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Isolation and Functional Characterization of *ZmDBP2* Encoding a Dehydration-Responsive Element-Binding Protein in *Zea mays*

Chang-Tao Wang · Quan Yang · Cheng-Tao Wang

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Abstract Dehydration-responsive element-binding proteins (DREBs) play vital regulatory roles in abiotic stress responses in plants. A member of the DREB gene family in maize, isolated and designated as *Zea mays* dehydration-responsive element-binding protein *2* (*ZmDBP2*), encoded a putative protein of 343 amino acids with a conserved DNA-binding domain. Transcription of the *ZmDBP2* gene was highly induced by drought and moderately induced by salt and exogenous abscisic acid. Isolation of the *ZmDBP2* promoter revealed some *cis*-acting elements responding to stresses. The ZmDBP2 protein activated C-repeat/dehydration responsive element-containing genes under normal growth conditions in transgenic *Arabidopsis* plants and accumulated throughout the cell. These results indicated that ZmDBP2 possibly interacts with another transcription factor(s) before acting via the nucleus. Overexpression of *ZmDBP2* improved drought stress tolerance in transgenic *Arabidopsis* plants. Our data showed that *ZmDBP2* could be a new member of DREB transcription factor family involved in activation of downstream genes in response to environmental stresses.

Chang-Tao Wang and Quan Yang contributed equally to this work.

The nucleotide sequences reported in this paper was submitted to the GenBank database under accession number FJ805750 (*ZmDBP2*).

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Abbreviations

ABA	Abscisic acid
DREB	Dehydration-responsive element-binding protein
CRT/DRE	C-repeat/dehydration responsive element
NLS	Nuclear localization signal
Q-RT-PCR	Real-time quantitative RT-PCR
SSH	Suppression subtractive hybridization

Introduction

Environmental stresses have adverse effects on plant growth. To survive, plants have developed defense mechanisms to perceive signals from their surroundings and appropriately respond to the different stresses by modulating the expression of response genes (Lee et al. 1999; Ramanjulu and Bartels 2002). Transcription factors function pivotally in activating defense gene expression (Shinozaki and Dennis 2003; Chen and Zhu 2004; Xu et al. 2008a). More than 1,500 genes encode transcription factors in the *Arabidopsis* genome (Riechmann et al. 2000). Among them, dehydration-responsive element-binding proteins (DREBs; also referred to as CBF) have received considerable attention in the past decade. A variety of DREB genes were identified and investigated in various species, including rice (*Oryza sativa*), maize (*Zea mays* L.), soybean (*Glycine max*), and wheat (*Triticum aestivum*; reviewed in Agarwal et al. 2006; Xu et al. 2008b).

DREBs play an important role in stress signal regulatory pathways by specifically binding to C-repeats/dehydration responsive elements (DREs/CRTs; A/GCCGAC) to control the expression of RD/COR genes, such as *rd29A*, *kin1*, *rab17*, *cor6.6*, and *cor15A*. DREB genes were found to be involved in response to abiotic stresses (Stockinger et al. 1997; Liu et al. 1998; Gilmour et al. 1998; Haarke et al. 2002). For example, *CBF1* was induced by low temperature and its overexpression activated downstream cold-responsive genes and improved freezing tolerance in plants (Jaglo-Ottosen et al. 1998). *Arabidopsis DREB1A/CBF3* and rice *OsDREB1A* conferred drought and freezing tolerance in transgenic *Arabidopsis* plants (Dubouzet et al. 2003). Recently, it was reported that overexpression of maize *ZmDREB2A* and wheat *TaAIDFa* resulted in increased thermotolerance and/or drought tolerance in plants (Qin et al. 2007; Xu et al. 2008b). Isolation and functional characterization of more DREB genes could facilitate understanding the mechanisms by which plants perceive and transduce adverse environmental stimuli to trigger adaptive responses.

Only few DREB transcription factors have been described in maize (*Z. mays*), an important food and feed crop in the world (Jiang et al. 2004; Qin et al. 2004, 2007; Wang and Dong 2009). In this paper, we report the cloning and characterization of the *ZmDBP2* gene. The expression pattern, subcellular localization, and putative *cis*-acting elements in the promoter of *ZmDBP2* were investigated. Importantly, overexpression of the *ZmDBP2* gene improved drought tolerance in transgenic *Arabidopsis* plants.

Materials and Methods

Plant Materials and Stress Treatments

Seedlings of maize (X178) grown at 25°C for 10 days were subjected to various abiotic stresses. All treatments were applied for 5 h or for times otherwise indicated. Seedlings were exposed to air on filter paper for induction of rapid drought conditions, or placed in a 4°C chamber for cold stress. To mimic salinity and for abscisic acid (ABA) treatments, seedling roots were soaked into solutions containing 2% salt and 200 μM ABA, respectively.

Suppression Subtractive Hybridization

A suppression subtractive hybridization (SSH) cDNA library was constructed using the PCR-Select^{TM} cDNA Subtraction kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Total RNA was extracted from drought- and salt-treated leaves harvested at 5 h. Poly $(A)^+$ RNA was extracted from total RNA using the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). Equal amounts of two mRNA samples were pooled and used to make tester cDNAs. Similarly, driver cDNAs were derived from nontreated maize leaves harvested concomitantly. After two rounds of subtractive hybridization and two rounds of PCR amplification, the second round PCR amplification product was ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA), and ligation mixtures were transformed into *Escherichia coli* JM109. Cloned products were sequenced and results were compared with GenBank database sequences using the BLAST homology search program.

Isolation of Full-Length cDNA and Genomic Sequences

Several homologous cDNA fragments were obtained using the target sequence as a query to search the maize EST database. Based on the putative sequences, the full-length cDNA sequence or genomic sequences of *ZmDBP2* were amplified using the primer set (5′-GCTCCATCCAAGCTGTAGTCCT-3′) and (5′-GCTCATGACAGGATGGAATCC-3′).

First-Strand cDNA Synthesis and Quantitative RT-PCR

Total RNA was extracted from leaves using Trizol Reagents (TianGen, Beijing, China). The real-time quantitative RT-PCR (Q-RT-PCR) were conducted using the ABI Prism 7000 system (Applied Biosystems, USA). The actin gene was used as an internal reference. The transcript analysis of the *ZmDBP2* gene was performed using gene-specific primers (5′-GCCCGATGGCATTTTA GACG-3′ and 5′-AACCAGGAGATTAGCACGCA-3′), which were located in the 3′-untranslated region of the gene. Validation experiments were performed to demonstrate that amplification efficiency of the *ZmDBP2* specific primers were approximately equal to the amplification efficiency of the endogenous reference primers. Quantification of the target gene expression was carried out by the comparative CT method (Livak and Schmittgen 2001). Average CT values for the target gene from at least three PCRs were normalized to average CT values for actin from the same cDNA preparations and analyzed using Microsoft Excel.

Subcellular Localization Analysis

The full-length cDNA fragment containing the coding region of *ZmDBP2* was amplified and fused to the N terminus of the *hGFP* gene under control of the 2× CaMV35S promoter. Subcellular localization of the *ZmDBP2::hGFP* fusion protein and *hGFP* control in onion epidermal cells was performed as described by Xu et al. (2007).

Arabidopsis Transformation

To construct an expression vector for *Arabidopsis*, the full-length *ZmDBP2* cDNA was ligated into the modified vector pBI121 under the control of the CaMV35S promoter. Columbia (Col-0) ecotype *Arabidopsis* plants were transformed using the vacuum infiltration method. Transformants were selected on MS medium containing 50 μg mL^{-1} kanamycin. T2 generation plants were used for further analysis.

Drought and Salt Stress Treatment of Transgenic Plants

Sixty *Arabidopsis* plants of two independent lines grown for 3 weeks at 22°C were subjected to stress treatments. For drought stress treatment, plants were grown at 22°C without watering for 12 days and then rewatered under normal growing conditions. For salt stress, plants were irrigated with 200 mM NaCl under normal growing conditions. Survival rates were calculated 10 days later.

Results

Isolation and In Silico Analysis of *ZmDBP2* cDNA

We constructed a maize SSH cDNA library to isolate new genes that mediate drought stress responses. One cDNA fragment of 349 bp (MF245) showed sequence homology to DREB proteins in the BLAST search (BLASTX). BLASTN was performed on the maize EST database using the MF245 sequence. Using sequence assembly and RT-PCR, we obtained the full-length cDNA sequence and designated it as *ZmDBP2* (GenBank accession no. FJ805750). The genomic sequence was isolated using PCR primers corresponding to the 5′- and 3′-ends of *ZmDBP2* cDNA. Sequence alignment of *ZmDBP2* with its genomic sequence indicated that *ZmDBP2* has no intron.

ZmDBP2 comprised a 1,032-bp ORF encoding a 36.7-kD protein with p*I* 6.6 (Fig. 1). The deduced amino acid sequences of *ZmDBP2* contain a highly conserved AP2/ERF domain. Two conserved amino acids, 14th Val and 19th Leu, within the AP2/ERF domain are characteristic of DREB proteins (Sakuma et al. 2002; Figs. 1 and 2a). ZmDBP2 contains two acidic amino acid regions that might act as transcriptional activation domains and two potential *N*-linked glycosylation sites (NXS/T, where X denotes any amino acid), but has no clearly basic amino acid region that potentially acts as a nuclear localization signal (NLS; Fig. 1). Phylogenic analysis confirmed that ZmDBP2 belongs to the A-6 subgroup of the DREB subfamily (Fig. 2b). Among members, ZmDBP2 shares 53% and 47% identity with maize ZmDBF1 and soybean GmDREBb proteins, respectively (Fig. 2a).

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1     ttttttttttctttcttggcacaaacccacctcgtttctctacttttattgaggaacagagctggtttttcacaaaaaaaaattctactt
91    tgattttccataaagaaaacctctcagcctctttcttctagctccatccaagctgtagtcctaATGGCCGCAGCCATCGACATGTACAAG
1                                                                     M  A  A  A  I  D  M  Y  K
181   TACTACAATACCAGCGCACACCAGATCCCCTCCTCATCCCCCTCGGATCAGGAGCTCGCGAAAGCACTCGAGCCTTTTATAACGAGTGCT
10     Y  Y  N  T  S  A  H  Q  I  P  S  S  S  P  S  D  Q  E  L  A  K  A  L  E  P  F  I  T  S  A
271   TCCTCCTCTTCATCCTCCTCCCCCTACCATGGCTACTCGTCCTCTCCATCCATGTCCCAAGATTCTTACATGCCTACACCCTCTTACACC
40     S  S  S  S  S  S  S  P  Y  H  G  Y  S  S  S  P  S  M  S  Q  D  S  Y  M  P  T  P  S  Y  T
361   AGCTACGCCACCTCGCCTCTTCCCACTCCCGCCGCCGCCTCCTCCTCGCAGTCGCAGCTTCCGCCGCTCTACTCGTCGCCTTATGCGGCG
70     S  Y  A  T  S  P  L  P  T  P  A  A  A  S  S  S  Q  S  Q  L  P  P  L  Y  S  S  P  Y  A  A
451   CCGTGCATGGCCGGCCAGATGGGCCTGAACCAGCTCGGCCCGGCCCAGATCCAGCAGATCCAGGCCCAGTTCATGTTCCAGCAGCAGCAG
100    P  C  M  A  G  Q  M  G  L  N  Q  L  G  P  A  Q  I  Q  Q  I  Q  A  Q  F  M  F  Q  Q  Q  Q
541   CAGCAGCAGAGGGGCCTGCACGCGGCGTTCCTGGGCCCGCGGGCGCAGCCGATGAAGCAGTCAGGGTCGCCGTCGCCGCCGCCGCCGCTG
130    Q  Q  Q  R  G  L  H  A  A  F  L  G  P  R  A  Q  P  M  K  Q  S  G  S  P  S  P  P  P  P  L
631   GCGCCGGCGCAGTCGAAGCTGTACCGCGGCGTGCGGCAGCGCCACTGGGGCAAGTGGGTGGCGGAGATCCGGCTCCCGAAGAACCGCACG
160    A  P  A  Q  S  K  L  Y  R  G  V  R  Q  R  H  W  G  K  W  V  A  E  I  R  L  P  K  N  R  T
721   CGGCTGTGGCTCGGCACCTTCGACACCGCGGAGGACGCGGCGCTCGCCTACGACAAGGCGGCCTTCCGCCTCCGCGGCGACACGGCGCGC
190    R  L  W  L  G  T  F  D  T  A  E  D  A  A  L  A  Y  D  K  A  A  F  R  L  R  G  D  T  A  R
811   CTCAACTTCCCGGCCCTCCGGCGCGGCGGCGCGCACCTCGCCGGCCCGCTGCACGCCTCCGTGGACGCCAAGCTGACCGCCATCTGCCAG
220    L  N  F  P  A  L  R  R  G  G  A  H  L  A  G  P  L  H  A  S  V  D  A  K  L  T  A  I  C  Q
901   TCCCTGTCGGAGTCCAAGTCCAAGAGCGGCTCGTCCGGCGACGAGTCGGCCGCGTCCCCGCCGGACTCCCCCAAGTGCTCGGCGTCGACG
250    S  L  S  E  S  K  S  K  S  G  S  S  G  D  E  S  A  A  S  P  P  D  S  P  K  C  S  A  S  T
991   ACGGAGGGAGAGGGGGAGGAGGAGTCGGGCTCCGCCGGCTCCCCTCCTCCTCCTCCTCCTCCCCCGACGCTGGCGCCGCCCGTGCCGGAG
280    T  E  G  E  G  E  E  E  S  G  S  A  G  S  P  P  P  P  P  P  P  P  T  L  A  P  P  V  P  E
1081  ATGGCGAAGCTGGACTTCACGGAGGCGCCGTGGGACGAGACGGAGGCCTTCCACCTGCGCAAGTACCCGTCCTGGGAGATCGACTGGGAT
310    M  A  K  L  D  F  T  E  A  P  W  D  E  T  E  A  F  H  L  R  K  Y  P  S  W  E  I  D  W  D
1171  TCCATCCTGTCATGAgcaataacagctccgtgtaattttctactgtttggtttttgcggctgcgttggcccgatggcattttagacgtcg
340    S  I  L  S  *
1261  gccatggcggctgcaagtagcaagtgagtaactagctagctagtacatcgtcgtcgtccagtgttgtgaagcagagttaagtacatgcgt
1351  gctaatctcctggttgagctgccggttgtttttttctcacggcacggccagtcgagaagagtcagtagtgtaatctcgtggtttttatgatc
1441  atctgttgcagcttatgtatggaactttgtctatctagtactatttattggtctaaaaaa
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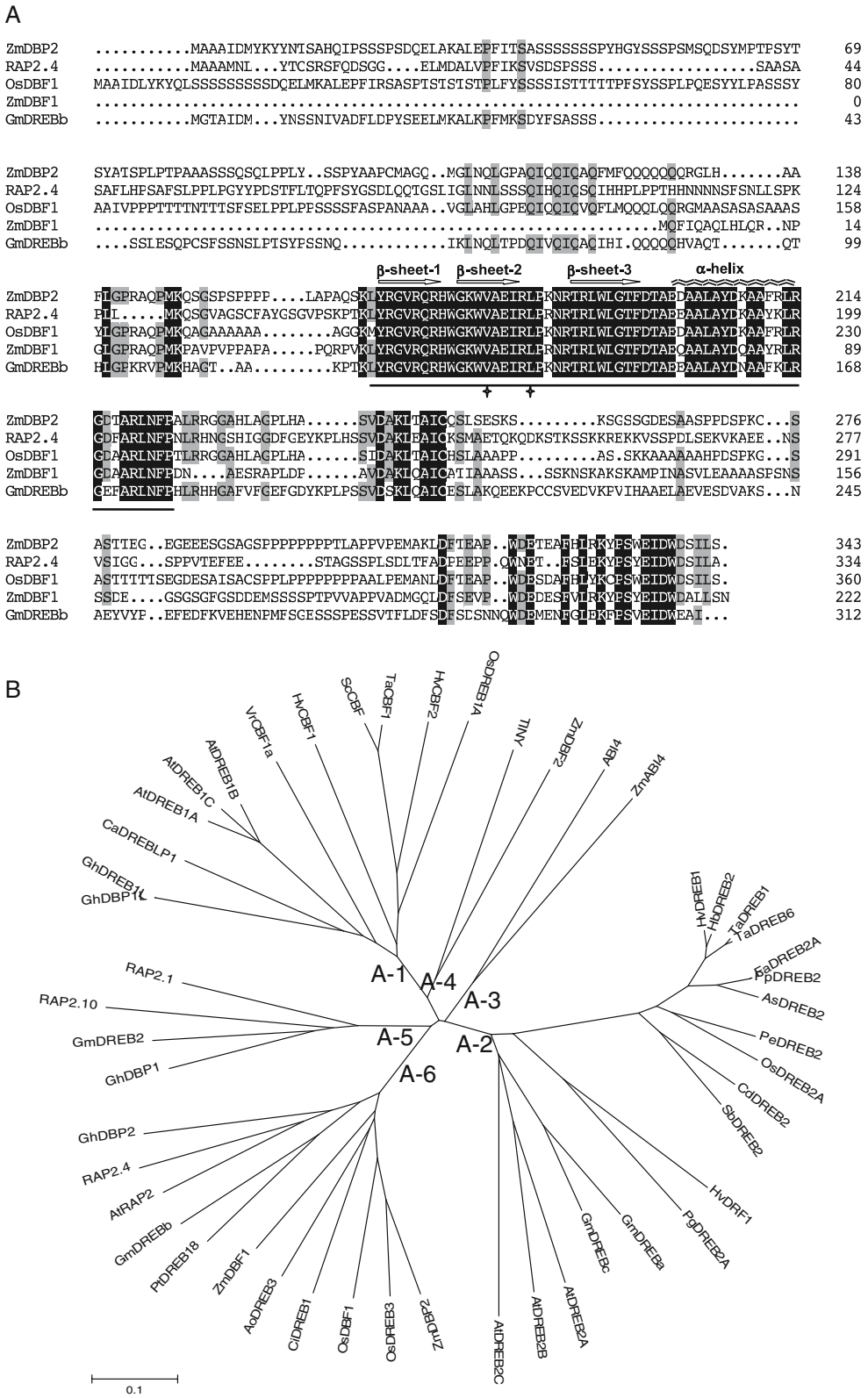
Fig. 1 Nucleotide and deduced amino acid sequences of the *ZmDBP2* gene. The ORF sequence is represented by *uppercase letters*, and untranslated regions are indicated by *lowercase letters*. The deduced amino acid sequence is shown below the DNA sequence. The AP2/ERF domain is *single underlined*; acidic and Ser/Thr-rich regions that may act as transcriptional activation domains are shown in *bold italics*, and two potential *N*-linked glycosylation sites are in *bold script*



ZmDBP2 Protein Is Not Localized in the Nucleus

Transcription factors are commonly expected to localize to the nucleus. As shown in Fig. 3, ZmDBP2::hGFP fusion protein, like *hGFP*, was uniformly distributed throughout the onion epidermal cell, suggesting that ZmDBP2 probably has no nuclear localization activity, which agrees with the results of protein subcellular

◀ **Fig. 2** Comparison and phylogenetic relationship of maize ZmDBP2 with other plant DREBs. **a** Amino acid alignment of DREB proteins. RAP2.4 (NP_177931), OsDBF1 (BAD37688), ZmDBF1 (AAM80486), and GmDREBb (AY296651). The conserved AP2/ERF domain is *underlined* and *asterisks* indicate the conserved V_{14} and L_{19}. Three β-sheets and one amphipathic α-helix are marked over the corresponding sequences. **b** Phylogenetic tree of ZmDBP2 with DREBs from other plants. The phylogenetic tree was constructed by the MEGA program. The accession number of each appended protein is as follows: OsDBF1: BAD37688, CiDREB1: ABR53728, AoDREB3: ABB89754, PtDREB18: EEE94094, HvDRF1: AY223807, HvCBF1: AF418204, HvCBF2: AF442489, FaDREB2A: AJ717396, HbDREB2: AY728807, TaDREB1: DQ195068, TaCBF1: AF376136, OsDREB1: AY064403, ZmDBF1: AAM80486, ZmDBF2: AF493799, ZmABI4: AY125490, GhDBP1: AY174160, GhDREB1L: DQ409060, AtDREB1A: AB007787, AtDREB1B: AB007788, AtDREB1C: AB007789, AtDREB2A: AB007790, AtDREB2B: AB007791, AtDREB2C: NM_129594, GmDREBa: AAT12423, GmDREBb: AAQ57226, GmDREBc: AAP83131, PgDREB2A: AAV90624, ScCBF: AAL35759, VrCBF1a: AAR28671, OsDREB2A: AF300971, CaDREBLP1: AY496155, TaDREB6: AAX13289, PpDREB2: AAS59530, AsDREB2: ABS11171, PeDREB2: ABY19375, CdDREB2: AAS46285, SbDREB2: ABX45045, ABI4: A0MES8, GmDREB2: ABB36645, RAP2.10: Q9SW63, RAP2.1: Q8LC30, AtRAP2: AAP04063, RAP2.4: NP_177931, GhDBP2: AAT39542, OsDREB3: NP_001048142, GhDBP1L: ABD65473, and TINY: Q39127

localization prediction (WoLF PSORT; http://wolfpsort.org/).

ZmDBP2 Is Induced by Drought, Salt, and Exogenous ABA

To assess responses to various abiotic stress conditions, we qualitatively surveyed the transcript expressions of *ZmDBP2* using Q-RT-PCR. Total RNA was extracted from maize seedlings subjected to drought, salt, cold, and exogenous ABA. As shown in Fig. 4a, *ZmDBP2* transcripts accumulated greatly in response to drought and moderately to salt and exogenous ABA, but cold treatment had no effect.

We studied the kinetics of mRNA accumulation in maize seedlings over 48 h. Under drought condition, *ZmDBP2* mRNA began to accumulate at 1 h and reached its maximum at 5 h after treatment (Fig. 4b). Under high-salt and exogenous ABA treatment, the expression pattern of *ZmDBP2* was similar to that with drought treatments, but the peak time for salt-induced transcription was later than for drought treatment (Fig. 4c, d).

ZmDBP2 Promoter Contains Several Putative Stress-Responsive Elements

To further investigate the mechanism responsible for regulation of *ZmDBP2* gene expression, we cloned a 1.62-kb promoter region upstream of the *ZmDBP2* ATG start codon from maize genomic DNA. Putative *cis*-acting elements in the promoter region were identified using PLACE (a database of plant *cis*-acting elements, http://www.dna.affrc.go.jp/PLACE/). Several distinct regulatory motifs homologous to *cis*-acting elements involved in responses to abiotic stresses and plant hormones were identified (Fig. 5). These included two DPBF binding core sequences (ACACNNG), two ABRE elements (TACGTGKC, K represents G or T), two RAV binding sequences (CAACA), a ARF site (TGTCTC), a MBS site (CAACTG), and a W-box element (TTGACC). In addition, there were sequences resembling a TC-rich repeat (GTTTTTTTAC), a Py-rich stretch (TTTCTCTTCT), CGTCA motif (CGTCA), and a GCC-box element (GCCGCC).

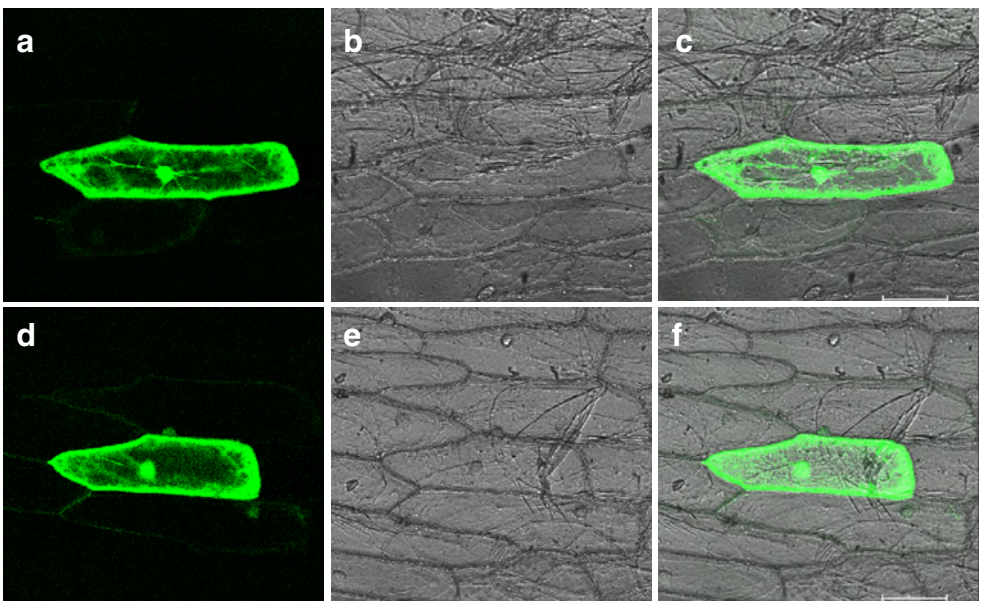


Fig. 3 Subcellular localization of the ZmDBP2 protein in onion epidermis cells. The fusion construct for *ZmDBP2::*hGFP and the control plasmid hGFP were introduced into onion epidermis cells by particle bombardment. Transformed cells were cultured on MS medium at 25°C for 16 h and observed under a confocal microscope. Photographs were taken in dark field for green fluorescence (**a** and **d**), in bright light for morphology of the cells (**b** and **e**), and in combination (**c** and **f**)

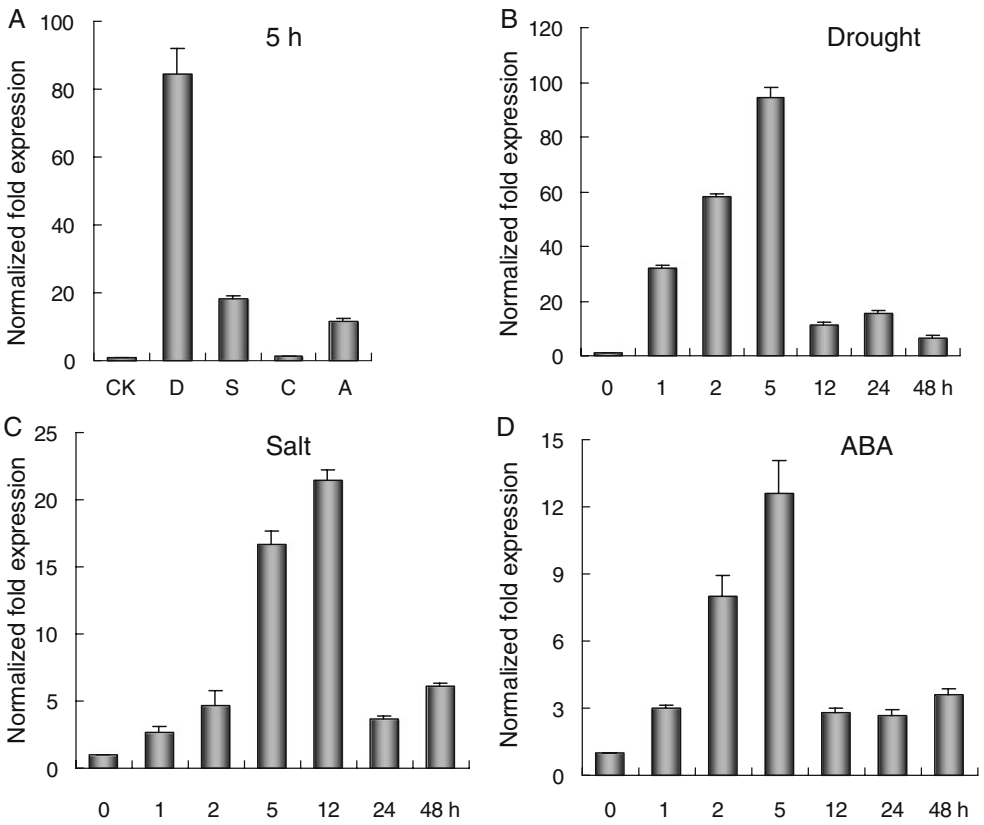


Fig. 4 Expression of *ZmDBP2* under different abiotic stress conditions. **a** All treatments were applied for 5 h. In **a**, CK, D, S, C, and A indicate control, drought, salt, cold, and ABA treatment, respectively. **b**–**d** The kinetics of mRNA ZmDBP2 accumulation of under drought, salt, and exogenous ABA over 48 h

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-1620  tcatagacaataagctggaaaaggtttaatttacatcatatttcgaccttacaattatgggtaaaagaaaacatttgataaaaaagcatc
-1530  cttgctagttatcagtttattaaggattatttgaaccctactaaaagatgtgaagtacttcaggcgttttgttcaaaacacgatttgaaa
-1440  tggcatggtcttctaaataacact[ttgacc]attcatttactatatcttacatttcatatttatttataaaaatatgattattggatagta
                                W-box
-1350  caattacttataaatctaaccatatcaatttgtgttataatagttaaaaattgctagtcagattattagttaaaacttttaaagtccgat
-1260  tcttgatatgaaagagaatggtggtagcaggacgcaaagttccagtaatattaatatccttccgt[acgt]attgagctcttgagggcatgt
                                                                        ABRE
-1170  acaacccagatactgctgcacggtactccaagtataagatacaactaaaacacaacataatacagtggtcatgtataaaacatgtgtctt
-1080  acgatattcattgtaccaatcagagcattcaataaattaaagtgaccaatcagctagtctcc[tgtctc]gaacatacagctaagacactgt
                                                                      ARF
-990   gtctt[cgtca]aga[tacgtgtc]ttga[gttttttttac]attcacccccctagacacgctgtaagacacaacttaaaacaccctctgtacatgcc
           CGTCA     ABRE          TC-rich
-900   catgcaatgaaaaggcaaattactcacaagaagggctccagcgttggcgagtcaatccagcccagcacgcgagaccaggcg[cacacgtgg]
                                                                                        ABRE/DPBF
-810   [c]agaccagaactggctgcgacttggcattccccgcgtgctacctcgcgtgggatgacgccatggacgagagcaacaggcctccttccttc
-720   caaactgcatttggtaattttcattcacgggaatattgct[cagttg]attaatcgaatcattttgtgctaatcccccacggagaagaaatc
                                                  MBS
-630   gcaaacagcggcgagcaagagatgcccgcttggcttcgcgtgcacggccccggtgccaagcctggcactatataaaggcctggccgagag
-540   acctttcttccccagagagacagacacgcctcacccccaccacaagaacaccaaagccacatcccatccatctccacggtgaggcttgag
-450   cgatccgagcaaaaaaaatcatccgcgacgcagcgacgcagg[tttgttctct]gatctctctggttcttcttttagcagcagggacgcaca
                                                        Py-rich
-360   [acacaag]aaaaggtccggttcaagaggctcttctgttgggtttttttagaatcctcaagagaagcgtgttcttgggttaggatttttttc
        DPBF
-270   tcacctcataacacctctcctaggaggaacagagatccagagaacctttt[tttctcttct]ggatccttg[ccgcc]ttttggcgttcgaaagg
                                                                  Py-rich          GCC-box
-180   gggttgttttgcaataccctctctctgttttttttttctttcttggcacaaacccacctcgtttctctact[tttatt]gaggaacagagct
                                                                                    TATA
-90    ggtttttcacaaaaaaaaattctactttgattttccataaagaaaacctctcagcctctttcttctagctccatccaagctgtagtccta
1      [ATG]GCCGCAGCCATCGACATGT
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Fig. 5 The promoter sequence of the *ZmDBP2* gene. The ATG translation initiation codon is assigned as position 1 in the nucleotide sequence, and the nucleotide positions upstream of position 1 are presented as *negative numbers*. The putative *cis*-acting elements are indicated in *bold*, and the names are given under the elements. *DPBF* DPBF binding core sequence, *ABRE* ABRE element, *ARF* ARF site, *MBS* MBS site, *W-box* W-box element, *TC-rich* TC-rich repeat, *Py-rich* Py-rich stretch, *CGTCA* CGTCA motif, *GCC-box* GCC-box element

Improved Drought Tolerance in Transformed *Arabidopsis* Plants

The *ZmDBP2* gene under the control of CaMV35S was transformed into *Arabidopsis* plants to further investigate its functions. The transcription of *COR/RD* genes with the CRT/DRE element in their promoters probably regulated by *ZmDBP2* gene were analyzed. As shown in Fig. 6a, overexpression of *ZmDBP2* in the transgenic *Arabidopsis* plants under normal growth conditions resulted not only in the accumulation of its own mRNA but also of transcripts corresponding to *rd29A* and *rab17*. Thus, it is likely that the presence of *ZmDBP2* leads to improved stress tolerance in transgenic *Arabidopsis* plants.

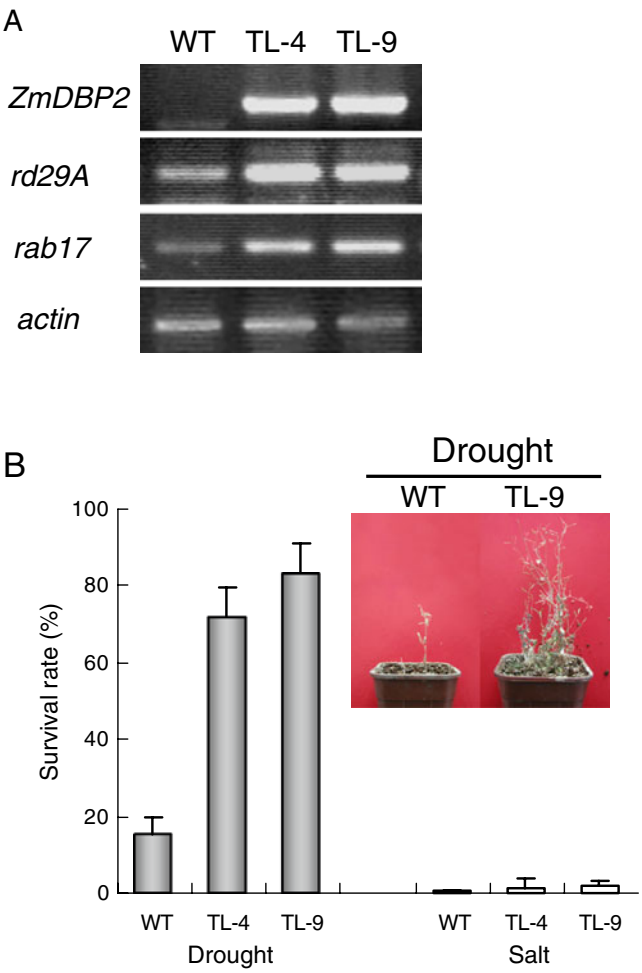

Fig. 6 Effect of *ZmDBP2* expression on drought and salt tolerance in transgenic *Arabidopsis* plants. **a** Expression patterns of *ZmDBP2*, *rd29A*, and *rab17* in wild-type (WT) and *35S::ZmDBP2* transgenic *Arabidopsis* plants (*lines TL-4* and *TL-9*) under normal growing conditions. Parallel reactions using *actin* in primers were carried out to normalize the amounts of added template. **b** Survival rates of WT and *CaMV35S::ZmDBP2* transgenic *Arabidopsis* lines (*TL-4* and *TL-9*) were estimated after stress treatments. Stress treatments were applied to transgenic *Arabidopsis* and WT 3-weeks-old plants under common conditions. Results are averages of three replicates ± SD

Responses to abiotic stresses were analyzed using *35S::ZmDBP2* transgenic *Arabidopsis* plants. Seedlings of transgenic and wild-type *Arabidopsis* grown for 3 weeks at 22°C were used for analyses of survival rates under drought and NaCl stresses (Fig. 6b). Under water-free conditions, most (more than 80%) wild-type plants were dead after 12 days, whereas 71.7–83.3% of the transgenic plants survived and continued to grow after rewatering. Ten days after 200 mM NaCl treatment, almost all of the transgenic plants, like the wild-type plants, had died, although the loss of greenness of transgenic plants was slower than that of wild-type plants (data not shown). These experiments indicated that overexpression of *ZmDBP2* resulted in enhancement of drought tolerance in *Arabidopsis* plants.

Discussion

The transcription of DREB genes are strongly and rapidly upregulated in response to abiotic stresses in numerous plant species (reviewed in Agarwal et al. 2006). Therefore, it is important to elucidate the mechanisms underlying the transmission of DREB-mediated abiotic stress signals in order to understand enhanced crop stress tolerance. DREBs, which show variations in some conserved motifs and in biological functions in different species, were divided into six groups (A-1, A-2, A-3, A-4, A-5, and A-6) based on different response profiles after a range of stresses (Dubouzet et al. 2003; Agarwal et al. 2006). For example, expression of A-1 group genes is specifically induced by low-temperature stress in *Arabidopsis* and rice (Liu et al. 1998; Sakuma et al. 2002; Dubouzet et al. 2003), whereas A-2 group genes respond to dehydration and high-salt stresses (Liu et al. 1998; Dubouzet et al. 2003). However, gene expression in the other groups has been studied in only a limited number of plant species and most of their functions remain undetermined. We therefore isolated an A-6 group transcription factor gene, *ZmDBP2*, from maize.

The ZmDREB2 protein showed no nuclear localization activity (Fig. 3). Most transcription factors have one or two putative NLSs. Xu et al. (2007) isolated a wheat AP2/ERF factor, TaERF1, containing three NLSs that synergistically controlled its subcellular localization. Interestingly, the AP2/ERF domain of TaERF1 plays dual roles in DNA-binding affinity and nuclear localization. As a transcription factor, ZmDBP2 possibly enters the nucleus but after interaction with other transcription factors.

There are ABA-dependent and ABA-independent regulatory systems for stress-responsive gene expression in plants (Yamaguchi-Shinozaki and Shinozaki 2006). A-2 subgroup members are key regulators in ABA-independent gene expression under abiotic stress conditions (Nakashima

et al. 2000; Sakuma et al. 2002; Chinnusamy et al. 2006; Nakashima and Yamaguchi-Shinozaki 2006). Expression pattern analyses revealed that *ZmDBP2* transcripts were induced by exogenous ABA (Fig. 4). Putative ABA-responsive motifs, such as DPBF binding core sequences, ARF site, and ABRE elements, were also found in the promoter of *ZmDBP2*. These results suggest that *ZmDBP2* may act in an ABA-mediated signal transduction pathway.

ZmDBP2 transcription was activated by drought and by salt (Fig. 4). Putative drought- and salt-responsive motifs were found in the promoter of *ZmDBP2*, such as an MBS site (responsive to drought), W-box element (responsive to drought and salt), DPBF binding core sequences, and ABRE elements (responsive to drought as well as ABA). A maize A-2 group gene, *ZmDREB2A*, was shown to accumulate under heat stress in maize seedlings (Qin et al. 2007). Clearly, DREB genes are involved in multiple stress responses, and more of them need to be isolated and tested in order to gain a more comprehensive understanding of their similarities and differences in response to various stresses.

Overexpression of *ZmDBP2* activated CRT/DRE-containing genes under normal growth conditions and conferred drought tolerance in transgenic *Arabidopsis* plants (Fig. 6). However, overexpression of *ZmDBP2* did not dramatically enhance resistance to salt. Although a gene may respond to a stress, such a response does not necessarily imply that the gene will play a positive role in increasing resistance to the stress (Liu et al. 1998; Xiong and Yang 2003). It is possible that some gene products might require posttranslational modifications or interaction with other factors to achieve such a response, or on the other hand, overexpression of some genes might not be enough to enhance resistance to clearly detectable or adequate levels (Xu et al. 2007).

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References

Agarwal PK, Agarwal P, Reddy MK, Sopory SK (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep 25:1263–1274

Chen WJ, Zhu T (2004) Networks of transcription factors with roles in environmental stress response. Trends Plant Sci 9:591–596

Chinnusamy V, Zhu J, Zhu JK (2006) Gene regulation during cold acclimation in plants. Physiol Plant 126:52–61

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J 33:751–763

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. Plant J 16:433–442

Haarke V, Cook D, Riechmann JL, Omaira P, Thomashow MF, Zang JZ (2002) Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. Plant Physiol 130:639–648

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis CBF1* overexpression induces cor genes and enhances freezing tolerance. Science 280:104–106

Jiang Y, Qin F, Li Y, Fang X, Bai C (2004) Measuring specific interaction of transcription factor ZmDREB1A with its DNA responsive element at the molecular level. Nucleic Acids Res 32: e101

Lee H, Xiong L, Ishitani M, Stevenson B, Zhu JK (1999) Cold-regulated gene expression and freezing tolerance in an *Arabidopsis thaliana* mutant. Plant J 17:301–308

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10:1391–1406

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method method. Methods 25:402–408

Nakashima K, Yamaguchi-Shinozaki K (2006) Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants. Physiol Plant 126:62–71

Nakashima K, Shinwari ZK, Sakuma Y, Seki M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2000) Organization and expression of two *Arabidopsis* DREB2 genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. Plant Mol Biol 42:657–665

Qin F, Sakuma Y, Li J, Liu Q, Li YQ, Shinozaki K, Yamaguchi-Shinozaki K (2004) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L. Plant Cell Physiol 45:1042–1052

Qin F, Kakimoto M, Sakuma Y, Maruyama K, Osakabe Y, Tran LS, Shinozaki K, Yamaguchi-Shinozaki K (2007) Regulation and functional analysis of *ZmDREB2A* in response to drought and heat stresses in *Zea mays* L. Plant J 50:54–69

Ramanjulu S, Bartels D (2002) Drought- and desiccation-induced modulation of gene expression in plants. Plant Cell Environ 25:141–151

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science 290:2105–2110

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem Biophys Res Commun 290:998–1009

Shinozaki K, Dennis ES (2003) Cell signalling and gene regulation: global analyses of signal transduction and gene expression profiles. Curr Opin Plant Biol 6:405–409

Stockinger EJ, Gilmour SJ, Thomashow MF (1997) Arabidopsis thaliana CBF1 encodes an AP2 domain-containing a transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA 94:1035–1040

Wang CT, Dong YM (2009) Overexpression of maize ZmDBP3 enhances tolerance to drought and cold stress in transgenic Arabidopsis plants. Biologia 64:1108–1114

Xiong L, Yang Y (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell 15:745–759

Xu ZS, Xia LQ, Chen M, Cheng XG, Zhang RY, Li LC, Zhao YX, Lu Y, Ni ZY, Liu L, Qiu ZG, Ma YZ (2007) Isolation and molecular characterization of the *Triticum aestivum* L. ethylene-responsive factor 1 (*TaERF1*) that increases multiple stress tolerance. Plant Mol Biol 65:719–732

Xu ZS, Chen M, Li LC, Ma YZ (2008a) Functions of the ERF transcription factor family in plants. Botany 865:969–977

Xu ZS, Ni ZY, Liu L, Nie LN, Li LC, Chen M, Ma YZ (2008b) Characterization of the TaAIDFa gene encoding a CRT/DRE-binding factor responsive to drought, high-salt, and cold stress in wheat. Mol Genet Genomics 280:497–508

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular response and the tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803