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Cloning and Functional Analysis of a Novel DREB1/CBF Transcription Factor Involved in Cold-Responsive Gene Expression in *Zea mays* L.

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The transcription factors DREB1s/CBFs specifically interact with the DRE/CRT *cis*-acting element (core motif: G/ACCGAC) and control the expression of many stress-inducible genes in *Arabidopsis*. We isolated a cDNA for a DREB1/CBF homolog, ZmDREB1A in maize using a yeast one-hybrid system. The ZmDREB1A proteins specifically bound to DRE and the highly conserved valine at the 14th residue in the ERF/AP2 DNA binding domain was a key to determining the specific interaction between this protein and the DRE sequence. Expression of ZmDREB1A was induced by cold stress and slightly increased by high-salinity stress. This gene was also transiently expressed by mechanical attack. ZmDREB1A activated the transcription of the GUS reporter gene driven by DRE in rice protoplasts. Overexpression of ZmDREB1A in transgenic *Arabidopsis* induced overexpression of target stress-inducible genes of *Arabidopsis* DREB1A resulting in plants with higher tolerance to drought and freezing stresses. This indicated that ZmDREB1A has functional similarity to DREB1s/CBFs in *Arabidopsis*. The structure of the ERF/AP2 domain of ZmDREB1A in maize is closely related to DREB1-type ERF/AP2 domains in the monocots as compared with that in the dicots. ZmDREB1A is suggested to be potentially useful for producing transgenic plants that is tolerant to drought, high-salinity and/or cold stresses.

Keywords: Abiotic stress tolerance — DREB/DRE regulon — Maize — Target stress-inducible genes — Transcription factor — Transgenic *Arabidopsis*.

Abbreviations: DRE, dehydration responsive element; CBF, C-repeat binding factor; ABRE, ABA responsive element; ERF, ethylene responsive factor; GST, glutathione *S*-transferase; bZIP, basic domain / leucine zipper; Zm, Zea mays; AP2, APETALA2; CaMV35S, cauliflower mosaic virus 35S; NLS, nuclear localization signal; 3-AT, 3-aminotriazole.

The nucleotide sequence (*ZmDREB1A* gene) reported in this paper has been submitted to the DDBJ/EMBL/GenBank databases under the accession number AF450481.

Introduction

Drought, high salinity and low temperature are environmental conditions that cause adverse effects on the growth of plants and the productivity of crops. Plants respond and adapt to these stresses at the molecular and cellular levels as well as at the physiological and biochemical levels. Expression of a variety of genes has been demonstrated to be induced by these stresses in a variety of plants (Ingram and Bartels 1996, Thomashow 1999, Shinozaki and Yamaguchi-Shinozaki 2000). The products of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress response (Xiong et al. 2002, Shinozaki et al. 2003). The *cis*- and *trans*-acting elements that function in stress-responsive gene expression have been precisely analyzed to elucidate the molecular mechanisms of gene expression in response to drought stress. The dehydration-responsive element (DRE) with the core sequence A/GCCGAC was identified as a *cis*-acting promoter element in regulating gene expression in response to drought, high-salinity and cold stresses in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994). A similar motif was identified as C-repeat (CRT) and low temperature-responsive element (LTRE) in cold-inducible genes (Baker et al. 1994, Jiang et al. 1996).

Arabidopsis cDNAs encoding ERF/AP2 type DRE-binding proteins, *C-repeat binding factor* (*CBF*) *1*, *DRE-binding protein* (*DREB*) *1A*, and *DREB2A*, have been isolated by using a yeast one-hybrid screening (Stockinger et al. 1997, Liu et al. 1998). These proteins specifically bind to the DRE/CRT sequence and activate the transcription of genes driven by the DRE/CRT sequence in *Arabidopsis*. There are three DREB1/CBF proteins that are encoded by genes that lie in tandem on chromosome 4 in the order DREB1B/CBF1, DREB1A/CBF3 and DREB1C/CBF2. There are two DREB2 proteins, DREB2A and DREB2B (Liu et al. 1998, Gilmour et al. 1998). Expression of the *DREB1/CBF* genes is induced by cold, but not by dehydration or high-salinity stress (Liu et al. 1998, Shinwari et al. 1998). By contrast, expression of the *DREB2* genes

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is induced by dehydration and high-salinity stresses but not by cold stress (Liu et al. 1998, Nakashima et al. 2000). Then, three novel DREB1/CBF- and six novel DREB2-related genes in the *Arabidopsis* genome were reported but the expression levels of these genes were low under the stress conditions (Sakuma et al. 2002). The DREB1A, DREB1B and DREB1C, and DREB2A and DREB2B proteins are probably major transcription factors that function in cold-, and high-salinity- and drought-inducible gene expression in *Arabidopsis*, respectively. However, one of the *CBF/DREB1* genes, *CBF4/DREB1D*, is induced by osmotic stress, suggesting the existence of cross-talk between the CBF/DREB1 and the DREB2 pathways (Haake et al. 2002).

Strong tolerance to freezing stress was observed in transgenic *Arabidopsis* plants that overexpress *CBF1/DREB1B* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Jaglo-Ottosen et al. 1998). Overexpression of the *DREB1A/CBF3* under the control of the CaMV 35S promoter also increased the tolerance of drought, high-salinity, and freezing stresses (Liu et al. 1998, Kasuga et al. 1999, Gilmour et al. 2000). Six genes have been identified as the target stress-inducible genes of DREB1A using RNA gel blot analysis (Kasuga et al. 1999). Recently, more than 40 target genes of CBF/DREB1 have been identified using both cDNA and GeneChip microarrays (Seki et al. 2001, Fowler and Thomashow 2002, Maruyama et al. 2004). Most of these target genes contained the DRE or DRE-related core motifs in their promoter regions (Maruyama et al. 2004). However, overexpression of the DREB1A protein also caused severe growth retardation under normal growth conditions. Use of the stress-inducible *rd29A* promoter instead of the constitutive 35S CaMV promoter for the overexpression of DREB1A minimizes the negative effects on plant growth (Kasuga et al. 1999).

There are 145 distinct genes encoding the ERF/AP2-type proteins in *Arabidopsis* and these proteins were classified into five groups, APETALA2 (AP2) subfamily, RAV subfamily, DREB subfamily, ERF subfamily, and one very specific gene AL079349 based on the similarity of their ERF/AP2 DNA binding domains (Sakuma et al. 2002). The proteins of the DREB subfamily were further divided into six groups, among which DREB1 (A-1) and DREB2 (A-2) were the two largest subgroups (Sakuma et al. 2002). Overexpression of the DREB1-type proteins in *Arabidopsis* directly gave high stress tolerance to transgenic plants, but plants overexpressing the DREB2-type proteins failed to show any stress tolerance (Liu et al. 1998, Dubouzet et al. 2003). These results indicate that the DREB1-type proteins are constitutively active in plants but that the DREB2-type proteins probably require modification in response to stress for its activation in plants.

DRE has been shown to function in gene expression in response to stress in tobacco plants, which suggests the existence of similar regulatory systems in tobacco and other crop plants (Yamaguchi-Shinozaki and Shinozaki 1994). The DRE-related motifs have been reported in the promoter region of cold-inducible *Brassica napus* and wheat genes (Jiang et al. 1996, Ouellet et al. 1998). CBF/DREB1 transcription factors were also identified in *B. napus*, wheat, rye, tomato and rice, and all of them showed a rapid response to cold stress (Jaglo et al. 2001, Dubouzet et al. 2003). On the other hand, overexpression of the *Arabidopsis DREB1/CBF* genes in transgenic *B. Napus* or tobacco plants induced expression of orthologs of *Arabidopsis* DREB1/CBF-targeted genes and increased the freezing tolerance of the transgenic plants (Jaglo et al. 2001, Kasuga et al. 2004). These observations suggest that the DRE/DREB regulon can be used to improve the tolerance of various kinds of agriculturally important crop plants to drought, high-salinity and freezing stresses by gene transfer.

Maize is currently produced in most countries in the world and is the third most planted field crop after wheat and rice. This plant is used to produce grain and fodder that are the basis of many foods, feed, pharmaceuticals and industrial products. Thus, the enhanced stress tolerance in this plant is of great importance. Previously, two DRE-binding proteins, named DBF1 and DBF2, were cloned in maize (Kizis and Pages 2002). However, RNA-gel blot and structural analyses showed that these two proteins were not DREB1-type transcription factors (Kizis and Pages 2002). As DREB1-type transcription factors are quite useful for generating transgenic plants that are tolerant to drought, high-salinity and freezing stresses, we examined whether or not such a transcription factor existed in the plant. In this study we report the identification of a *DREB1*-type gene from maize, named *ZmDREB1A*, which can be greatly induced by cold stress. We analyzed the effect of overexpression of ZmDREB1A in transgenic *Arabidopsis* plants and compared those plants with those overexpressing *Arabidopsis* DREB1A. We also discuss the function of ZmDREB1A in the cold-responsive gene expression in maize.

Results

Isolation of a cDNA encoding a DRE-binding protein in maize

To isolate a cDNA encoding a DRE-binding protein, we carried out yeast one-hybrid screening. We used a parental yeast strain carrying as reporter genes integrated copies of *HIS3* and *lacZ* with a four-times tandemly repeated 75-bp DNA fragment of the *rd29A* promoter containing a DRE motif at the center. As the DRE/DREB regulon has been identified in many kinds of plant species and it is probably conserved in the plant kingdom, we used the *Arabidopsis* DRE sequence as a bait for isolating DREB transcription factors in maize. Approximately 1.0×10^6 yeast transformants were screened using a maize cDNA library according to the manufacturer's protocol (Clontech Mathchmaker one-hybrid system). Yeast clones resistant to 20 mM 3-aminotriazole (3-AT) and forming blue colonies were isolated and submitted for sequencing. By this procedure we identified one positive clone which could grow well even on the 60 mM 3-AT plates and had a relatively high *lacZ* activity on the filter (data not shown). To analyze the transactivation activity of the isolated clone in yeast cells, we transferred the

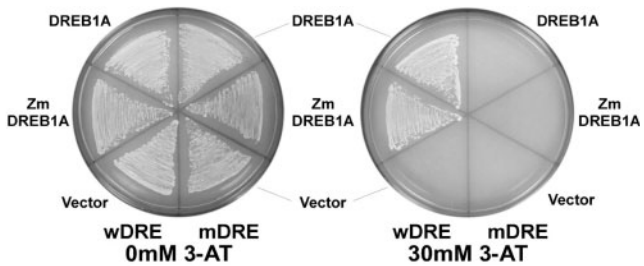

Fig. 1 Activation of the *HIS* gene in yeast reporter cells by ZmDREB1A. Two -His-Ura-Trp SD medium plates containing 0 mM 3-AT or 30 mM 3-AT were equally divided into six parts. Clones in the left three parts of each plate were yeast reporter cells carrying the wild-type DRE sequence (wDRE) transformed by three plasmids containing *DREB1A* (as a positive control), *ZmDREB1A* and the YepGAP empty vector (as a negative control). Clones in the right three parts were yeast reporter cells carrying the mutated DRE sequence (mDRE) transformed by three plasmids containing *DREB1A*, *ZmDREB1A* and the YepGAP empty vector.

insert cDNA fragment to YepGAP, a yeast expression vector without the yeast GAL4 activation domain (Liu et al. 1998). When the YepGAP plasmid containing the insert cDNA was introduced to yeast reporter cells carrying the reporter gene driven by the DRE sequence, the yeast cells grew on the 30 mM 3-AT plates. However, the yeast cells carrying the reporter gene driven by the mutated DRE sequence (mDRE) did not show 3-AT resistance (Fig. 1).

To measure the β-galactosidase activity, we used *Arabidopsis DREB1A* as the positive control and the YepGAP vector as a negative control. The β-galactosidase activity assay showed that *lacZ* expression in the isolated yeast clone carrying the DRE element was stimulated 21.8-fold more than the control. By contrast, there was no significant change in *lacZ* expression compared to the control in the yeast clone carrying mDRE (Table 1). These data indicate that the isolated cDNA clone encodes a polypeptide that specifically binds to the DRE sequence and activates the transcription of the dual reporter genes in yeast.

Structural and phylogenetic analysis of the ZmDREB1A

We sequenced the 1.08-kb inserted DNA fragment of the positive cDNA clone. This cDNA contained a single open reading frame of 267 amino acids and encoded a putative protein with a predicted molecular mass of 29.9 kDa. The sequence was submitted to the GenBank under the accession number of AF450481. The isolated cDNA clone encoded an ERF/AP2 DNA binding protein. The alignment of the ERF/AP2 DNA binding domain against various ERF/AP2 proteins suggested that this cDNA encoded a DREB1/CBF-type protein in maize. Therefore, the isolated cDNA clone was designated *ZmDREB1A*. Besides the highly conserved ERF/AP2 DNA binding domain, ZmDREB1A contained a typical DREB1-type nuclear localization signal (NLS) consensus, KR/KPAGRT/KKFRETRHP, before the ERF/AP2 domain and a DSA sequence after the domain, which were identified in all DREB1-type proteins from various species. Multiple full-length sequence alignment of these proteins also revealed some conserved regions at the C-terminal (Fig. 2).

Since the first identification of the *DREB1*/CBF and *DREB2* genes from *Arabidopsis*, many *DREB/CBF* genes have been reported from a variety of plants. Two other DRE-binding proteins, DBF1 and DBF2 were cloned from maize. In order to clarify the relationship of these three proteins in the superfamily of the ERF/AP2 transcription factors in plants. A systematic phylogenetic analysis of the ERF/AP2 domains of these proteins was carried out on the basis of the classification of 145 ERF/AP2 transcription factors in *Arabidopsis* (Sakuma et al. 2002, Dubouzet et al. 2003). We analyzed the similarities of three cloned maize DRE-binding proteins together with proteins from other species, including those from monocots and dicots. The amino acid sequences of the ERF/AP2 domains of these proteins from maize, rice, tomato, barley, cotton, tobacco, and *B. napus*, were appended to the previous list of *Arabidopsis*

Table 1 *LacZ* expression in yeast cells transformed with *ZmDREB1A*

Reporter strains	β-Galactosidase activity, u [a]	Relative activity
(YepGAP +DREB1A)/wDRE	8.89±0.28	21.2
(YepGAP +DREB1A)/mDRE	0.44±0.14	1.0
(YepGAP +ZmDREB1A)/wDRE	9.15±0.47	21.8
(YepGAP + ZmDREB1A)/mDRE	0.78±0.21	1.9
YepGAP/wDRE	0.42±0.17	1.0
YepGAP/mDRE	0.37±0.07	0.9

Genes constructed in YepGAP, a yeast expression vector without the yeast GAL4 activation domain (Liu et al. 1998), were transformed into a yeast YM4271/His^+Ura^+ reporter containing the wild-type DRE or mutated DRE elements. Yeast cells were grown on a selective medium of SD/-His-Ura-Trp for 20 h. The β -galactosidase relative activity was calculated by defining the activity of the negative control as 1.0. The data were obtained after three independent experiments (± indicates standard error). wDRE and mDRE indicate yeast reporter cells carrying the *lacZ* gene driven by the wild-type and mutated DRE sequences, respectively.
[a] Miller units.

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Zm : MDTAGLVQHATSSSSTSTSASSSSSEQQSRKAAWPPSTASSPQQPPKKRPAGRTKFR : 57
Os :   MCGIKQEMSGES--SGSPCSSASAERQHQTVWT---------APPKRPAGRTKFR : 44
Ta :                 MDTAAAGSPREGHRTVCS---------EPPKRPAGRTKFR : 31
At : MNSFSAFS----EMFGSDYESSVSSGGDYIPTLAS---------SCPKKPAGRKKFR : 44
Bn : MNSVSTFS----ELLGSENESPV--GGDYCPMLAA---------SCPKKPAGRKKFR : 42
Le : MNIFETYYSDSLILTESSSSSSSSSFSEEEVILAS---------NNPKKPAGRKKFR : 48
                                                    ●●●●●● ●●●
                            *     *
Zm : ETRHPVFRGVRRRGAAGRWVCEVRVPGRRGARLWLGTYLAAEAAARAHDAAILALQG :114
Os : ETRHPVFRGVRRRGNAGRWVCEVRVPGRRGCRLWLGTFDTAEGAARAHDAAMLAINA :101
Ta : ETRHPLYRGVRRRGRLGQWVCEVRVRGAQGYRLWLGTFTTAEMAARAHDSAVLALLD : 88
At : ETRHPIYRGVRRR-NSGKWVCEVREPNKK-TRIWLGTFQTAEMAARAHDVAALALRG : 99
Bn : ETRHPIYRGVRLR-KSGKWVCEVREPNKK-SRIWLGTFKTAEIAARAHDVAALALRG : 97
Le : ETRHPIYRGIRKR-NSGKWVCEVREPNKK-TRIWLGTFPTAEMAARAHDVAALALRG :103
     ●●●●
Zm : RGAG-----RLNFPDSAR-LLAVPPP--SALPGLDDARRAALEAVAEFQRRSGSGSG :163
Os : GGGGGGGACCLNFADSAW-LLAVP----RSYRTLADVRHAVAEAVEDFFRRR----- :148
Ta : RAAC------LNFADSAWRMLPVLAAGSSRFSSAREIKDAVAIAVLEFQRQR----- :134
At : RSAC------LNFADSAW-RLRIP-----ESTCAKDIQKAAAEAALAFQDEMCDATT :144
Bn : RGAC------LNFADSAW-RLRIP-----ETTCAKDIQKAAAEAALAFEAEKSDTTT :142
Le : RSAC------LNFSDSAW-RLPIP-----ASSNSKDIQKAAAQAVEIFRSEEVSGES :148
                  ●●●
Zm : AADEATSGASPPSSSPSLPDVSAAGSPAAALEHVPVKADEAVALDLDGD-------- :212
Os : LADDALSATSSSSTTPSTPRTDDDEESAATDGDESSSPASDLAFELD---------- :195
Ta : ----------PVVSTSEMHDGEKDAQGSPTPSELSTSSD---LLDEH---------- :168
At : ----------------DHGFDMEETLVEAIYTAEQSE------------------- :165
Bn : NDHGMNMASQAEVNDTTDHGLDMEETMVEAVFTEEQRDGFYMAEETTVEGVVPEEQM :199
Le : ----------------------PETSEN---VQESSD------------------- :160
Zm : VFGPDWFGDMGLELDAYYASLAEGLLVEPPPPPAAWDHGDCCDSGAADVALWSYY  :267
Os : -----VLSDMGWDL--YYASLAQGMLMEPPS-AALGDDG---DAILADVPLWSY  :238
Ta : -----WFGGMDAGS--YYASLAQGMLMEPPSARTWSEDGG--EYSAVYTPLWN  :212
At : -NAFYMHDEAMFEMPSLLANMAEGMLLPLPS--VQWNHNHEVDGDDDDVSLWSY  :216
Bn : SKGFYMDEEWMFGMPTLLADMAAGMLLPPPS--VQWGHNDDFEG-DVDMNLWNY  :250
Le : ----FVDEEAIFFMPGLLANMAEGLMLPPPQ--CAEMGDHCVETDAYMITLWNYSI :210
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Fig. 2 Alignment of the CBF/DREB1 proteins. The ERF/AP2 DNA-binding domains are underlined. Asterisks show different amino acids in the consensus between DRE-binding proteins and GCC box-binding proteins (Liu et al. 1998). V14 is highly conserved in all the DREB proteins but E19 is conserved only in the DREB protein of dicot plants. The NLS sequence and DSA box are indicated by black circles. The conserved sequences in the C-terminal of these proteins are highlighted on a black background. The amino acid sequences of maize ZmDREB1A (Zm; AF045481), rice OsDREB1A (Os; AF300970), *Arabidopsis* DREB1A (At; AB007787), *B. napus* CBF1 (Bn; AY370733), tomato (*L. esculentum*) CBF1 (Le; AY034473) and wheat CBF (Ta; AF376136) were aligned.

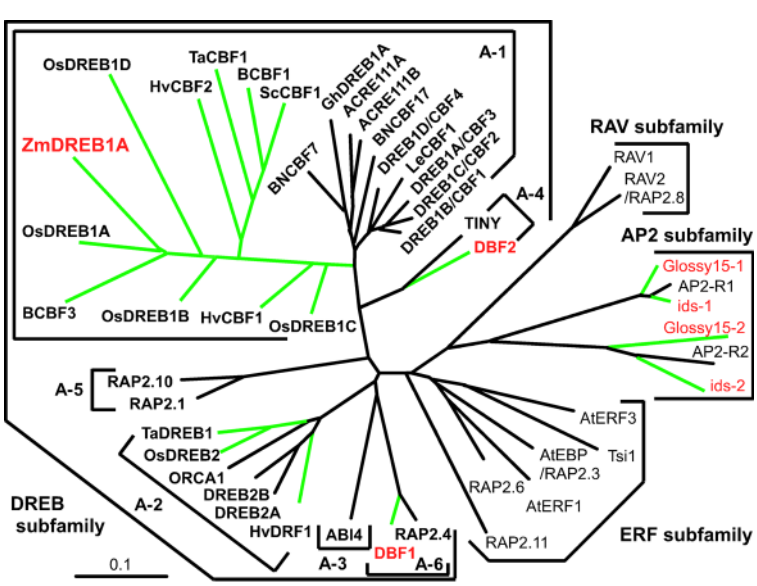

Fig. 3 Phylogenetic analysis of the ZmDREB1A protein. A phylogenetic tree of the ERF/AP2 domains was constructed by CLASTAX. The scale indicates branch lengths. A-1–A-6 indicate subgroups proposed by Sakuma et al. (2002). The accession number of each appended protein was: *LeCBF1* (AY034473); *BNCBF7* (AF499032); *BNCBF17* (AF499034); *GhDREB1A* (AY321150); *HvCBF1* (AF418204); *ZmDREB1A* (AF045481); *HvCBF2* (AF442489); *BCBF1* (AF298230); *HvDRF1.1* (AY223807); *ScCBF1* (AF370730); *BCBF3* (AF298231); *TaDREB1* (AF303376); *DBF1* (AF493800); *DBF2* (AF493799); *Glossy15* (U41466); *ids1* (AF048900). Genes from maize are shown in red; genes from other monocot plants in green (wheat, rice, rye, and barley); and genes from dicot plants in black (*B. napus*, cotton, tomato, and tobacco).

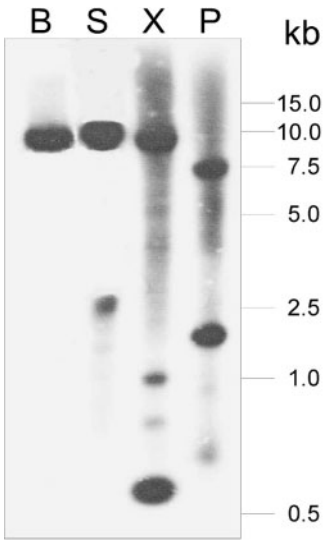

Fig. 4 DNA-gel blot analysis of *ZmDREB1A* cDNA. Five μg of maize genomic DNA, was digested with *Bam*HI (B), *Sal*I (S), *Xho*I (X) and *Pst*I (P) and fractioned in a 1.0% agarose gel. The full-length *ZmDREB1A* cDNA was used as a probe. Numbers on the right are molecular weight markers in kilo bases. Southern hybridization was carried out as described in Maniatis et al. (1982).

ERF/AP2 transcription factors, and the phylogenetic analysis was carried out by the ClustalX program (Fig. 3, Sakuma et al. 2002). As a result, ZmDREB1A was classified into the DREB1 type-transcription factor, A-1 subgroup (Sakuma et al. 2002), whereas, DBF1, like *Arabidopsis* RAP2.4, belonged to the A-6 subgroup in the DREB subfamily. DBF2 shared a high homology with the TINY protein, assigned to the A-4 subgroup. Moreover, DREB1 proteins from monocots, such as maize, rice, barley, wheat and rye formed a small group independent of those from dicots. CBF/DREB1 proteins from tomato, *B. napus*, cotton and tobacco showed a relative closer relationship with *Arabidopsis* DREB1 proteins (Fig. 3). Golssy15 and ids1 from maize, which contain two ERF/AP2 domains and function in maize flower development, were grouped into the AP2 subfamily (Moose and Sisco 1996, Chuck et al. 1998). Some DREB proteins, such as HvDRF1.1, OsDREB2 and TaDREB1, grouped into the DREB2-type transcription factors (Fig. 3, Xue and Loveridge 2004, Jaglo et al. 2001, Dubouzet et al. 2003).

DNA-gel blot analysis of ZmDREB1A

The copy number of *ZmDREB1A* in a maize genome was determined by DNA-gel blot analysis employing maize genotype P138. Genomic DNA was digested with *Bam*HI, *Sal*I, *Xho*I and *Pst*I, and transferred to the membrane. The hybridization was carried out under high-stringency conditions using the full-length *ZmDREB1A* cDNA as a probe. Digestion with *Bam*HI, which did not cut this gene, a single hybridization band of a high molecular mass was detected. For *Sal*I (one site at 227) or *Pst*I (one site at 453) treatment, two hybridization bands were detected. When the genomic DNA was digested by *Xho*I (two sites at 8 and 561), a specific hybridization band about 500 bp and upper bands were generated (Fig. 4). These results indicate that this gene has only one copy in the maize genome.

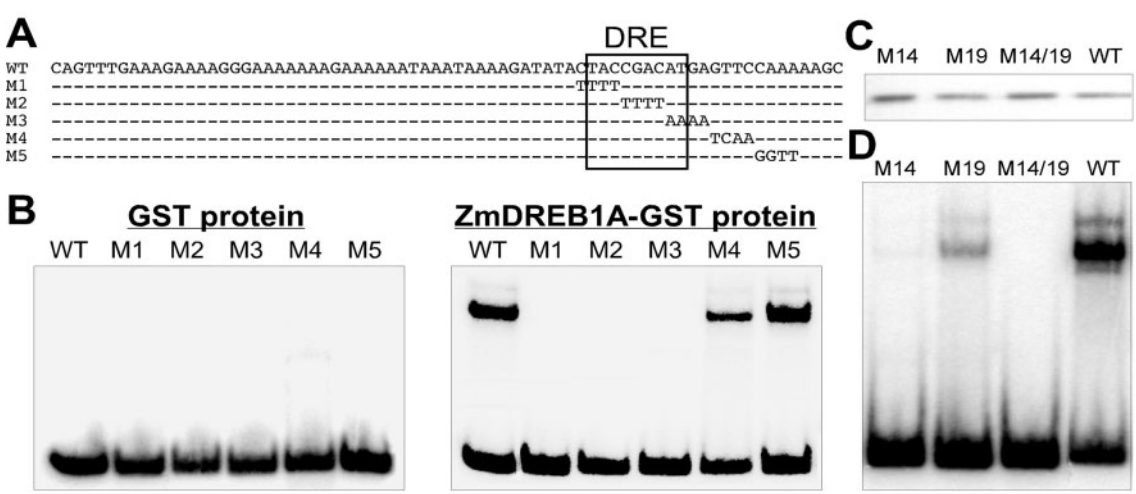

Fig. 5 ZmDREB1A DNA-binding affinity to DRE in the 75-bp fragment of the *rd29A* promoter. (A) Sequences of the 75-bp fragment of the *rd29A* promoter and its mutated fragments (M1–M5) used as probes (Liu et al. 1998). (B) Gel mobility shift assay of sequence specificity of the ZmDREB1A protein. WT and M1 to M5 indicate the wild-type and mutated DRE sequences. (C) SDS-PAGE of proteins used in the gel mobility shift assay. The same amount of proteins used in gel mobility shift assay was separated in 10% SDS-PAGE gel and stained by Coomassie Brilliant Blue. M14, M19 and M14/19 indicated single or double amino acid substitutions at corresponding sites. WT indicates the wild-type ZmDREB1A protein. (D) Gel mobility shift assay of the wild-type and mutant ZmDREB1A proteins using the wild-type DRE sequence as a probe.

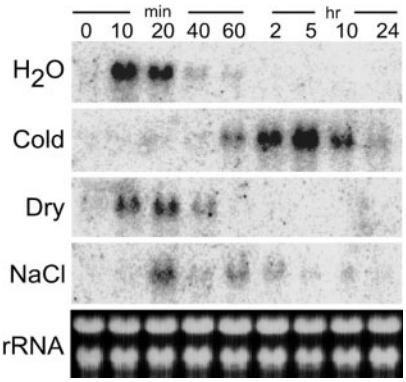

Fig. 6 RNA-gel bolt analysis of the *ZmDREB1A* transcripts under various stress conditions. (A) Seven-day-old seedlings were kept in water (dH_2O), in a refrigerator below 4°C (Cold), on filter paper in a clean bench (Dry), or in a 250 mM NaCl solution (NaCl) or 100 μM ABA solution (ABA) for 4 h. (B) Seedlings were given dH_2O, cold, dry, and NaCl treatments, for the periods indicated above each line. Total RNA (10 μg) was fractionated by electrophoresis in a formaldehyde agarose gel.

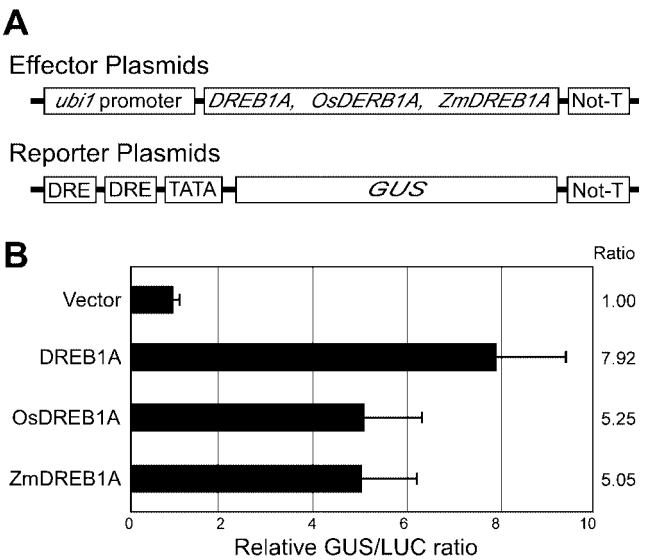

Fig. 7 Transactivation of the rd29A-GUS reporter gene by the DREB1A, OsDREB1A and ZmDREB1A proteins using rice protoplasts. For each individual experiment, 8×10^6 protoplasts were mixed with 20 μg of the reporter plasmid and 20 μg of the effector plasmid. (A) Schematic diagram of the effecter and reporter constructs used in the transactivation experiments. The reporter plasmid was constructed using two tandemly repeated 75-bp DNA fragments of the *rd29A* promoter containing the DRE sequence. The promoter was fused to the –61 rd29A minimal TATA promoter-GUS construct (Yamaguchi-Shinozaki and Shinozaki 1994). (B) Transactivation of the GUS reporter gene by the DREB1A, OsDREB1A or ZmDREB1A proteins. The cauliflower mosaic virus 35S promoter-LUC plasmid was cotransfected in each experiment to normalize for transfection efficiency. Transactivation activity of each protein was determined by the value of GUS/LUC, and the relative transactivation activity was calculated by defining the GUS/LUC value of vector control as 1.00.

DNA-binding property of ZmDREB1A according to gel mobility shift assay

The DNA-binding specificity of the ZmDREB1A protein was analyzed by expressing the 159 amino acids of the DNA-binding domain of ZmDREB1A as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli*. The ability of the ZmDREB1A fusion protein to bind the wild-type or base-substituted DRE sequences in the *rd29A* promoter was examined using the gel mobility shift assay. The recombinant protein bound the wild-type 75-bp DNA fragment, but not the base-substituted fragments M1, M2 and M3. By contrast, the protein still bound the M4 and M5 (Fig. 5). The DRE sequence was base-substituted in M1, M2 and M3 but not in M4 and M5 (Fig. 5A). As the negative control, no retardation band was detected when the pure GST protein was tested (Fig. 5B).

The 14th and 19th conserved amino acids in the ERF/AP2 domain have been reported to be important in determining the target DNA sequences (Sakuma et al. 2002). To confirm the importance of these conserved amino acids of *ZmDREB1A*, we performed a gel mobility shift assay using mutant ZmDREB1A proteins. We substituted the valine residue (V) at position 14 in the DNA-binding domain of ZmDREB1A with alanine (A). We also substituted the valine residue (V) at position 19 with aspartic acid (D). Proteins with substitutions at positions 14 and/or 19 were expressed as GST-fusion proteins in *E. coli* (Fig. 5C). The substitution at position 14 from V to A greatly reduced the binding ability to the DRE sequence of the protein, and only a very weak retardation band was detected. In contrast, when the V at position 19 was changed to D, the mutated protein could still bind the DRE sequence, but the band was weaker than that produced by the wild-type protein. When both the 14th and 19th amino acids in ZmDREB1A were substituted, the mutated protein completely lost the binding affinity to the DRE sequence (Fig. 5D).

Expression of ZmDREB1A

The expression pattern of the *ZmDREB1A* gene was analyzed by using RNA-gel blot analysis. The 3′-terminal region of *ZmDREB1A* was used as a probe to avoid cross hybridization due to homology in the ERF/AP2 DNA-binding domain. *ZmDREB1A* was strongly induced within 4 h after exposure to cold stress but hardly responded to dehydration or NaCl or ABA application at 4 h (data not shown). Then, we performed a time course experiment using four kinds of treatment (Fig. 6). The *ZmDREB1A* mRNA was accumulated within 60 min, reaching a maximum level at 5 h, and then decreased to the basal level after 24 h of cold stress. Additionally, ZmDREB1A was slightly increased by high-salinity stress. This expression pattern was similar to those of other reported *CBF/DREB1* genes that function in an ABA-independent pathway. Probably, *ZmDREB1A* was transiently expressed by the mechanical attack causing by transferring plants from soil into water, and

this induction appeared to be somehow suppressed by the following stress treatments especially under cold conditions.

The ZmDREB1A protein acts as a transactivator in rice protoplasts

It is important to show that the ZmDREB1A protein functions as a transcriptional activator in plants cells, especially monocot cells. Therefore, we performed transactivation assays using protoplasts prepared from cultured rice cells. A β-*glucuronidase* (*GUS*) gene driven by the two repeated 75-bp fragments of the *rd29A* promoter containing DRE sequence was used as a reporter plasmid. To overexpress the effector genes in monocot cells, the maize *ubiquitin1* promoter was fused to each coding region of the *ZmDREB1A*, *OsDREB1A* or *DREB1A* cDNA. To control the transfection efficiency, *ubiquitin1* promoter-*luciferase* (*LUC*) plasmids were cotransfected together with both reporter and effector plasmids. LUC activity was quantified as an internal control to normalize the transfection efficiency. The relative GUS/LUC activity was distinctly upregulated in the presence of AtDREB1A, OsDREB1A, or ZmDREB1A in rice protoplasts. *ZmDREB1A* and *OsDREB1A* exhibited almost the same transactivation activity in rice protoplasts, with about a 5-fold higher GUS/LUC ratio compared to the vector control. Although *AtDREB1A* was cloned from a dicot plant, it showed the highest GUS/LUC ratio even in rice protoplasts (Fig. 7). These results indicate that these three proteins function as transactivators in rice cells.

Overexpression of ZmDREB1A in Arabidopsis

To study the function of the ZmDREB1A protein in the cold stress signal transduction pathway, we generated transgenic *Arabidopsis* overexpressing *ZmDREB1A*, because this cold-responsive signaling pathway has been studied in this model plant and many cold accumulated genes can be used as the molecular markers to indicate this pathway. The *ZmDREB1A* cDNA was overexpressed by the enhanced CaMV 35S promoter (Mitsuhara et al. 1996) in *Arabidopsis*. Twenty-nine T1 transgenic plants were generated by using a vacuum infiltration method. Quantitative real-time PCR method was employed to screen the expression level of *ZmDREB1A* for each of 29 T1 plants. Among them, 17 plants expressed the transgene at an undetectable level, showing no obvious phenotype compared with the wild-type plants (transformed by pBE2113; Fig. 8A); three plants expressed *ZmDREB1A* at a medium level displaying dwarfed shape and delayed bolting time. 35S:ZmDREB1Aa represented this phenotype, which was a T2 line from one of this kind of plant as shown in Fig. 8A. The remaining nine plants expressed the gene at a considerably high level, bearing shorter petioles and darker green leaves, with a severe growth retardation and a significantly delayed bolting time. All of them produced very few T2 seeds. 35S:ZmDREB1Ab was a T2 line coming from one of this kind of plants (Fig. 8A). This indicated that the degree of growth retardation and dwarfed phenotype correlated with the expression level of the *ZmDREB1A* gene. The higher level of *ZmDREB1A* expression caused a more severe phenotype.

To investigate whether the ZmDREB1A transcription factor could also activate some downstream genes in the cold or dehydration pathway, we analyzed the expression level of some target genes of the DREB1A protein. Northern blot analysis was carried out in two transgenic T2 lines, 35S:ZmDREB1Aa and 35S:ZmDREB1Ab, which expressed the transgene at a medium and high level, respectively. The expression of some stress responsive genes was analyzed under unstressed normal, cold and dehydration conditions in the wild-type and 35S:ZmDREB1Aa plants. In addition, the expression patterns of these genes under control conditions were compared among the wild-type, 35S:ZmDREB1Aa and 35S:ZmDREB1Ab plants. As shown in Fig. 8B, expression of *COR15A*, *KIN1*, *KIN2*, in 35S:ZmDREB1Aa was significantly upregulated under normal conditions as compared with that in the wild-type plants. The transcripts of *RD29A*, *RD17* and *ERD10* were observed to be slightly increased in 35S:ZmDREB1Aa. For *COR15B*, *AtGolS3* and *At1g16850*, which were previously identified as target genes of the OsDREB1A protein (Dubouzet et al. 2003), the expression remained almost the same in 35S:ZmDREB1Aa and wild-type plants. However, in the 35S:ZmDREB1Ab line, all of these genes could be overexpressed without stress, and the expression levels of these genes were well correlated with that of the transgene, which indicated that all these genes were target genes of this transcription factor, but more ZmDREB1A protein was required to activate the expression of *COR15B*, *AtGolS3* and *At1g16850*.

Improved stress tolerance of the ZmDREB1A transgenic plants

To evaluate the effect of overexpression of *ZmDREB1A* on stress tolerance, transgenic *Arabidopsis* plants overexpressing *DREB1A* (35S:DREB1A; Kasuga et al. 1999) was used as a positive control. First we tried to explore the recovery differences after desiccation. The wild-type, 35S:ZmDREB1Aa and 35S:DREB1A transgenic plants were kept on dry plastic plate for 5 h (the relative humidity about 15%) followed by rehydration overnight. The wild-type plant had wilted and curved leaves with a dark color, whereas the transgenic plants had green leaves that were spread out and appeared to be healthier (Fig. 9A). The leakage of electrolytes is a sensitive measure of loss-of-membrane integrity and it is commonly used to assay osmolality injury (Gilmour et al. 1998, Vannini et al. 2004). We applied the ion leakage test to three kinds of plants using all of their rosette leaves (Fig. 9B). When three-week-old plants were removed from the agar plates and exposed to –6°C for 3 h, 31.6% electrolytes measured to be leaked out of the cells in wild-type plants. For the 35S:ZmDREB1Aa and 35S:DREB1A transgenic plants, the ion leakage was 13.9% and 12.1%, respectively. When plants were dehydrated on dry plastic plates for 2.5 h (the relative humidity about 30%), ion leakage of the wild-type plants was 88.6%, whereas for transgenic plants the

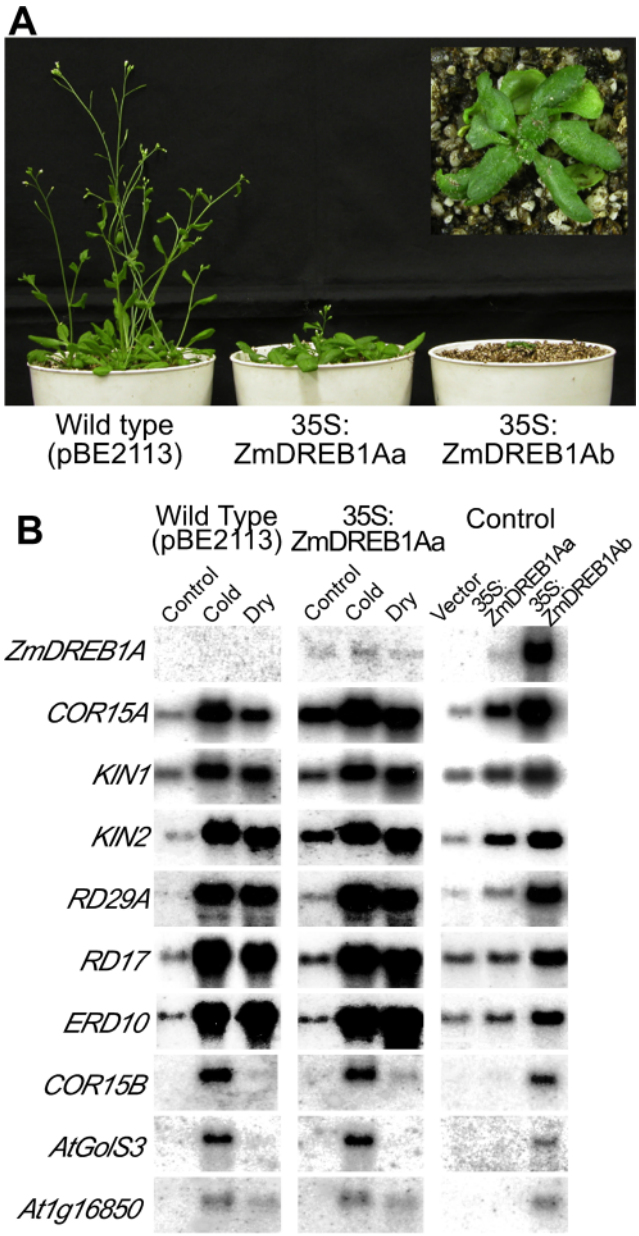

Fig. 8 (A) Comparison of growth retardation among the wild-type (transformed by pBE2113 vector), 35S:ZmDREB1Aa and 35S: ZmDREB1Ab plants. Plants were grown for 32 d under normal growth conditions. (B) Expression of *ZmDREB1A* and some DREB target genes in the wild-type (transformed by pBE2113 vector), 35S: ZmDREB1Aa and 35S:ZmDREB1Ab plants. Each lane was loaded with 5 μg of total RNA prepared form transgenic *Arabidopsis* plants that had been treated at 4°C for 5 h (cold), dehydrated for 5 h (dry) or were untreated (control). A 3′-terminal specific DNA fragment was used for each gene as a probe.

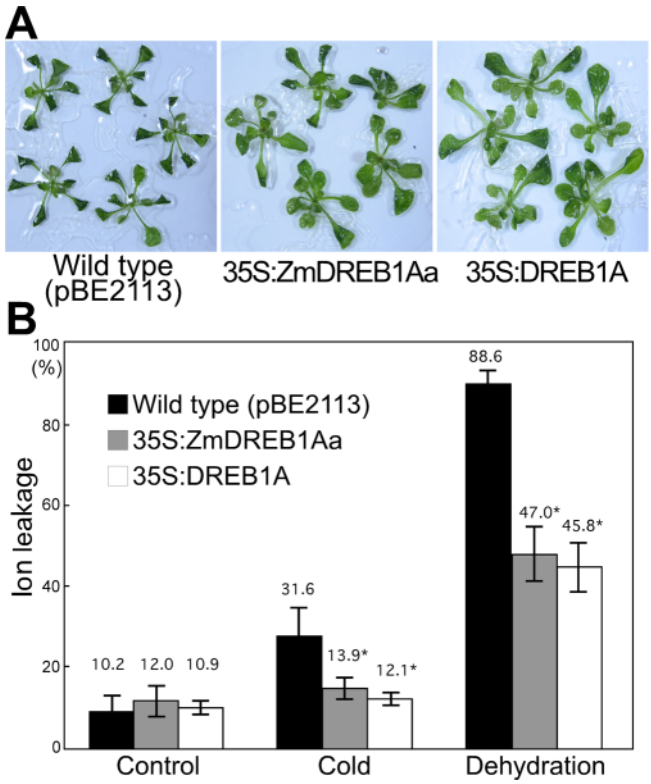

Fig. 9 Enhancement of stress tolerance of transgenic plants overexpressing ZmDREB1A. (A) Recovery difference after rehydration among the wild-type, 35S:*ZmDREB1Aa* and 35S:DREB1A plants. Photos show the plants dehydrated on a dry plastic plate in air for 5 h (the relative humidity is 15%), and then rehydrated overnight. (B) Electrolyte leakage was evaluated after cold or dehydration treatment. All the leaves of a 3-week-old plant were used in each experiment. Plants were removed from the agar plates and either exposed to –6°C for 3 h (cold) or dehydrated on a dry plastic plates for 2.5 h (dehydration). The values are means of five independent samples. Statistical significance compared with the value of the control plants was determined by Welch's test (*P <0.005).

ion leakage was only 40.7% and 45.8%. In normal conditions, all three kinds of plants had an equivalent electrolytes leakage percentage (around 10%; Fig. 9B). These results indicated that electrolyte leakage of plants carrying *ZmDREB1A* or *DREB1A* after cold and dehydration stress treatments was reduced significantly compared with that of the wild-type plants.

Discussion

We identified a maize *DREB1*-like transcription factor, *ZmDREB1A*. The deduced amino acid sequence of ZmDREB1A contained an AP2 DNA-binding domain, a typical DREB1-type NLS consensus and a DSA sequence flanking this domain, which was conserved in all the other DREB1 transcription factors (Jaglo et al. 2001). A gel mobility shift assay demonstrated that this protein specifically bound the DRE sequence (Fig. 5) and RNA-gel blot analysis of *ZmDREB1A* showed that this gene was significantly induced by cold stress (Fig. 6). This expression pattern was also observed in other *DREB1* genes by cold treatment (Liu et al. 1998, Jaglo et al.

2001, Dubouzet et al. 2003). *ZmDREB1A* did not respond to exogenous ABA, implying that it functioned in an ABA-independent pathway like other DREB1 proteins. *ZmDREB1A* was slightly induced by high-salinity stress (Fig. 6) which was observed in *DREB1C* (Nakashima et al. 2000) and *OsDREB1A* (Dubouzet et al. 2003). We also found that *ZmDREB1A* was transiently accumulated by wounding. This expression pattern of *ZmDREB1A* implies that its role in the transduction of abiotic stress signals in maize is more similar to *DREB1C* than to *DREB1A* like OsDREB1A.

Previously, two DBF1 and DBF2 transcription factors belonging to the ERF/AP2 superfamily were isolated and characterized in maize (Kizis and Pages 2002). These two proteins could bind the DRE sequence in the promoter region of a maize *rab17* gene. *DBF1* was induced by dehydration, NaCl, or ABA treatment and during maize embryogenesis, but not by cold stress, while *DBF2* showed a constitutive expression profile. Although they were classified into the same superfamily and had a similar DNA binding affinity, their functions were different from those of the *Arabidopsis* DREB1 and DREB2 proteins. In order to clarify the phylogenetic relationship among *DBF1*, *DBF2* and *ZmDREB1A* in the ERF/AP2 superfamily, we performed the phylogenetic analysis of the AP2/ERF domain on the basis of the classification of 145 ERF/AP2-type transcription factors in *Arabidopsis*. ZmDREB1A was grouped into A-1 subgroup, which consisted of the DREB1-type proteins, and especially gathered with the DREB1 proteins from monocots. DBF2 was classified into the A-4 subgroup, which was represented by a *TINY* gene (Wilson et al. 1996). The *TINY* mRNA accumulated slightly in only cold-treated roots, and this expression was detected to be very low (Sakuma et al. 2002). Unlike the *TINY* gene, *DBF2* was observed to be constitutively expressed (Kizis and Pages 2002). DBF1 was grouped with *RAP2.4*, which was designated the A-6 subgroup. Previously, the DBF1and DBF2 proteins have been suggested to have evolved in a way non-parallel to the DREB proteins (Kizis and Pages 2002).

In this research we substituted V14 of ZmDREB1A by alanine (A), and tested the DNA binding affinity of the mutant protein by gel mobility shift assay. We found that this substitution directly led to a significant loss of DNA-binding ability of this protein, which suggested that this residue was very important in determining the protein and DNA recognition, whereas, the mutation at the 19th amino acid from V to aspartic acid (D) had only a minor effect (Fig. 5D). An earlier study suggested that two conserved amino acids, V14 and E19, in the AP2/EREBP domain of the DREB proteins might play important roles in the recognition of the *cis*-element in the promoter region of target genes, and of these two residues valine at 14th was more important (Sakuma et al. 2002, Cao et al. 2001). In *Arabidopsis* DREB1A and DREB2A, the substitution of the 19th amino acid from E to D had little effect on protein and DNA interaction (Sakuma et al. 2002). In many of the DREB proteins in monocot plants, this amino acid was V, while this amino acid was conserved as E in dicot DREB1 proteins. The variety in the 19th residue supported the inference that E19 was not so important as V14 for the interaction between protein and DNA, but such a difference at the 19th residue between monocot and dicot DREB1 proteins has any biological significance remains unknown.

Transactivation experiment of the ZmDREB1A protein in rice protoplasts demonstrated that this protein could activate the expression of the reporter gene driven by promoters containing DRE. This result indicates that this protein acts as a transcriptional activator, and it is constitutively active in plant cells like DREB1A and OsDREB1A. It was proposed that the activation domain of the DREB proteins was located in the acid C-terminal (Liu et al. 1998). The full-length alignment of several DREB1-type proteins showed that there were two highly conserved sequences in the C-terminal and their consensuses were GMLM/L and LWS/NY (Fig. 2). Further experimental analyses are necessary to show whether these two sequences are important for the activation activity of the DREB1 proteins. Unlike the DREB1 proteins, the DREB2-type transcription factors, involved in dehydration and high-salinity stress signal transduction, had very low activation activity under normal conditions (Liu et al. 1998). Thus it was supposed that some modification mechanism might be employed to activate it under stress conditions.

Transgenic *Arabidopsis* overexpressing *ZmDREB1A*, displayed a dwarf and growth retardation phenotype, and it became more severe with higher expression of this gene. A similar phenomenon was observed in transgenic plants overexpressing *DREB1A* and *OsDREB1A*, which indicated that the biological function of these proteins resembles that in *Arabidopsis*. Thus the DREB1/CBF cold-responsive pathway may be conserved in maize, rice and dicot plants. Many stress-inducible genes were identified and designated to be target genes of DREB1/CBF in *Arabidopsis*, which allowed us to compare the function of different DREB1 proteins. The expressions of nine genes were checked in two transgenic lines. All of them can be upregulated due to the overexpression of *ZmDREB1A*, which indicated that all these genes were target genes of this transcription factor. However, *KIN1*, *KIN2* and *COR15A* were highly expressed in normal conditions without stress with a medium expression level of *ZmDREB1A*, but the expression of *COR15B*, *AtGolS3* and *At1g16850*, required a higher expression of ZmDREB1A (Fig. 8B). This implied that the regulation of ZmDREB1A on different downstream genes was different and a higher expression of this protein inevitably led to a higher expression of the target gene. We previously reported that OsDREB1A preferred to bind GCCGAC to ACCGAC in the promoter region of target genes, so that overexpression of *OsDREB1A* in *Arabidopsis* could not increase the expression of *KIN1*, *KIN2* and *ERD10* which contain only the ACCGAC sequence as DRE in their promoters (Dubouzet et al. 2003). Nevertheless, ZmDREB1A, unlike OsDREB1A, could increase the transcription of *KIN1*, *KIN2* and *ERD10* under

normal conditions. The single molecular interaction force of ZmDREB1A with GCCGAC and ACCGAC was determined by atomic force microscope, and as a result this protein can bind these two sequences with an equivalent force (Qin et al. unpublished). ZmDREB1A seem to resemble DREB1A in the DNA-binding specificity more than its homolog in rice. The comparison of amino acid sequence of three proteins revealed that OsDREB1A contains an additional amino acid sequence of GGGACC at the end of DNA-binding domain (Fig. 2). Whether this additional sequence resulted in the difference in DNA-binding affinity of this protein was unclear.

DREB1A/CBF3, *CBF1*, *CBF4*, and *OsDREB1A* have been overexpressed in *Arabidopsis* and all overexpressors showed improved stress tolerance to drought, high salinity and cold (Liu et al. 1998, Stockinger et al. 1997, Haake et al. 2002, Dubouzet et al. 2003). In this research we also evaluate the effect of *ZmDREB1A* overexpression on *Arabidopsis* tolerance to cold or dehydration stresses using 35S:ZmDREB1Aa, because we could not obtain enough seeds of 35S:ZmDREB1Ab for a statistic test. The 35S:ZmDREB1Aa plants revealed a high recovery rate after desiccation followed by rehydration and a greatly reduced ion leakage after cold or dehydration treatment (Fig. 9B). On the other hand, the DREB1/CBF genes have been shown to improve agriculturally important plants, such as *B. napus*, tomato and tobacco (Jaglo et al. 2001, Kasuga et al. 2004). Therefore the DREB1-type genes including ZmDREB1A should increase the stress tolerance in transgenic maize. Furthermore, for a better understanding of the function of the DREB1-type proteins in monocot, transgenic rice or maize should be generated and the target genes of the proteins in these crops should be determined.

Materials and Methods

Plant materials and stress treatments

Plants (*Zea mays* L. pure inbred line P138) were sterilized and planted as described (Kizis and Pages 2002) for 7 d and submitted to stress treatments. For cold, NaCl and ABA treatments, plants were pulled out from soil and put into water or 250 mM NaCl or 100 μM ABA solution, respectively. For cold stress treatment, the plants were kept in a 4°C refrigerator for the designated period. For drought stress treatment, the plants were placed on filter paper on a clean bench under dim light. A maize cDNA library was a generous gift from Dr. Zhao Jun (Biotechnology Research Center, Chinese Academy of Agriculture Sciences, Beijing 100081, China).

Yeast one-hybrid screening of maize cDNA library

Construction of reporter plasmids and selection of the yeast reporter strain were performed as described (Liu et al. 1998). Yeast one-hybrid screening was carried out as described in the Yeast Protocols Handbook (CLONTECH, U.S.A.).

DNA- and RNA-gel blot analyses

DNA- and RNA-gel blot analyses were carried out as described previously (Liu et al. 1998).

Preparation of mutant ZmDREB1A fragments

The 14th and 19th residues in ZmDREB1A were singly or doubly replaced by alanine and aspartic acid named M14, M19 and M14/19, respectively. PCR strategy was used to introduce point mutations into the wild-type sequence by mutant primer pairs, mutation sites are underlined: 14-For 5′-GGTGGGCGTGCGAGGTGCGCGTCCCGG-3′; 14-Rev 5′-CCGGGACGCGCACCTCGCACGCCCACC-3′; 19-For 5′-GGTGGGTGTGCGAGGTGCGCGACCCGG-3′; 19-Rev 5′-CCGGGTCGCGCACCTCGCACACCCACC-3; 14/19-For 5′-GGTGGGCGTGCGAGGTGCGCGACCCGG-3′; 14/19-Rev 5′-CCGGGTCGCGCACCTCGCACGCCCACC-3′. Each of the above forward mutant primers was paired with the *ZmDREB1A* full-length forward primer 5′-AAAGAATTCATGGACACGGCCGGCCTC-3′ to generate a 5-terminal smaller fragment of *ZmDREB1A*. Each of the reverse mutant primers was paired with ZmDREB1A full-length reverse primer 5′-AAAGCGGCCGCCTAGTAGTAGCTCCAGAGCG-3′ to produce a 3′-terminal larger fragment of *ZmDREB1A*. These pairs of fragments were used as the second PCR templates to generate the full-length and mutant *ZmDREB1A* fragments.

GST fusion proteins preparation and gel mobility shift assays

The wild type and three mutants of 479-bp fragments of *ZmDREB1A* containing DNA-binding domain were prepared by the primer pair: 5′-TGACGAATTCGCTTCCTCACCAC-3′ (forward); 5′-TGACGTCGACGCACGTGCTCAAG-3′ (reverse), respectively. Each of these fragments was cloned into the *Eco*RI–*Sal*I sites of the pGEX-4T-1 vector and transformed into *E. coli* to produce the GST-fusion proteins. The GST fusion proteins were prepared using a Glutathione Sepharose 4B column (Pharmacia Biotech) according to the manufacturer's instructions. Gel mobility shift assays were conducted as described previously (Liu et al. 1998). The 71-bp DNA fragment was labeled by filling in 5′ overhangs with [^{32}P]dCTP and the Klenow fragment. The DNA-binding reaction was allowed to proceed for 20 min at 25°C in 20 μl of binding buffer (25 mM HEPES/KOH, PH7.9, 50 mM KCl, 0.5 mM DTT. 0.5 mM EDTA, 5% glycerol, 5 μg μl^{-1} BSA) that contained 20,000 dpm of the ^{32}P-labeled probe, 2 μg of poly(dI-dC) and bacterially produced fusion proteins as described previously (Liu et al. 1998).

Transient expression experiment

Suspension culture of a rice cell line (Oc), protoplast isolation, and electroporation were performed according to Hattori et al. (1995). The maize *ubiquitin1* promoter was a gift from Dr. Yusuke Ito of Japan International Research Center of Agricultural Science (JIRCAS) to overexpress the gene in rice cells.

Electrolyte leakage assay

Three-week-old *Arabidopsis* plants of wild type, 35S:ZmDREB1Aa and 35S:DREB1A were removed from the agar plates and exposed to –6°C for 3 h or dehydrated on empty plates for 2.5 h (the relative humidity about 30%). All the leaves of each plant were used for one measurement. Results were shown as the means of 10 plants of each line. Electrolyte leakage assay was performed as described by Fan et al. (1997).

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