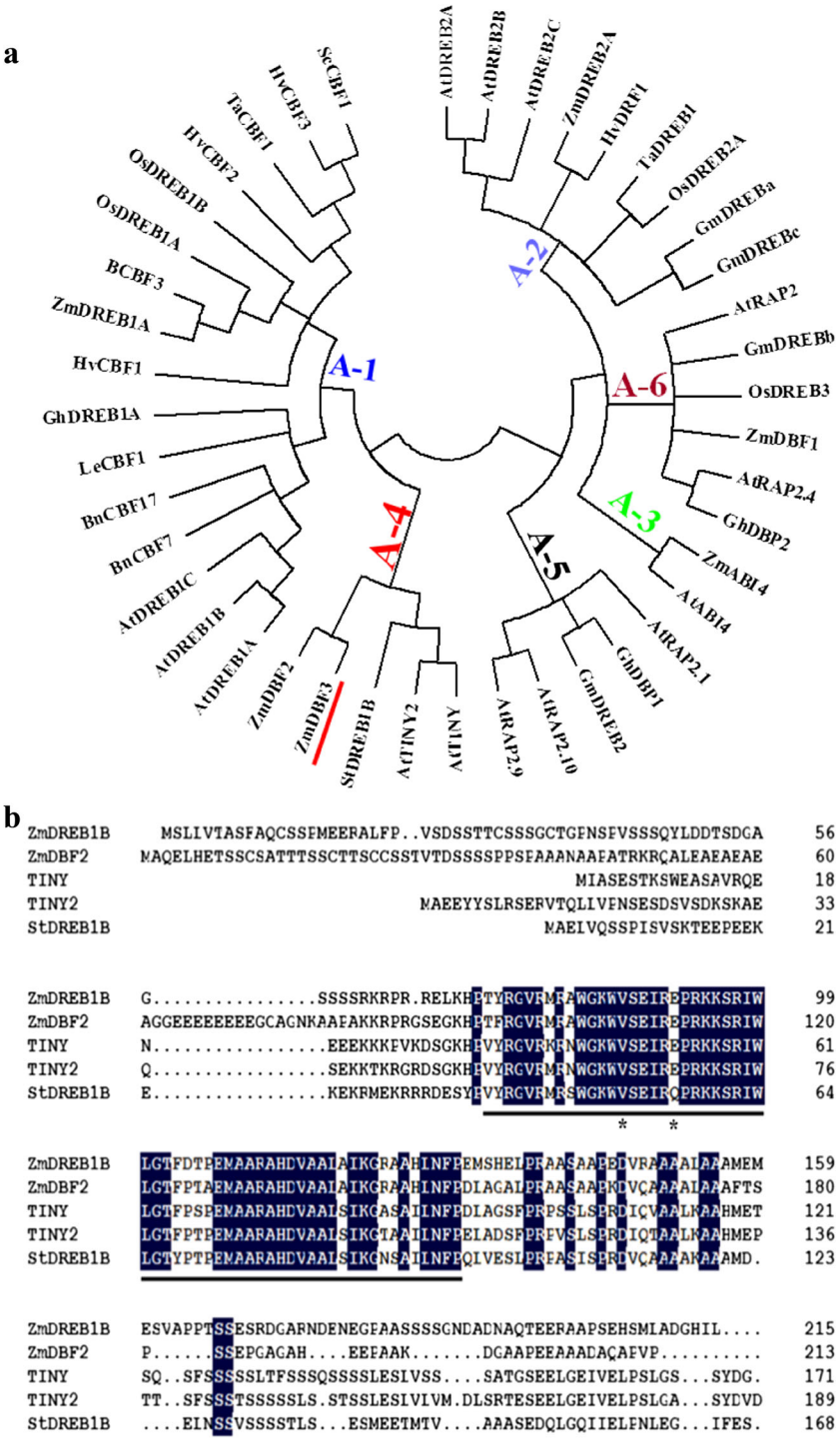


Fig. 1 Sequence analysis of *ZmDREB1B*. **a** Phylogenetic analysis of ZmDBF3 with AP2/ERF transcription factors from other plant species. The phylogenetic analysis was conducted by MEGA 4.0 software. The appended proteins are as follows: ZmDREB1A (AF450481), HvCBF3 (AF298230), ZmDREB2A (NP_001105876), HvDRF1 (AY223807), HvCBF1 (AF418204), HvCBF2 (AF442489), TaDREB1 (DQ195068), TaCBF1 (AF376136), OsDREB1A (AF300970), ZmDBF1 (AAM80486), ZmDBF2 (AF493799), ZmABI4 (AY125490), GhDBP1 (AY174160), AtDREB1A (AB007787), AtDREB1B (AB007788), AtDREB1C (AB007789), AtDREB2A (AB007790), GmDREBa (AAT12423), GmDREBb (AAQ57226), GmDREBc (AAP83131), OsDREB2A (AF300971), PeDREB2 (ABY19375), AtABI4 (A0MES8), GmDREB2 (ABB36645), AtRAP2.10 (Q9SW63), AtRAP2.1 (Q8LC30), AtRAP2 (AAP04063.1), AtRAP2.4 (NP_177931), GhDBP2 (AAT39542), OsDREB3 (NP_001048142), AtTINY (Q39127), AtTINY2 (AY940160.1), LeCBF1 (AY034473), BnCBF7 (AF499032), BnCBF17 (AF499034), GhDREB1A (AY321150), OsDREB1B (AF300972), HvBCBF3 (AF298231), AtDREB2B (NM_111939.2), AtRAP2.9 (NM_179009.1), ScCBF1 (AF370730), GhDBP3 (DQ224382), StDREB1B (JN125862.1), and AtDREB2C (At2g40340). **b** Multiple alignments of the amino acid sequences of DREB proteins. The AP2/ERF domain is underlined, and two conserved residues of V14 and E19 are indicated over *asterisks*. Identical sequences are *shaded in black*

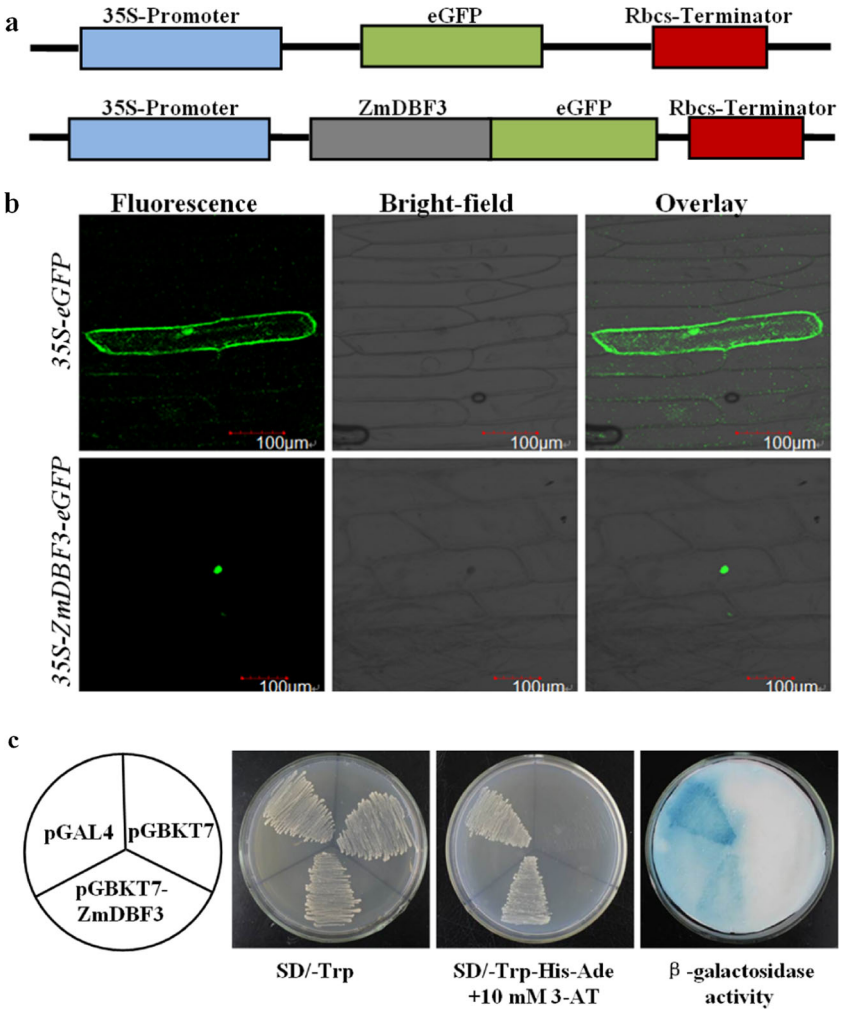


Fig. 2 Subcellular localization and transcriptional activity analysis of ZmDBF3. **a** Schematic structures of *35S::eGFP* and *35S::ZmDBF3-eGFP* vectors. **b** Subcellular localization of ZmDBF3 protein in onion epidermal cells. The transformed cells with *35S::eGFP* and *35S::ZmDBF3-eGFP* were observed under a confocal microscope after inoculation for 36 h. The photographs were taken in dark field for green fluorescence, bright light to illustrate the morphology of the cells and in overlay. The *bar* represents 100 μm. **c** Transcriptional activity analysis of ZmDBF3. The full-length ORF of *ZmDBF3* was fused to the GAL4 DNA-binding domain in the vector pGBKT7, and the construct was transformed into yeast strain AH109 (Invitrogen). pGBKT7 and pGAL4 plasmids were used as negative and positive control, respectively

200 mM mannitol. However, when supplemented with 200 mM NaCl, over 70 % Col-0 plants showed etiolation or death, whereas the transgenic plants exhibited significantly higher survival rate and longer primary roots relative to control (Fig. 5a, c, d). Similarly, *ZmDBF3*-overexpressing lines exhibited longer roots than Col-0 on MS plates containing 300 mM mannitol (Fig. 5b, d), indicating overexpression of *ZmDBF3* has decreased NaCl and mannitol sensitivity. On MS containing 300 mM NaCl or 400 mM mannitol, both transgenic lines and control displayed sign of death or severely inhibited root length (Fig. 5a, b), suggesting that the concentration of NaCl is beyond the reach of tolerance.

To further investigate the role of *ZmDBF3* in improvement of plant salt and drought tolerance at adult stage, 3-week-old soil-grown plants were irrigated with 350 mM NaCl or without irrigation for 2 weeks, respectively. The survival percentage of transgenic plants was 47–73 % under salt stress, which was significantly higher than that of Col-0 (7 %) (Fig. 5e, g). After drought treatment for 2 weeks, Col-0 plants became severely wilted, whereas *ZmDBF3* overexpression lines appeared relatively healthy (Fig. 5f, h). After re-watering for 3 days, only 20 % Col-0 plants survived, whereas the survival rates of overexpressing lines ranged from 25 to 80 %. These results indicated that the transgenic plants are more tolerant to salt and drought than the Col-0 plants.

Overexpression of *ZmDBF3* in *Arabidopsis* Increases Freezing Tolerance

To evaluate the contribution of *ZmDBF3* to plant tolerance to low temperature, 3-week-old *ZmDBF3*-overexpressing lines and Col-0 seedlings were exposed to −6 °C for 6 h. After 1 day of recovery at normal conditions, over 80 % of the Col-0 seedlings were dead; conversely, several of those from the transgenic lines (OE3, OE8, and OE15) were green and regrew normally after recovery (Fig. 6a). The survival percentages of the three transgenic lines ranged from 43 to 46 % (Fig. 6b), significantly higher than the control (19 %) under the freezing condition, indicating that overexpression of *ZmDBF3* in *Arabidopsis* improves tolerance to freezing stress.

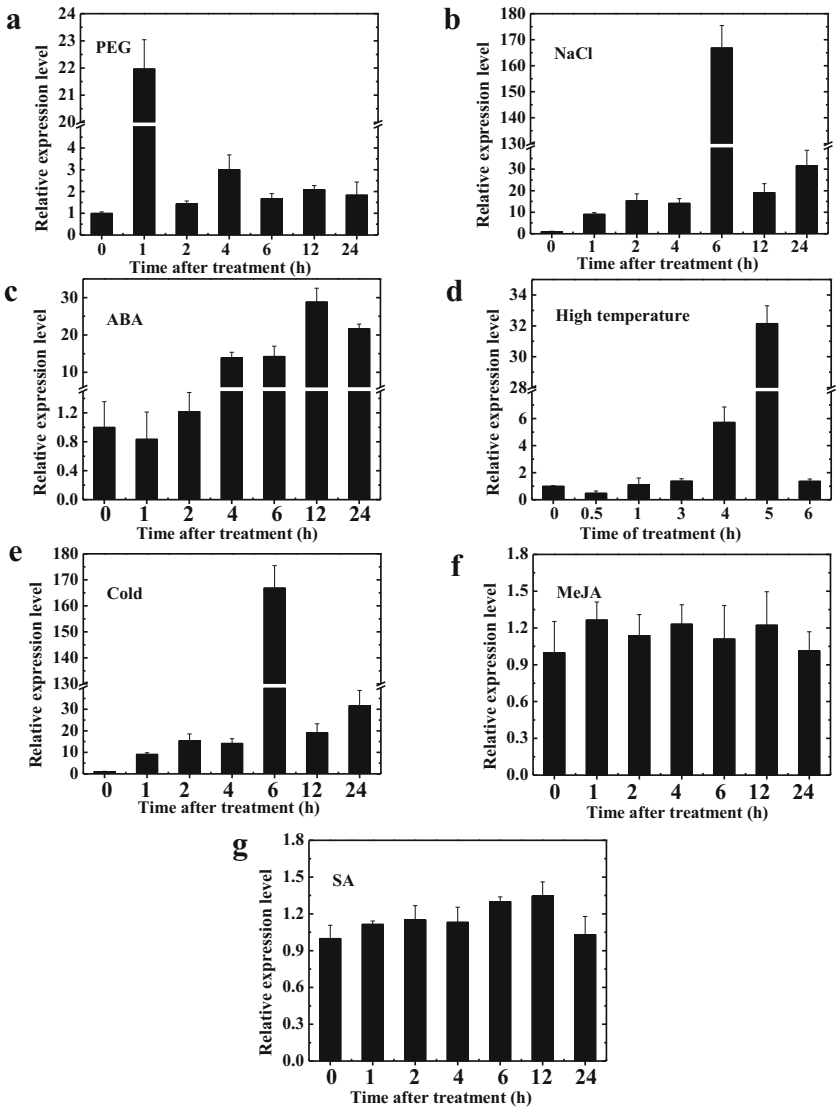


Fig. 3 Transcript accumulation of *ZmDBF3* in maize leaves in response to different abiotic stresses and signaling molecules as determined by qRT-PCR. Maize seedlings were treated with 20 % PEG 6000 (**a**), 250 mM NaCl (**b**), 100 μM ABA (**c**), 40 °C (**d**), 4 °C(**e**), 100 μM MeJA (**f**), and 2 mM SA (**g**). Total RNA was isolated from leaves at indicated times after different treatments. *Bars* indicate mean relative expression values and three replicates

Discussion

Plant growth and yield are severely decreased by abiotic stresses. Identification of the transcription factors that regulate responses to abiotic stress is considered as a key target for improving stress tolerance in crop plant species. DREBs play a vitally important role in abiotic stress tolerance through binding to the DRE/CRT element in the promoter regions of a large number of stress-responsive genes in plants (Yamaguchi-Shinozaki and Shinozaki 2006). In the present study, we isolated *ZmDBF3*, a novel member of DREB transcription factor family gene from maize, and studied the function of this gene by overexpression in yeast cells and *Arabidopsis*.

Identification and classification of genes are significant for the functional analysis of a gene family. According to the conserved AP2/ERF domain, the subfamily of DREB transcription factors can be further divided into six subgroups from A-1 to A-6 (Sakuma et al. 2002). Although much information has elucidated the regulatory function of the A-1 (DREB1/CBF) and A-2 (DREB2) subfamily in *Arabidopsis*, there is still less learn about the species roles of DREBs belonging to A-3 to A-6. Phylogenetic analysis suggested that *ZmDBF3* is classified into A-4 group (Fig. 1a). Each DREB protein contains a highly conserved AP2/ERF domain with 14th valine (V14) and the 19th glutamic acid (E19), whereas alanine and aspartic acid are conserved in the corresponding positions of the other ERF proteins (Sakuma et al. 2002). The conserved valine and glutamic acid residues in the AP2/ERF domain play an important role in binding to the target DNA sequences (Liu et al. 1998; Sakuma et al. 2002; Sakuma et al. 2006). It was also reported that 14th residue is more conserved and important than the 19th residue in the DRE-binding activity of DREB (Cao et al. 2001; Sun et al. 2014). The analysis of ZmDBF3 protein revealed that it contains a conserved ERF/AP2 domain of 64 amino acids with conserved V14

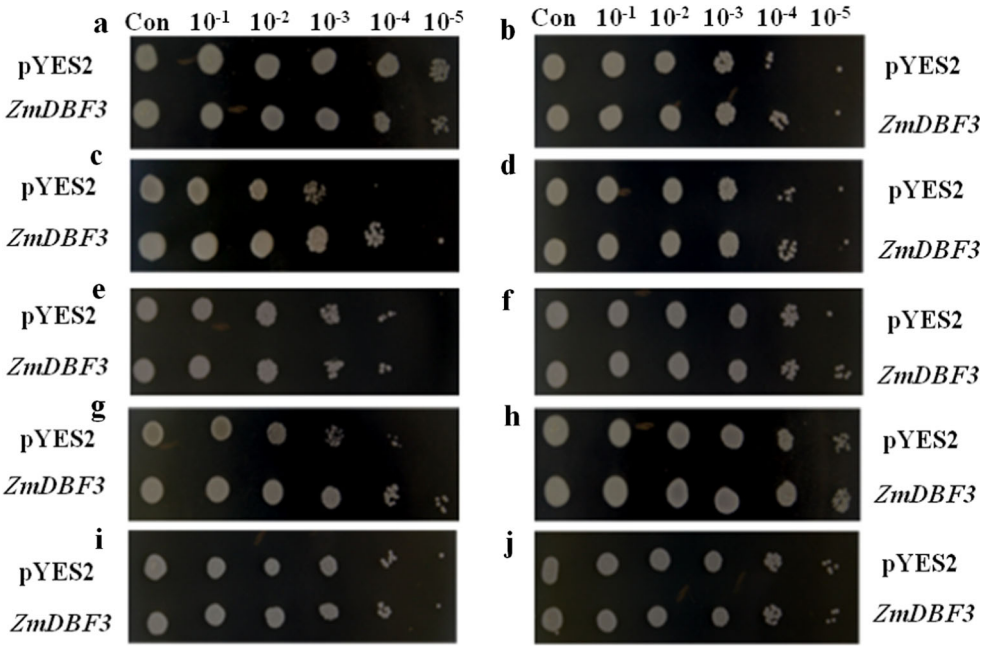


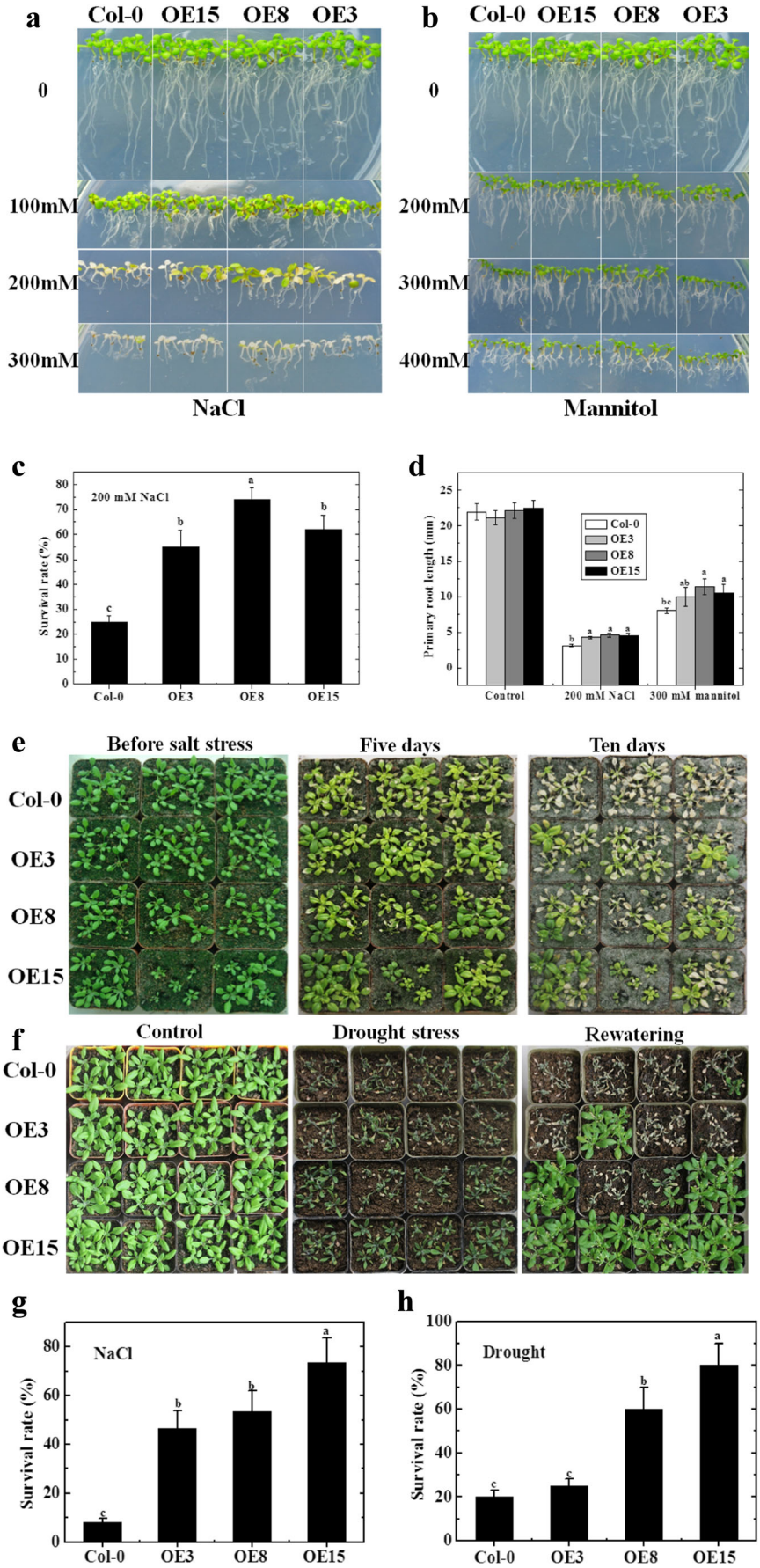
Fig. 4 Stress tolerance analysis of *ZmDBF3* in yeast cells. The transformed yeast *INVSc1* harboring *ZmDBF3* (pYES2-*ZmDBF3*) and *INVSc1* harboring empty pYES2 were diluted into different ratio and treated with various stress conditions, and their survival rates were compared. **a** Non-stress, **b** 5 M NaCl, **c** 5 M KCl, **d** 6 % Na_2CO_3, **e** 8 % Na_2CO_3, **f** 6 % $NaHCO_3$, **g** 8 % $NaHCO_3$, **h** 40 % PEG6000, **i** 5 M sorbitol, and **j** −20 °C

and E19 residues (Fig. 1b), which was consistent with the characteristics of DREB1 subclass. In addition, the results of transient expression assays and transcriptional activity analysis demonstrated that ZmDBF3 is a nuclear localized protein with transcriptional activity (Fig. 2a–c). Above data indicated that ZmDBF3 is one new member of the A-4 subfamliy of DREB1 transcription factors.

Previous studies showed that *DREB1s* and *DREB2s* are the largest groups that involved in ABA-independent pathways (Liu et al. 1998). Generally, *DREB1* genes are induced by cold, whereas *DREB2* genes are induced by dehydration, high salinity, and heat stresses in *Arabidopsis* (Lata and Prasad 2011). However, many *DREB1* genes isolated from various plant species exhibited different expression pattern following abiotic stresses induction. *CaDREBLP1* from hot pepper was rapidly induced by dehydration and salt, but not by cold stress (Hong and Kim 2005). *PpDBF1* from *Physcomitrella patens* and *HvDREB1* from *Hordeum vulgare* were significantly induced by salt, cold, and drought, respectively (Liu et al. 2007; Xu et al. 2009). In this study, *ZmDBF3* was induced by drought, salt, cold, and high temperature, indicating that *ZmDBF3* is an abiotic stress-responsive gene and may contribute to plant stress tolerance. ABA is an important hormone signal to improve plants to overcome various environment stresses (Fujita et al. 2011). The ABA-inducible expression pattern of *ZmDBF3* suggests that *ZmDBF3* may be involved in the plant response to ABA (Fig. 3c). Consistent with our results, previous studies indicated that ABA plays a role in the regulation of ZmDBF activity and the existence of an ABA-dependent pathway for the regulation of genes through the C-repeat/DRE element (Kizis D and Pagès 2002). As important signaling molecules, JA and SA play important roles in wounding and defense responses against pathogens, respectively. In our study, it was also showed that *ZmDBF3* was not affected by MeJA or SA treatment (Fig. 3f, g), suggesting that it may not be involved in wounding or biotic stress response, although previous study has showed that expression of some *DREB1* genes is correlated with wounding (Dubouzet et al. 2003; Hong and Kim 2005).

Gene expression in yeast cells is a good model system for screening the candidate gene or studying the mechanisms underlying stress tolerance. For example, expression of *ThVHAc1* in yeast increased salt, drought, ultraviolet (UV), oxidative, heavy metal, cold, and high temperature tolerance (Gao et al. 2011). The transgenic yeast expressing *GhBCP1* and *GhBCP4* significantly increased cell growth rate under Cu^{2+}, Zn^{2+}, and high salinity stresses (Ruan et al. 2011). Using this expression system, we also have successfully identified and characterized a heavy metal-associated protein gene,

Fig. 5 Salt and drought tolerance assay of transgenic *Arabidopsis* plants ▶ overexpressing *ZmDBF3*. **a**, **b** Phenotype of transgenic lines (OE15, OE8, and OE3) and control (Col-0) on MS medium containing different concentrations of NaCl (100, 200, or 300 mM) and mannitol (200, 300, or 400 mM), respectively. Survival rates of *ZmDBF3* transgenic lines (OE3, OE8, and OE15) and Col-0 on MS medium containing 200 mM NaCl (**c**). **d** Primary root length of *ZmDBF3*-overexpressing lines (OE3, OE8, and OE15) and Col-0 on MS medium containing 200 mM NaCl or 300 mM mannitol. Data are shown as mean±SD of three replicates. The statistical significance was determined by Duncan's multiple comparison tests. *Different letters above bars* indicate significant differences ($P<0.05$) among different genotypes in the same treatment. **e**, **f** Performance of Col-0 and *ZmDBF3*-overexpressing seedlings under salt and drought stresses. Survival rates of *ZmDBF3* transgenic lines and Col-0 under 350 mM NaCl and drought stresses. Data are shown as mean±SD of three replicates


a
Col-0 OE15 OE8 OE3
0
100mM
200mM
300mM
NaCl
b
Col-0 OE15 OE8 OE3
0
200mM
300mM
400mM
Mannitol
c
200 mM NaCl
Survival rate (%)
Col-0 OE3 OE8 OE15
d
Primary root length (mm)
Col-0
OE3
OE8
OE15
Control
200 mM NaCl
300 mM mannitol
e
Before salt stress
Five days
Ten days
Col-0
OE3
OE8
OE15
f
Control
Drought stress
Rewatering
Col-0
OE3
OE8
OE15
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Survival rate (%)
Col-0 OE3 OE8 OE15
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Drought
Survival rate (%)
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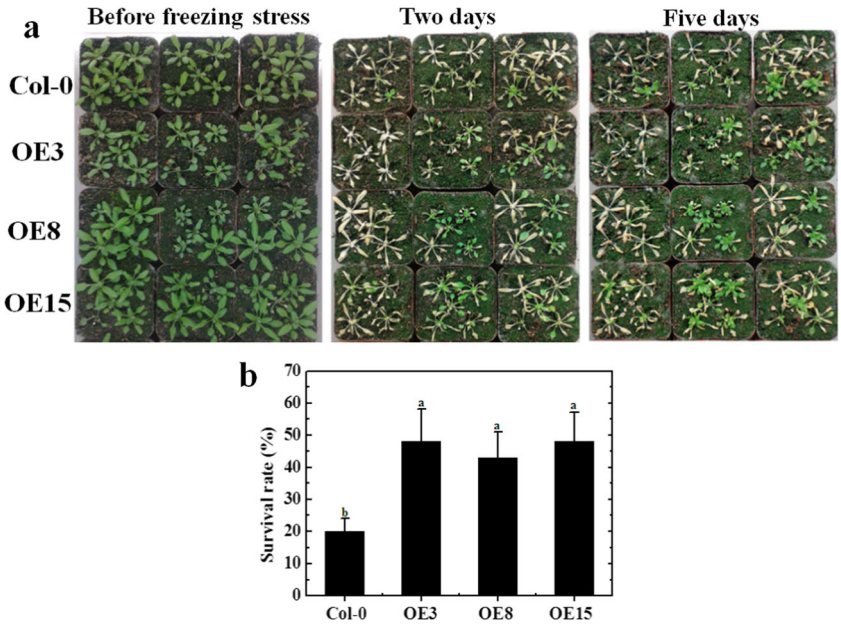

Fig. 6 Performance of Col-0 and *ZmDBF3*-overexpressing seedlings under freezing stress. **a** Phenotypic comparison between Col-0 and *ZmDBF3* transgenic *Arabidopsis* plants (OE3, OE8, and OE15) after freezing treatment. **b** Survival rates of *ZmDBF3* transgenic lines and Col-0 after freezing treatment

AcHMA1, from *Atriplex canescens*, in metal stress tolerance (Sun et al. 2014). In this study, *ZmDBF3*-overexpression yeast cells exhibited increased growth rate compared with the control under NaCl, KCl, Na_2CO_3, $NaHCO_3$, PEG6000, and freezing stresses (Fig. 4a–j), suggesting that this gene is sufficient to enhance abiotic stress tolerance in yeast.

To further investigate the stress tolerance of *ZmDBF3*, we obtained *ZmDBF3* transgenic overexpressing lines of *Arabidopsis*. In most cases, *DREB* transgenic plants under constitutive promoter showed dwarfism or abnormal phenotype, which limits the practical use of *DREB* genes (Agarwal et al. 2006). The retard growth phenotype is thought to be related to interference in gibberellic acid (GA) metabolism (Achard et al. 2008). In contrast to the phenotype in yeast cells (Fig. 4a), *ZmDBF3*-overexpressing *Arabidopsis* plants exhibited normal growth rate compared with Col-0 under non-stressed conditions (Fig. 5a, b). The results also indicated the existence of difference between two expression systems. Similar results were obtained from *AtCBF3* overexpression in diverse plant species (Gilmour et al. 2000; Oh et al. 2005). Overexpression of *AtCBF3* in *Arabidopsis* plants caused severe growth retardation (Gilmour et al. 2000); however, *AtCBF3* transgenic plants exhibited growth retardation when expressed in rice (Oh et al. 2005). Overexpression of the *TINY* or *HARDY* gene, two genes belong to A-4 DREB subgroup, resulted in stunted growth and thick leaves (Wilson et al. 1996; Karaba et al. 2007), suggesting that distinctive DREB genes may have various functions, although sharing high sequence similarity. Therefore, selecting proper DREB transcription factors or using stress-inducible promoters may be good choices to minimize the negative effects of DREB on plant growth and development.

Previous studies have showed the role of DREB transcription factors in tolerance to abiotic stress. Transgenic tobacco plants overexpressing *PpDBF1* showed higher tolerance to salt, drought, and cold stresses (Liu et al. 2007). Similarly, overexpression of *DREB1A* under the control of the CaMV 35S promoter also increased the tolerance to drought, high-salt, and freezing stresses (Liu et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000). It was also reported that *Arabidopsis* plants overexpressing *AtDREB2A* did not improve stress tolerance, suggesting that a posttranslational modification is necessary for the function of *AtDREB2A* (Liu et al. 1998; Sakuma et al. 2006). In this study, *ZmDBF3* transgenic *Arabidopsis* plants showed reduced salt and drought sensitivity, and enhanced tolerance to drought and salt (Fig. 5a–h). Moreover, overexpression of *ZmDBF3* in *Arabidopsis* also exhibited enhanced tolerance to freezing stress (Fig. 6a, b). In addition, we also noted that the three transgenic lines showed different stress tolerance under stress conditions. The overexpressing line OE15 showed stronger tolerance to drought and high-salt than the others (Fig. 5e–h). The difference may be due to the various expression levels of transgenic lines or the different insertion site in plant genome.

In conclusion, we identified a novel maize DREB A-4 group gene, *ZmDBF3*. The results clearly showed that *ZmDBF3* was upregulated at the transcription level by cold, high salinity, cold, and ABA but was not affected by exogenous JA and SA. Furthermore, overexpression of *ZmDBF3* in yeast cells and *Arabidopsis* enhanced tolerance to salt, drought, and freezing stresses, suggesting that ZmDBF3 may be a positive regulator of multiple abiotic stress tolerance. Therefore, in the future, *ZmDBF3* might be a candidate gene for genetic engineering in plants to improve stress tolerance.

Acknowledgments This study was supported by grants of the 12th Five-Year Plan Project of Science and Technology Support, China (2012BAD19B04, 2014BAD14B02), by the Research and Development of Industrial Technology Special at Jilin Provincial Development and Reform Commission (2013C001), and by the Ministry of Agriculture Key Project of GM Cultivation of New Varieties (2013ZX08004004), P.R. China.

The Ethical Statement We all have read the Instructions for Authors carefully and fully comply with the Ethical Standards.

1. The manuscript has not been submitted to more than one journal for simultaneous consideration.
2. The manuscript has not been published previously (partly or in full).
3. A single study is not split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time.
4. No data have been fabricated or manipulated (including images) to support our conclusions.
5. No data, text, or theories by others are presented as if they were the author's own ("plagiarism"). Proper acknowledgements to other works have been given (this includes material that is closely copied (near verbatim), summarized, and/or paraphrased), quotation marks are used for verbatim copying of material, and permissions are secured for material that is copyrighted.
6. Consent to submit has been received explicitly from all coauthors, as well as from the responsible authorities—tacitly or explicitly—at the institute/organization where the work has been carried out, before the work is submitted.
7. Authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

   Conceived and designed the experiments: Hongyu Pan, Wei Zhou, Chengguo Jia; Performed the experiments: Wei Zhou, Chengguo Jia; Analyzed the data: Wei Zhou, Gang Yu, Jinliang Liu, Xianghui Zhang; Wrote the paper: Chengguo Jia, Wei Zhou, Hongyu Pan; Contributed reagents/materials/analysis tools: Others.
8. We will not change authorship or the order of authors after acceptance of this manuscript.
9. We will provide relevant documentation or data in order to verify the validity of the results if needed.

Compliance with Ethical Standards

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

Research without involving human participants and/or animals.

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