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Regulation and functional analysis of *ZmDREB2A* in response to drought and heat stresses in *Zea mays* L

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Summary

DREB1/CBFs and DREB2s are transcription factors that specifically interact with a *cis*-acting element, DRE/CRT, which is involved in the expression of genes responsive to cold and drought stress in *Arabidopsis thaliana*. The function of DREB1/CBFs has been precisely analyzed and it has been found to activate the expression of many genes responsive to cold stress containing a DRE/CRT sequence in their promoters. However, the regulation and function of DREB2-type transcription factors remained to be elucidated. In this research, we report the cloning of a *DREB2* homolog from maize, *ZmDREB2A*, whose transcripts were accumulated by cold, dehydration, salt and heat stresses in maize seedlings. Unlike Arabidopsis *DREB2A*, *ZmDREB2A* produced two forms of transcripts, and quantitative real-time PCR analyses demonstrated that only the functional transcription form of *ZmDREB2A* was significantly induced by stresses. Moreover, the ZmDREB2A protein exhibited considerably high transactivation activity compared with DREB2A in Arabidopsis protoplasts, suggesting that protein modification is not necessary for ZmDREB2A to be active. Constitutive or stress-inducible expression of *ZmDREB2A* resulted in an improved drought stress tolerance in plants. Microarray analyses of transgenic plants overexpressing *ZmDREB2A* revealed that in addition to genes encoding late embryogenesis abundant (LEA) proteins, some genes related to heat shock and detoxification were also upregulated. Furthermore, overexpression of *ZmDREB2A* also enhanced thermotolerance in transgenic plants, implying that ZmDREB2A may play a dual functional role in mediating the expression of genes responsive to both water stress and heat stress.

Keywords: transcription factor, DRE/CRT, ZmDREB2A, drought tolerance, heat stress.

Introduction

Plant growth and productivity are affected by various abiotic stresses such as heat, cold, drought and high salinity, and plants must respond and adapt to these stresses in order to survive. Exposure to these stresses induces various biochemical and physiological changes in the process of acquiring stress tolerance. A number of genes have been described that respond to these stresses at the transcriptional level (Bartels and Sunkar, 2005; Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu, 2002). The *cis*- and *trans*-acting factors involved in the expression of stress-responsive genes have been extensively analyzed as a means to elucidate the molecular mechanisms of gene expression in response to the stresses of cold, drought and high-salinity (Yamaguchi-Shinozaki and Shinozaki, 2005). The dehydration-responsive element (DRE) containing the core sequence A/GCCGAC was identified as a *cis*-acting

promoter element which regulates gene expression in response to drought, high salinity and cold stresses in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1994). A similar motif was identified as the CRT (C-repeat) and LTRE (low-temperature-responsive element) in cold-inducible genes (Baker *et al.*, 1994; Jiang *et al.*, 1996). Complementary DNAs encoding DRE-binding proteins, *CBF*/*DREB1*s and *DREB2*s, have been isolated (Liu *et al.*, 1998; Stockinger *et al.*, 1997) and their corresponding gene products showed significant sequence similarity to the conserved DNA-binding domain found in ERF/AP2 proteins. Functional analyses of DREB1/CBF transcription factors have established them as important components of the cold stress response in Arabidopsis (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998). In Arabidopsis *DREB1*/*CBF* genes are induced by cold stress and their gene products activate the expression of >40 genes in the DREB1/CBF regulon (Fowler and Thomashow, 2002; Maruyama *et al.*, 2004). The expression of this regulon results in an improved tolerance not only to freezing, but also to drought and high salinity. DREB1/CBF orthologs have been reported and shown to function in cold stress tolerance from various species, such as *Brassica napus*, tomato, barley, maize, rice, rye, and wheat (Choi *et al.*, 2002; Dubouzet *et al.*, 2003; Gao *et al.*, 2002; Jaglo *et al.*, 2001; Qin *et al.*, 2004; Skinner *et al.*, 2005; Xue, 2002).

Although DREB1/CBF and DREB2 share a high homology in the ERF/AP2 DNA-binding domain and can bind the same DRE core sequence (A/GCCGAC), these two proteins differentially transmit cold or dehydration stress. Expression of the *DREB1*/*CBF* genes is induced to high levels specifically in response to a low-temperature stimulus. In contrast, Arabidopsis *DREB2A* is gradually induced by dehydration and high salinity stresses over 24 h, but hardly responds to cold stress (Liu *et al.*, 1998; Sakuma *et al.*, 2002). Multiple amino acid sequence alignment of DREB1 and DREB2 proteins shows that the sequences of PKK/RPAGRxKFxETRHP and DSAWR, which are located upstream and downstream of the DNA-binding domain, are conserved in DREB1-type transcription factors. These sequences are designated as DREB1/CBF 'signature sequences' (Jaglo *et al.*, 2001) and are absent in DREB2-type proteins. However, a serine- and threonine-rich region adjacent to the DNA-binding domain is found to be unique to the DREB2A protein. Overexpression of *DREB2A* in transgenic plants does not activate downstream genes under normal growth conditions, which implies that post-translational regulation may be involved in its activation (Liu *et al.*, 1998). Recently, a negative regulatory domain was identified in the central region of DREB2A and deletion of this region transforms DREB2A to a constitutive active form (*DREB2A CA;* Sakuma *et al.*, 2006a,b). Transgenic Arabidopsis overproducing DREB2A CA showed increased expression of many stress-responsive genes and improved tolerance to drought stress (Sakuma *et al.*, 2006a).

Because of the significance of gene regulation under dehydration and high salinity stress, multiple research efforts have been initiated to isolate drought- and osmotic stress-inducible transcription factors in other species. In rice, *Oryza sativa* L., one homolog, named *OsDREB2A*, was identified as a DREB2-type protein. Similar to Arabidopsis *DREB2A*, *OsDREB2A* was gradually induced by dehydration and high salinity stresses, but hardly increased under cold stress (Dubouzet *et al.*, 2003). In wheat, *Triticum aestivum* L., the *TaDREB1* gene was found to be induced by cold, salinity and drought and was classified into the DREB2-type transcription factors by phylogenetic analysis (Shen *et al.*, 2003). A barley, *Hordeum vulgare* L., gene for the DREB2-type protein, *HvDRF1*, was reported to accumulate under drought and salt stresses and was involved in ABA-mediated gene regulation (Xue and Loveridge, 2004). However, the function of these genes in plants under stress conditions is still unclear.

In the field, heat stress and water deficit often occur in a parallel manner, especially in tropical areas. The effects of these stresses may cause oxidative damage of cellular components or result in the misfolding or denaturation of cellular proteins. Heat shock proteins (HSPs) are synthesized and accumulated in the heat shock response and are correlated with thermotolerance in the plant (Li and Werb, 1982). Heat shock proteins act as molecular chaperones by maintaining the homeostasis of protein folding and thus help to maintain the metabolic and structural integrity of cells (Sung and Guy, 2003; Vierling, 1991; Wang *et al.*, 2004). Heat shock transcription factors (HSFs) have been found to primarily control the expression of HSP genes through the binding of the conserved heat-shock element (HSE) in the promoter region of these genes (Wu, 1995).

We previously isolated a DREB1-type transcription factor from maize (*Zea mays*; ZmDREB1A) by yeast one-hybrid screening (Qin *et al.*, 2004). Analysis of gene expression, phylogenetic studies and functional characterization of *ZmDREB1A* in transgenic plants demonstrated that it encodes a typical DREB1-type protein in maize. In the current study, we were interested to determine if a dehydration and salt stress signal transduction pathway mediated by DREB2-type transcription factor(s) also exists in this agriculturally important plant. We isolated a DREB2-type transcription factor ZmDREB2A from maize and identified its two transcription forms, *ZmDREB2A-L* and *ZmDREB2A-S*. Overexpression of *ZmDREB2A* in Arabidopsis resulted in an enhanced tolerance to drought stress. Microarray analyses of the 35S:ZmDREB2A transgenic plants revealed the upregulation of genes not only encoding late embryogenesis abundant (LEA) proteins, but also genes related to heat shock stress, detoxification and seed maturation. Overexpression of *ZmDREB2A* also resulted in thermotolerance in transgenic plants, suggesting that ZmDREB2A has a dual function in the expression of genes responsive to water and heat stress.

Results

Isolation of ZmDREB2A *and identification of its two transcription forms*

To determine whether DREB2-type transcription factors exist in maize, we performed a TBLASTN search with the amino acid sequence of the ERF/AP2 DNA-binding domain of Arabidopsis DREB2A. As a result, a new maize sequence was identified and found to potentially encode an ERF/AP2 DNA-binding domain (accession no. AY108198; http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Translation of the DNA according to the coding frame of the potential DNA-binding domain produced a putative protein containing 274 amino acid residues. Using a gene-specific primer pair designed from the AY108198 nucleotide sequence, we successfully obtained a 1034-bp fragment containing a coding sequence and a polyadenylation signal by using cold-, heat-, dehydration- or salt-treated maize cDNA samples. Multiple sequence alignment with DREB2A, OsDREB2A and other DREB2 proteins found that the putative protein did not contain a full DNA-binding domain and nuclear localization signal (NLS) which is normally found in the N-terminal of DREB proteins.

Subsequently six maize expressed sequence tag (EST) and mRNA sequences were identified in the UniGene database which potentially covered the 5′-end of this gene. Using a newly designed primer, in combination with the original AY108198 reverse primer, two different sized transcripts for this gene were amplified. These transcripts were subsequently named *ZmDREB2A-L* (accession no. AB218833) and *ZmDREB2A-S* (accession no. AB218832) according to their length. Both transcripts shared a 100% identity over a 1283-bp long nucleic acid sequence even on the 5′- and 3′-untranslated regions (UTRs). These transcripts only differed by 53 additional base pairs which were found in *ZmDREB2A-L* and lacking in *ZmDREB2A-S* (Figure 2c). *ZmDREB2A-S* was 1283 bp in length, encoding 318 amino acids, with a potential NLS and a typical ERF/AP2 DNA-binding domain (Figure 1a). In contrast, *ZmDREB2A-L* was 1336 bp long. Because of a premature termination caused by the 53-bp insertion and a frame shift *ZmDREB2A-L* encoded only 89 amino acids. This short peptide chain stopped before the ERF/AP2 DNA-binding domain and probably rendered it as a non-functional transcription form. Sequence alignment also showed that ZmDREB2A shared a homology not only within its DNA-binding domain but also in its C-terminal region with OsDREB2B (AK099221), HvDRF1 (AY223807) and PgDREB2A (AY829439) proteins (Figure 1a, dotted line). Thus, we named the protein encoded by *ZmDREB2A-S* as ZmDREB2A. As expected, phylogenetic study of the ERF/AP2 proteins according to the previous classification of this superfamily (Dubouzet *et al.*, 2003; Qin *et al.*, 2004; Sakuma *et al.*, 2002) assigned this protein to the DREB2 subgroup (Figure 1b). Two additional AP2/ERF transcription factors, DBF1 and DBF2, cloned in maize (Kizis and Pages, 2002), were classified to the A-4 and A-6 subgroup in DREB group proteins and share a high homology with RAP2.4 and TINY proteins, respectively.

Stress-inducible expression profiles of ZmDREB2A

The expression pattern of *ZmDREB2A* was examined in maize seedlings treated by cold (4°C), dehydration, salt (250 mM NaCl) and heat (42°C) stresses. Firstly, RNA gel blot analysis was used to study accumulation of *ZmDREB2A* mRNA in 7-day-old maize leaves, stems and roots, respectively. An accumulation of the *ZmDREB2A* mRNA was observed after 5 h of cold treatment in maize leaf, stem and root tissues. A relatively stronger induction was observed in root tissues during the cold treatment. Under dehydration stress, a rapid induction of *ZmDREB2A* was observed after 10 min which then remained unchanged in leaf tissue. In root tissue, *ZmDREB2A* expression was detected under normal conditions and was gradually upregulated. Under salt stress, *ZmDREB2A* was remarkably induced in root tissues up to 24 h, but in leaves and stems it was only slightly induced. Notably, a significant and transient induction of *ZmDREB2A* was observed with heat stress treatments in maize leaf, stem and root tissues. Subsequent to this induction, its mRNA rapidly became undetectable in stems 10 min after the induction and it gradually decreased in leaf and root tissues (Figure 2a). Exogenous application of ABA to maize seedlings did not result in the induction of *ZmDREB2A* (data not shown). Due to the small size difference of only 53 bp, these two fragments could not be clearly separated and distinguished by RNA gel blot analysis.

In order to verify the two kinds of transcription forms under stress conditions, a common pair of primers was designed according to the flanking sequence of the 53-bp insertion. These primers were then used in reverse transcription PCR (RT-PCR) with stress-treated maize cDNA templates. As shown in Figure 2(b), two kinds of transcript could be clearly identified under both heat and salt stresses. Under cold or dehydration stress *ZmDREB2A-S* was less induced than *ZmDREB2A-L*. Furthermore, we used quantitative real-time RT-PCR with two pairs of primers specific for either *ZmDREB2A-S* or *ZmDREB2A-L* in order to clarify the expression pattern of these two transcripts under different stresses (Figure 2c). Under cold stress, both kinds of transcripts displayed a similar pattern of induction, but *ZmDREB2A-L* appeared to be more abundant and increased to greater levels than *ZmDREB2A-S*. Under drought stress, both kinds of transcript appeared to be slightly increased. When the plants were treated by NaCl, both *ZmDREB2A-L* and *ZmDREB2A-S* were significantly upregulated to an equivalent amount. Upon exposure to a 42°C stimulus, both types of transcript displayed a transient induction pattern.

However, *ZmDREB2A-S* increased to a higher level within 10 min of the treatment (Figure 2d). Taken together, under normal conditions *ZmDREB2A-L* was present but *ZmDREB2A-S* transcripts were always undetectable. The expression of *ZmDREB2A-S* was only induced subsequent to stress treatments. Furthermore, the induction ratios of *ZmDREB2A-S* were always higher than *ZmDREB2A-L* under all stresses (Figure 2e). Quantitative analysis revealed that under heat or salt stress the amount of *ZmDREB2A-S* transcript was increased >100-fold compared with the non-stressed condition. On the other hand, the highest induction ratio of *ZmDREB2A-L* was obtained under cold or salt stress and was increased by only about 10-fold. These data seem to indicate that splicing mechanisms play an important role in regulating *ZmDREB2A* gene activity under stress conditions.

ZmDREB2A functions as a transcriptional activator and the region of amino acids 235–272 is important for its activation

Since only *ZmDREB2A-S* could be translated into a potential functional protein, in order to determine whether the ZmDREB2A protein was capable of transactivating DRE-dependent transcription in plant cells, we performed transactivation assays using Arabidopsis T87 protoplasts. A β-*glucuronidase* (GUS) gene driven by the trimeric 75-bp fragments containing the DRE sequence was used as a reporter (Figure 3a; Dubouzet *et al.*, 2003). As expected, transfection with *ZmDREB2A-L* could not activate GUS gene expression because it did not encode a functional DRE-binding protein. In contrast, the relative GUS/LUC activity was distinctly upregulated when *ZmDREB2A-S*, *DREB2A* (*DREB2A-wt*), or the constitutive active form of *DREB2A* (*DREB2A-CA*), were transfected in T87 protoplasts. Furthermore, the ZmDREB2A protein exhibited a rather high transactivation activity, which led to a 40.46-fold increase of GUS/LUC activity in comparison with the vector-only transfection control. In a parallel experiment, DREB2A-wt and DREB2A-CA protein resulted in 5.73- and 22.34-fold increased GUS/LUC activity, respectively (Figure 3b). These data suggested that ZmDREB2A functioned as a stronger transactivator than Arabidopsis DREB2A.

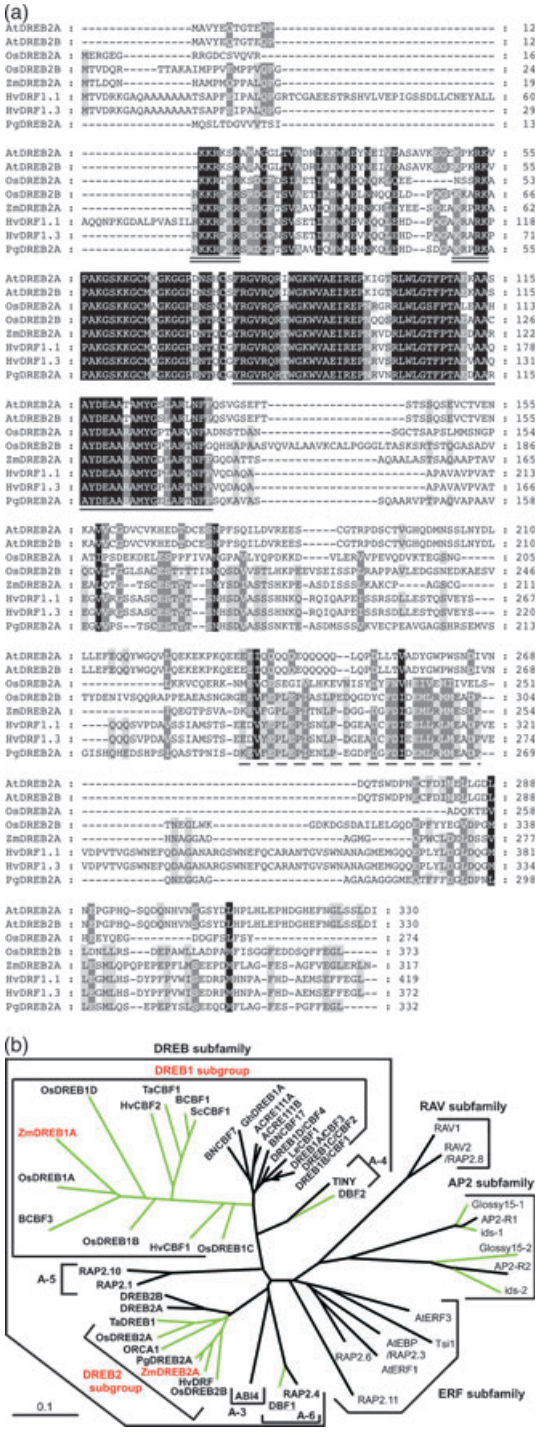

Figure 1. Sequence alignment of ZmDREB2A and DREB2 proteins from different species and phylogenetic analysis of the AP2/ERF DNA-binding domain of ZmDREB2A in the AP2/ERF supertranscription factor family.
(a) The entire protein sequences for DREB2A (AB007790), DREB2B (NM111939), OsDREB2A (AF300971), OsDREB2B (AK099221), ZmDRBE2A (AB218832), HvDRF1.1/1.3 (AY223807) and PgDREB2A (DQ227697) were aligned by the ClusterX program. The conserved amino acid residues among all sequences are highlighted with a black or grey background; nuclear localization signals, as predicted by psort (http://psort.nibb.ac.jp/) are double underlined; ERF/AP2 DNA-binding domains are underlined; and a conserved region in the potential activation domain among monocot DRBE2A proteins is indicated by a dashed line.
(b) A phylogenetic tree of the ERF/AP2 domains was constructed by ClustarX. The scale indicates branch lengths. A-1 to A-6 indicate subgroups proposed by Sakuma *et al.* (2002). The accession number of each appended protein is: *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) and *ids1* (AF048900). Genes belonging to the DREB subgroup are bold-faced; branches for genes from other monocot plants are designated in green. ZmDREB1A and ZmDREB2A were classified into the A-1 and A-2 subgroup, respectively. DBF1 (AF493800) and DBF2 (AF493799) from maize classified to the A-4 and A-6 subgroup and are underlined.

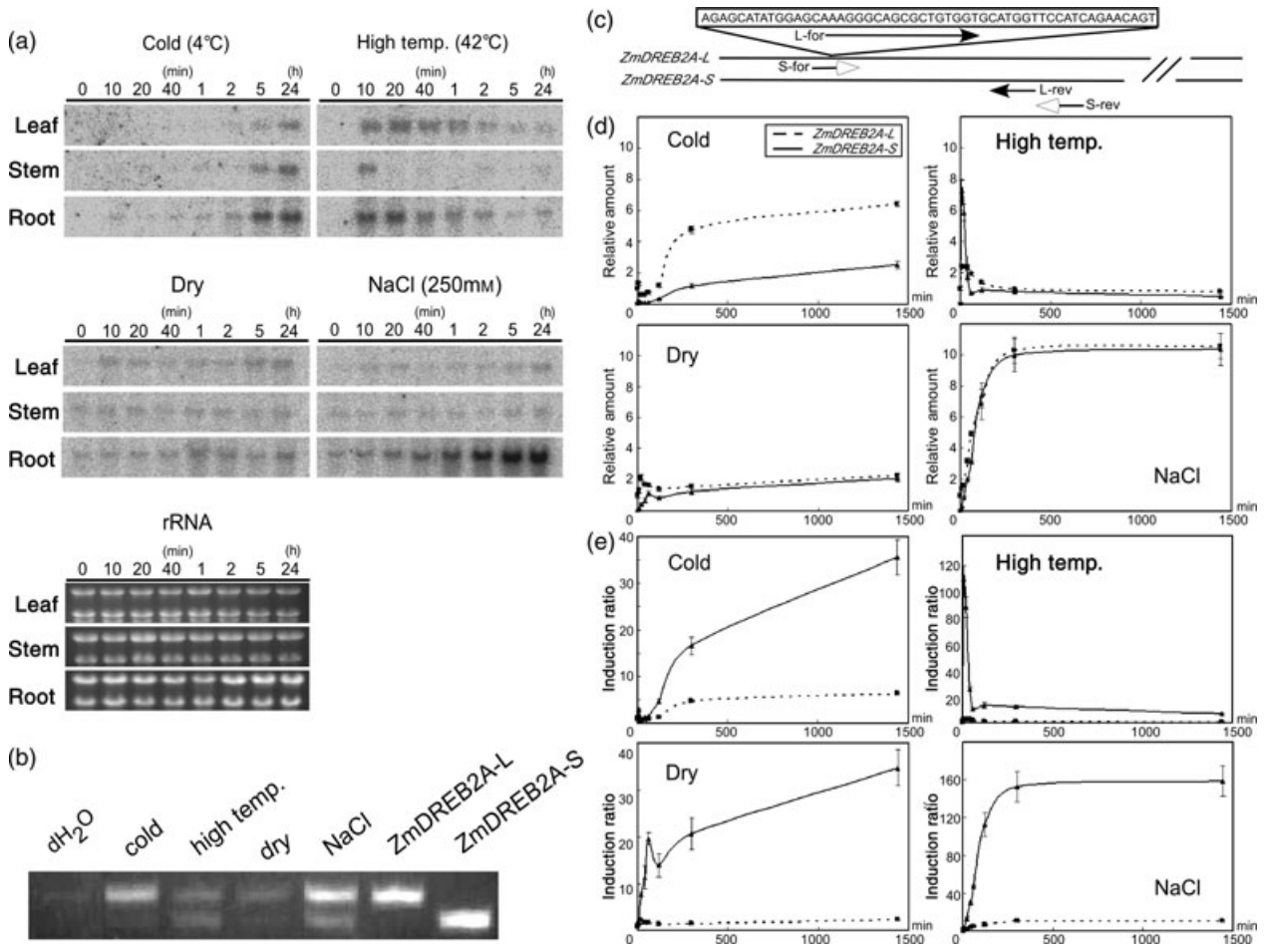

Figure 2. Expression analysis of *ZmDREB2A* under various stress conditions.
(a) Seven-day-old maize seedlings were treated by cold (4°C), heat (42°C), dehydration or NaCl (250 mM) for the time course indicated above each line. Ethidium bromide-stained total rRNAs were shown to indicate equal loading of samples.
(b) Seven-day-old maize seedlings treated by cold (4°C, 24 h), heat (42°C, 10 min), dehydration (1 h) and NaCl (250 mM, 24 h) were used for RNA preparation. As a negative control, dH_2O was used as template in reverse transcription and PCR. Plasmids containing *ZmDREB2A-L* or *ZmDREB2A-S* were amplified by the same primers to indicate the length of the two kinds of transcripts.
(c) A schematic diagram to indicate the position of two pairs of sequence-specific primers designed accordingly for *ZmDREB2A-L* and *ZmDREB2A-S*.
(d) Root samples from 7-day-old maize seedlings treated by cold (4°C), heat (42°C), dehydration or NaCl (250 mM), for indicated time courses, were used for quantitative real-time RT-PCR analysis. The relative amount of two kinds of transcripts was calculated, when the amount of *ZmDREB2A-L* was defined as 1.0. (Bars indicate the standard error from three individual experiments.)
(e) Root samples from 7-day-old maize seedlings treated by cold (4°C), heat (42°C), dehydration or NaCl (250 mM), for the indicated time courses, were used for quantitative real-time RT-PCR analysis. The induction ratios of two kinds of transcripts were calculated, when the amount of each transcript under control condition was defined as 1.0 respectively. Bars indicate the standard error from three individual experiments.

We performed mutational transactivation domain analysis for two reasons. First, we aimed to identify which region brought a high transactivation activity to the ZmDREB2A protein. We also wanted to determine whether the removal of any internal region would confer a higher transactivation activity to the ZmDREB2A protein. A series of C-terminal deletions of ZmDREB2A, ZmDREB2A (amino acids (aa) 1–272), ZmDREB2A (aa 1–253), ZmDREB2A (aa 1–208), ZmDREB2A (aa 1–191) and ZmDREB2A (aa 1–141) were constructed as effectors. As a result, removal of the zC-terminal end of the protein from aa 272 to aa 318 significantly reduced GUS/LUC activity. Further deletion from the C-terminal end to aa 236 completely abolished the ZmDREB2A transactivation capability by reducing GUS/LUC activity to 1.49-fold. As a positive control, a parallel experiment was conducted with ZmDREB2A protein and it always showed a high transactivation activity (Figure 3c). These data suggested that the C-terminal region from aa 236 to the C-terminal end was necessary for ZmDREB2A activity. In order to further localize its activation domain, an additional series of internal deletion fragments of ZmDREB2A was generated and constructed as effectors. Removal of the region of aa 236 to 272 greatly reduced its GUS/LUC activity. On the other hand, none of the other internal deletions

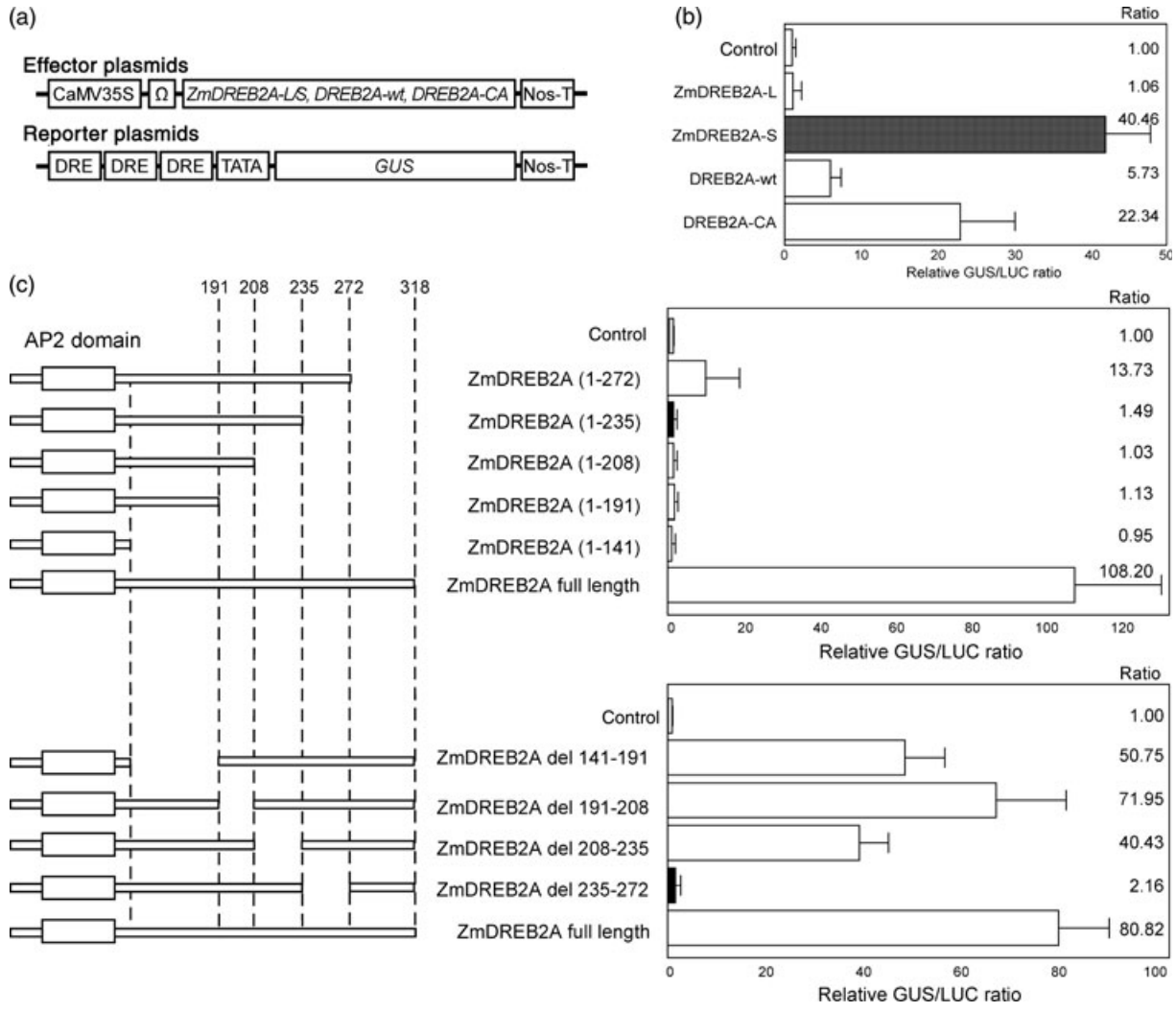

Figure 3. Transactivation domain analyses of ZmDREB2A in Arabidopsis T87 protoplasts.
(a) A schematic diagram of the effectors and reporter. Effectors were constructed with the enhanced CaMV 35S promoter and either *ZmDREB2A-L*, *ZmDREB2A-S*, *DREB2A-wt* or *DREB2A-CA* sequences. The reporter construct contained a three-tandem repeat of the DRE sequence and the GUS reporter gene (Dubouzet *et al.*, 2003).
(b) Protoplasts were co-transfected with the reporter plasmid and different effector constructs (indicated on the left). *35S-LUC* constructs were used as a control for transfection efficiency. Transactivation activity of each construct was given as a GUS/LUC ratio, when GUS/LUC activity of the empty vector was defined as 1.0. Average and standard deviation were given from six replicates.
(c) Domain analysis of the ZmDREB2A protein. Two series of C-terminal deleted fragments and internal deletions (as indicated by numbers of amino acid residues) were constructed as effectors (shown on the left). The open boxes indicated the AP2/ERF DNA-binding domain. Transactivation activity of each effector was obtained as described in (b).

showed such a critical effect to ZmDREB2A transactivation activity and these constructs still possessed at least 50% activity of the full-length protein of ZmDREB2A (Figure 3c). These data suggested that unlike Arabidopsis DREB2A, any internal deletions of ZmDREB2A could not enhance the protein transactivation activity, and the region of aa 236 to 272 might be essential for the high transactivation capability of ZmDREB2A. Interestingly, multisequence alignment of ZmDREB2A, OsDREB2B, HvDRF1.1, HvDRF1.3 and PgDREB2A proteins revealed that they all shared a high homology near this region (Figure 1a, dotted line); however, Arabidopsis DREB2A, DREB2B and rice OsDREB2A proteins did not.

Functional analysis of ZmDREB2A in planta

Previous studies showed that overexpressing Arabidopsis DREB2A protein hardly led to obvious morphological or physiological changes to transgenic plants (Liu *et al.*, 1998). As a result, we were curious to determine whether ZmDREB2A functions directly *in planta*, or whether modification is necessary for its activation. In order to answer this question, we generated transgenic Arabidopsis overproducing this protein by using an enhanced *CaMV35S* promoter (Gallie *et al.*, 1987; Mitsuhara *et al.*, 1996). Fifty-one independent transgenic lines were obtained by

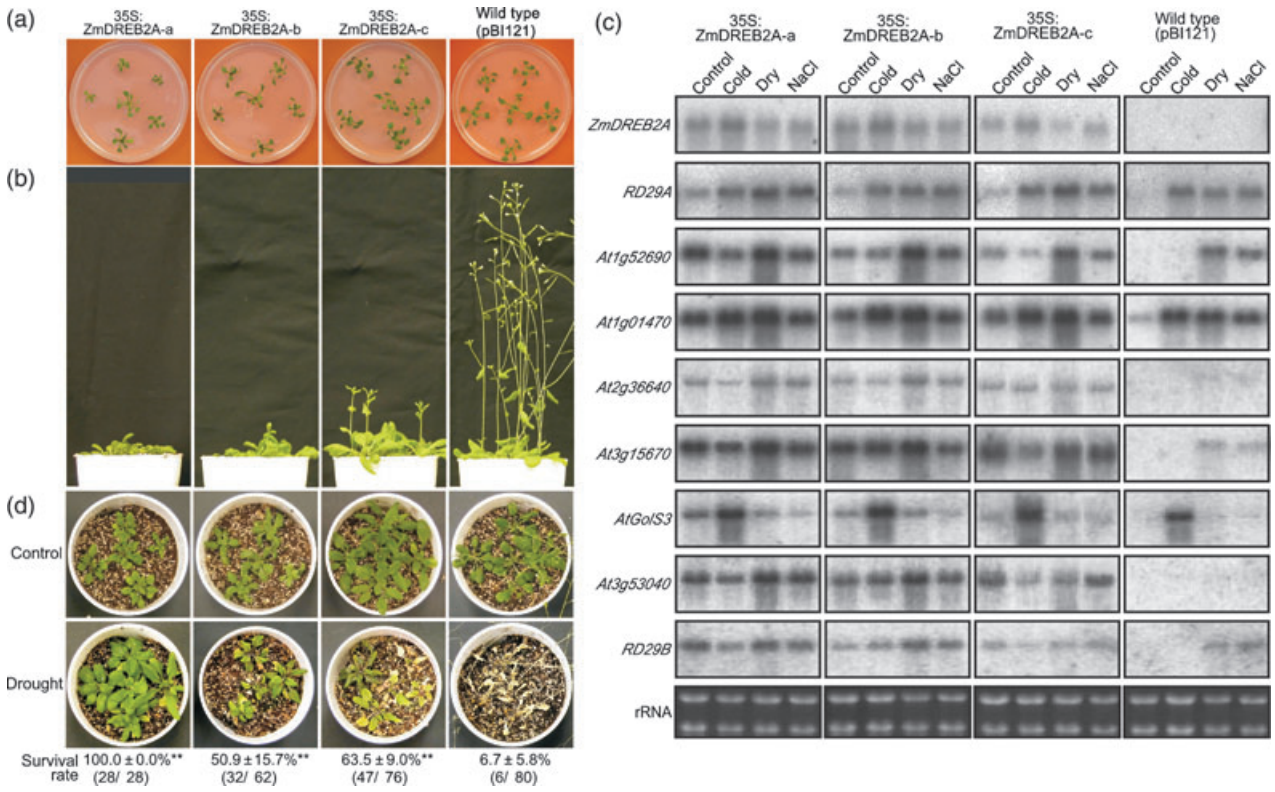

Figure 4. Phenotype, stress tolerance and gene expression analyses of the *35S:ZmDREB2A* transgenic plants.
(a) Three-week-old *35S:ZmDREB2A* plants of lines a, b, c and wild type (transformed by pBI121 empty vector) growing on selective germination medium (GM).
(b) Plants were transferred to pots and grown for two additional weeks.
(c) Ribonucleic acid gel blot analysis of transgene and some target gene expressions in the *35S:ZmDREB2A-a*, *35S:ZmDREB2A-b*, *35S:ZmDREB2A-c* and wild-type plants under normal, cold (4°C, 5 h), dehydration (5 h) and NaCl (250 mM, 5 h) conditions. Five micrograms of total RNA was loaded in each lane and ethidium bromide-stained total rRNAs were shown to indicate an equal loading of samples.
(d) Drought tolerance tests of the *35S:ZmDREB2A* plants. *35S:ZmDREB2A-a, 35S:ZmDREB2A-b, 35S:ZmDREB2A-c* and wild-type plants were investigated as described in the methods. Control, 4-week-old plants growing under normal conditions. Drought, water withheld from 4-week-old plants for 10 days; photographs were taken after rewatering for 7 days. Survival rates are indicated under the photographs. Means and SDs were obtained from three independent experiments. The plants with asterisks had significantly higher survival rates than the wild-type plants (χ^2-test, $*P < 0.05$, $**P < 0.001$).

kanamycin selection and expression levels of *ZmDREB2A* in each T_2 line were analyzed by RNA gel blot analysis. Transgenic plants displayed reduced rosette leaves and delayed bolting time in comparison to wild-type plants (transformed by the empty vector pBI121; Figure 4a, b). Reduction of rosette leaves and delay of bolting was dependent upon transgene expression levels (data not shown). This kind of phenotype was similar to those previously described for plants overexpressing DREB1A (Kasuga *et al.*, 1999; Liu *et al.*, 1998) and DREB2A-CA (Sakuma *et al.*, 2006a,b). Three comparatively high-expression lines, 35S:ZmDREB2A-a, 35S:ZmDREB2A-b and 35S:ZmDREB2A-c, were chosen for further analysis. Since *RD29A* has been identified as a target gene of the DREB1A and DREB2A proteins (Liu *et al.*, 1998; Sakuma *et al.*, 2006a,b), its expression level was studied in three lines under normal, dehydration and salt stresses. In all the lines, *RD29A* expression was elevated under non-stressed conditions. The level of expression of *RD29A* was correlated with that of *ZmDREB2A* (Figure 4c), indicating that the ZmDREB2A protein directly upregulated *RD29A* gene expression. In our next line of investigation, we carried out drought stress tolerance tests on these transgenic plants. Three-week-old plants grown on agar plates were transferred to soil and grown for an additional week. Water was subsequently withheld for a period of 10 days prior to rewatering. Only 6.7% of the wild-type plants survived under this condition, but 100% of the 35S:ZmDREB2A-a plants, 50.9% of the 35S:ZmDREB2A-b plants and 63.5% of the 35S:ZmDREB2A-c plants survived (Figure 4d). These data clearly demonstrate that overexpression of *ZmDREB2A* results in an enhanced drought tolerance to transgenic plants and suggest that ZmDREB2A is a fully functional protein *in planta*.

Microarray analysis of plants overexpressing ZmDREB2A

We were interested in assigning a function to ZmDREB2A and to understand the relationship between alteration of gene expression and stress tolerance. We therefore employed microarray analysis for *35S:ZmDREB2A-a* and

35S:ZmDREB2A-c plants using an Agilent Arabidopsis 2 Oligo Microarray (Agilent Technologies, http://www.agilent.com/) which covers >21 000 Arabidopsis genes. Two experiments were performed for each line using different labels, Cy3 or Cy5. A complete data set is available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-819. When using a statistical cutoff ($P < 0.001$, intensity >1000), 44 genes with increasing ratios above sevenfold were categorized into several groups according to putative gene functions (Table 1). Nine genes encoded LEA proteins, which were previously found to play a protective role in plant cells especially in response to desiccation and salt stresses. Among these LEA genes, seven contained DRE (A/GCCGAC) sequences in their promoters. According to the microarray results of 3-week-old Arabidopsis plants, four genes were identified to be inducible by dehydration or high salinity stress and one gene was inducible by cold (KM & KY-S unpublished data; Seki *et al.*, 2002).

Notably, four genes that are responsive to heat shock stress were increased above sevenfold in the 35S:ZmDREB2A plants. One of them, *At5G03720*, encoding a heat shock transcription factor-like protein (AtHsfA3), contains two DRE sequences in the promoter. Moreover, considering threefold upregulated genes by ZmDREB2A, among the total of 152 upregulated genes, 31 genes were shown to be inducible by heat stress (Table S1). Since *ZmDREB2A* was also shown to be transiently induced by heat stress (Figure 2), these data collectively suggest that it may function under heat stress by regulating heat shock-inducible gene expression in plants. In addition, two genes implicated in plant detoxification and four genes encoding seed proteins were also upregulated, but none of them contain the DRE sequence in their promoters. The lack of the DRE sequence implied that these genes might be indirectly controlled by this protein.

Upregulated expression was also observed for 10 genes implicated in metabolism. Six of these genes contain DRE sequences in their promoters, suggesting that various metabolites were also affected by overexpression of *ZmDREB2A*. Four genes with functions related to protein synthesis or degradation were found to be upregulated. Expression of another 11 unknown genes was also found to be increased. Seven of these unknown genes possess the DRE sequence in their promoters, and five of them were identified to be stress-inducible. Genes upregulated by ZmDREB2A and DREB2A-CA were also compared, and 28 of 44 genes were upregulated by both proteins (Sakuma *et al.*, 2006b). Ribonucleic acid gel blot analysis was carried out in three transgenic lines as a means to validate the microarray results. Five LEA genes, *At1g52690*, *LEA14*, *At2G36640*, *At3G15670*, *At3G53040* and a gene involved in metabolism, *AtGolS3*, were chosen for this analysis. As shown in Figure 4(c), all of these gene expressions were clearly upregulated under normal condition in the transgenic lines and under stress conditions their expression was also apparently higher than that of wild type. Since the *RD29B* gene is a target gene of the Arabidopsis DREB2A protein and it was also found to be upregulated 5.5-fold by ZmDREB2A referring to the microarray analysis the increased expression level of this gene was also confirmed by Northern analysis.

Stress-inducible expression of ZmDREB2A *enhanced drought stress tolerance and avoidance of dwarfism*

To minimize a negative effect of *ZmDREB2A* overexpression on plant growth, we made use of a stress-inducible *RD29A* promoter (Kasuga *et al.*, 1999) and used this to overexpress Zm*DREB2A* in Arabidopsis. Under the control of *RD29A* promoter, *ZmDREB2A* was introduced into Arabidopsis and 62 antibiotic-resistant transgenic lines were obtained. Almost all of the *RD29A:ZmDREB2A* plants grew normally as wild type. Three lines, *RD29A:ZmDREB2A-a, RD29A:ZmDREB2A-b* and *RD29A:ZmDREB2A-c*, were chosen for further analysis. With the exception that the *RD29A:ZmDREB2A-a* plants showed a slightly reduced size at rosette stage (data not shown), no obvious growth retardation was observed for the transgenic lines (Figure 5a,c). Ribonucleic acid gel blot analysis confirmed that the stress-inducible *RD29A* promoter could effectively induce *ZmDREB2A* expression by cold and drought stresses (Figure 5b). To verify that the transgenic plants exhibited enhanced drought tolerance, we carried out a drought tolerance test as described above. As a result, 30% of wild-type plants survived after water was withheld for 10 days, and the survival ratio of the *RD29A:ZmDREB2A-a*, *RD29A:ZmDREB2A-b* and *RD29A:ZmDREB2A-c* plants was 96.3, 88.8% and 81.3% respectively (Figure 5e). Moreover, freezing tolerance tests were also carried out on these transgenic lines. Four-week-old plants were treated under −6°C for 30 h and survival rates were calculated after plants recovered under normal condition for 5 days. As a result, 37.8%, 35.6% and 33.3% of the *RD29A:ZmDREB2A-a*, *RD29A:ZmDREB2A-b* and *RD29A:ZmDREB2A-c* plants, respectively, could grow normally after recovery. In the same experiment, 28.9% of the wild-type plants survived (Figure 5d,f). No remarkable difference could be observed between these transgenics and wild type. Similar results were also obtained in the *35S:DREB2A-a*, *35S:DREB2A-b* and *35S:DREB2A-c* plants (data not shown). These data indicate that ZmDREB2A apparently plays an important role in the plant's tolerance of drought stress but not in freezing tolerance.

Overexpression of ZmDREB2A enhanced plant thermotolerance

Expression analysis of *ZmDREB2A* showed that it was transiently and significantly induced by heat stress, and the microarray result indicated it may upregulate expression of some heat shock-inducible genes in plants. To determine

Table 1 Genes 7-time Upregulated in 35S:ZmDREB2A Plants, Identified by Microarray Analysis

AGI code	Description[a]	Median of fold change[b]	Inducibility[c]				DREB2A[d]	No. of DREs[e]
LEA protein								
AT3G53040	Late embryogenesis abundant protein - like	22.4	–	–	–	–		1
AT1G52690	Late embryogenesis-abundant protein, putative	21.1	D	S	–	–	+	1
AT2G36640	Late embryogenesis abundant protein (AtECP63)	19.2	–	–	–	–		–
AT3G15670	LEA76 homologue type2	18.0	D	–	–	–	+	3
AT2G40170	ABA-regulated gene (ATEM6)	14.6	–	–	–	–		–
AT5G06760	Late embryogenesis abundant protein LEA like	8.6	D	S	–	–		1
AT1G01470	LEA14	8.3	D	S	C	–	+	3
AT2G35300	Similar to late embryogenesis abundant proteins	8.1	–	–	–	–		1
AT1G32560	Late-embryogenesis abundant protein, putative	7.2	–	–	–	–		1
Heat shock responsive								
AT1G52560	Chloroplast-localized small heat shock protein, putative	18.6	–	–	–	H	+	(–)
AT5G03720	Heat shock transcription factor -like protein (AtHsfA3)	14.2	–	S		H	+	2
AT3G17790	Acid phosphatase type 5	7.5	–	–	–	H	+	–
AT3G12580	Heat shock protein 70	7.1	D	S	–	H	+	1
Detoxification								
AT1G49570	Peroxidase, putative	13.3	–	–	–	–	+	–
AT1G48130	Peroxiredoxin	7.4	–	–	–	–		–
Seed protein								
AT5G40420	Oleosin	26.0	–	–	–	–		–
AT3G27660	Oleosin	15.9	–	–	–	–	+	–
AT3G01570	Oleosin	13.1	–	–	–	–		–
AT5G39150	Germin-like protein	8.2	–	–	–	–	+	(–)
Protein fate								
AT4G36880	Cysteine proteinase	22.2	–	–	–	–	+	1
AT1G33540	Serine carboxypeptidase, putative	15.1	–	–	–	–	+	(–)
AT1G43780	Serine carboxypeptidase II, putative	8.2	–	–	–	–		(1)
AT3G54940	Cysteine proteinase	7.4	–	–	–	–		(–)
Enzymes in metabolism								
AT4G37990	Cinnamyl-alcohol Dehydrogenase ELI3-2	49.2	D	S	–	–	+	2
AT1G09350	Putative galactinol synthase (AtGolS3)	18.7	D	S	C	–	+	3
AT5G03860	Malate synthase -like protein	18.0	–	–	–	–		–
AT3G21720	Putative isocitrate lyase	12.2	–	–	–	–		1
AT5G42800	Dihydroflavonol 4-reductase	10.0	–	–	–	–	+	(–)
AT2G38530	Putative nonspecific lipid-transfer protein	8.7	D	–	–	–	+	2
AT3G55940	Phosphoinositide specific phospholipase C, putative	8.5	–	–	–	–	+	(1)
AT1G34580	Monosaccharide transporter, putative	7.9	–	–	–	–	+	3
AT4G22870	Leucoanthocyanidin dioxygenase, putative	7.7	–	–	–	–		(–)
AT3G10450	Putative glucose acyltransferase	7.6	–	–	–	–	+	(–)
Unknown protein								
AT3G02480	Expressed protein	46.0	D	S	–	–	+	1
AT3G17520	Unknown protein	37.5	D	S	–	–	+	1
AT1G17710	Hypothetical protein	25.3	–	–	–	–	+	(–)
AT4G21020	Putative protein	19.2	–	–	–	–	+	2
AT4G25580	Putative protein	15.7	–	S	–	–		–
AT5G20790	Putative protein	14.5	–	–	C	–	+	(3)
AT3G02040	Expressed protein	13.2	D	–	–	–		–
AT5G23220	Putative protein	11.3	–	–	–	–	+	1
AT1G23110	Unknown protein	11.2	–	–	–	–	+	(–)
AT4G25850	Putative protein	7.9	–	–	–	–	+	2
AT5G64080	Putative protein	7.6	–	–	–	–	+	1

[a]Descriptions as given by The Institute for Genomic Research database.
[b]Median values of fold change (Intensity of ZmDREB2A OX / vector) from two experiments.
[c]Data on inducibility were base on microarray analysis (Seki et al., 2002; Maruyama et al., unpublished data). D, drought (2 or 10h); S, high salinity (2 or 10h).; C, cold (2 or 10h); H, heat (0.5 or 5h); –, no induction.
[d]Genes upregulated by DREB2A-CA above 2-time (without restriction of intensity) (Sakuma et al., submitted).
[e]DRE sequences (A/GCCGAC) observed in 1000 nucleotides existing upstream of the full-length cDNA clones (http://rarge.gsc.riken.go.jp/). Numbers indicated the copies of DRE sequence. "()" indicated the full-length cDNA information of this gene was not available, and number of DRE sequence was decided from 1000 nucleotides upstream of start coden (http://www.arabidopsis.org/). "-" indicated no DRE sequence found in the sequence.

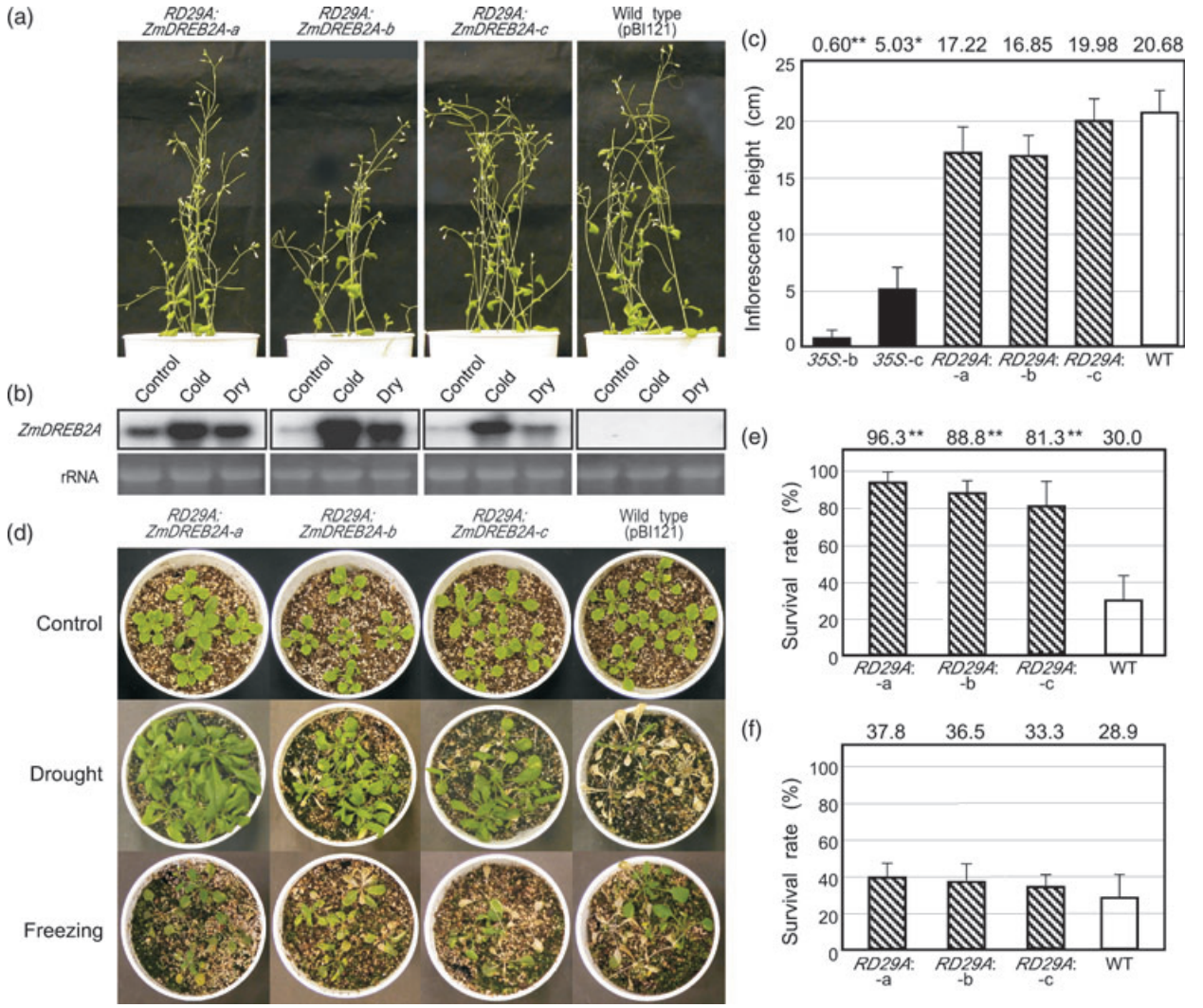

Figure 5. Phenotype and stress tolerance of the *RD29A:ZmDREB2A* transgenic plants.
(a) Photographs of wild-type and transgenic plants of *RD29A:ZmDREB2A-a*, *RD29A:ZmDREB2A-b* and *RD29A:ZmDREB2A-c*. The plants were grown on GM selective agar plates for 3 weeks and then transferred to soil and grown for 2 weeks.
(b) Ribonucleic acid gel blot analyses of ZmDREB2A expression under normal conditions (control), cold (4°C, 5 h) and dehydration (5 h). Five micrograms of total RNA was loaded in each lane and ethidium bromide-stained total rRNAs were shown to indicate an equal loading.
(c) Inflorescence height of 35-day-old *35S:ZmDREB2A-b*, *35S:ZmDREB2A-a*, *RD29A:ZmDREB2A-a*, *RD29A:ZmDREB2A-b* and *RD29A:ZmDREB2A-c* plants. Means and SDs were obtained from 20 plants for each line. The plants with asterisks were significantly different from the wild-type plants (χ^2-test, $*P < 0.05$, $**P < 0.001$).
(d) Drought and freezing tolerance of the *RD29A:ZmDREB2A* transgenic plants. *RD29A:ZmDREB2A-a*, *RD29A:ZmDREB2A-b*, *RD29A:ZmDREB2A-c* and wild-type plants were investigated as described in the methods. Control, 4-week-old plants growing under normal conditions. Drought, water withheld from 4-week-old plants for 10 days; photographs were taken after rewatering for 7 days. Freezing, 4-week-old plants exposed to a temperature of −6°C for 30 h; photographs were taken after freeze-stressed plants were returned to 22°C for 5 days.
(e, f) Survival rates. Means and SD were obtained from three independent experiments. Plants with asterisks had significantly higher survival rates than the wild-type plants (χ^2-test, $*P < 0.05$, $**P < 0.001$).

whether transgenic plants overexpressing *ZmDREB2A* had an improved tolerance to heat stress, we investigated the thermotolerance of the 35S:ZmDREB2A plants. Six-day-old plants germinating on selective agar plates were transferred to two layers of paper which was pre-moistened by liquid germination medium to avoid a dehydration effect. After conditioning the plants under 22°C for 2 days, wild-type and transgenic plants were subjected heat shock treatment at 44°C or 45°C. As shown in Figure 6(a) and (b), three transgenic lines, 35S:ZmDREB2A-a, b, c, and wild-type plants could grow very well under 22°C, but when the plants were exposed to 44°C for 1 h, about 85% of wild-type plants survived. The survival ratio of *35S:ZmDREB2A-a, 35S:ZmDREB2A-b* and *35S:ZmDREB2A-c* was 100%, 96% and 95% respectively. When the plants were exposed at 45°C for 1 h, only 2.4% of the wild-type plants survived, whereas 82% of 35S:ZmDREB2Aa; 48% of 35S:ZmDREB2Ab and 18% of 35S:ZmDREB2Ac survived during the subsequent 2-week

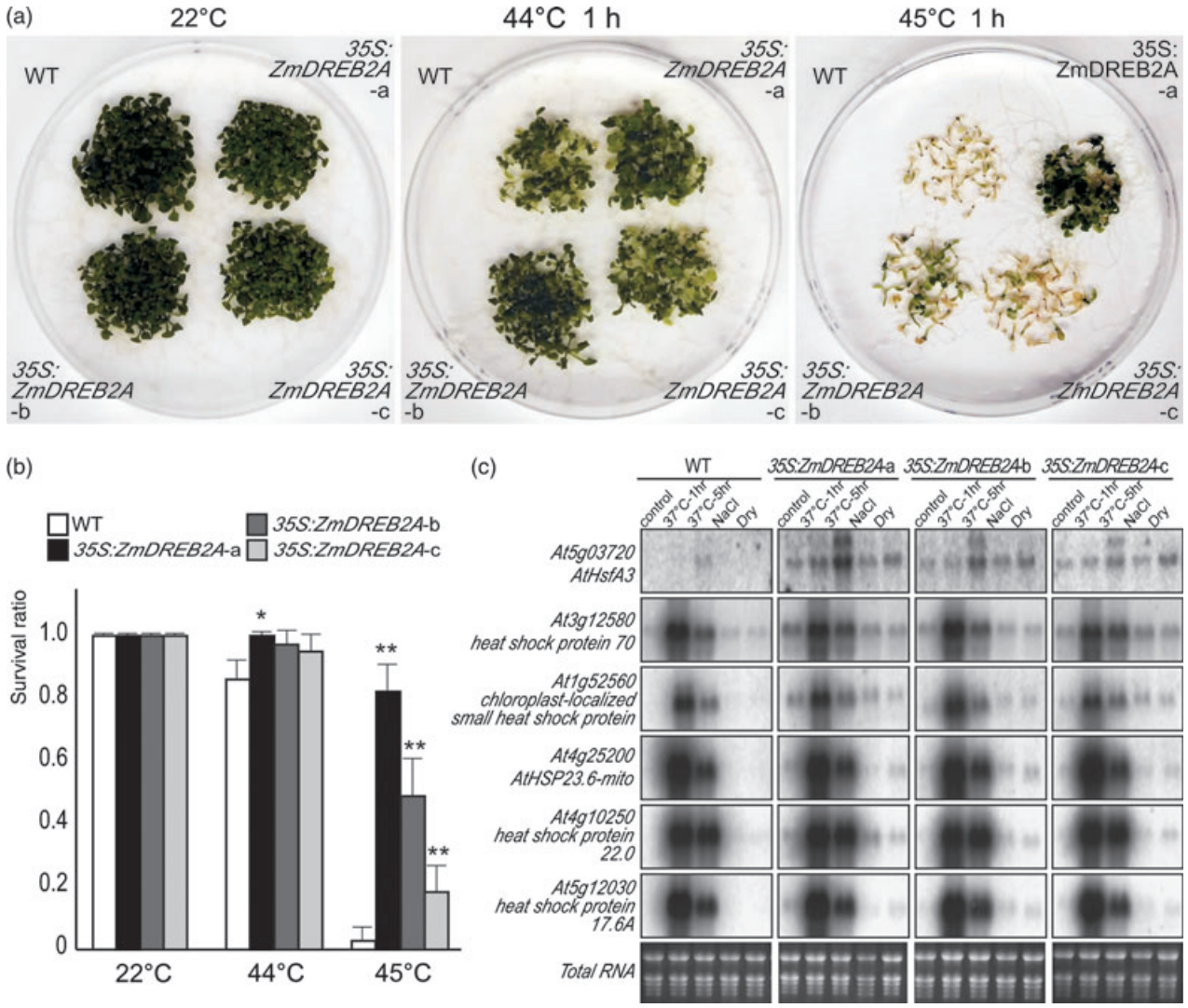

Figure 6. Thermotolerance and heat shock responsive gene expression in the *35S:ZmDREB2A* and wild-type plants.
(a) Six-day-old plants germinated on selective GM plates were transferred onto filter papers moistened with 4 ml GM in Petri dishes. After being maintained at 22°C for 2 days, plants were immediately exposed at 44°C or 45°C for 1 h and then returned to 22°C for recovery. Photographs show the representative results of plants after two further weeks of cultivation with liquid GM.
(b) Plant survival ratio of thermotolerance of (a). Mean and SD were obtained from three independent experiments. The plants with asterisks had significantly higher survival rates than the wild-type plants (χ^2-test, $*P < 0.05$, $**P < 0.001$).
(c) Ribonucleic acid gel blot analyses of heat shock-inducible gene expression under normal conditions (control), heat shock (37°C, 1 h or 37°C, 5 h), NaCl (250 mM 5 h) and dehydration (5 h) in wild-type and transgenic 35S:ZmDREB2A-a, b, c plants. Five micrograms of total RNA was loaded in each lane and ethidium bromide-stained total rRNAs are shown to indicate equal loading of samples.

recovery at 22°C. The plants which survived appeared to be developmentally delayed in comparison with the plants growing under normal conditions. The level of thermotolerance was observed to be consistent with the expression level of *ZmDREB2A*. Referring to microarray results, some heat shock-inducible genes were chosen for Northern blot analysis and were tested in both transgenic and wild-type plants. *AtHSFA3*, a heat shock transcription factor-like gene, was undetectable under non-stressed conditions but induced by exposure to 37°C for 5 h in wild type. In all three transgenic lines, this particular gene became constitutively expressed under non-stressed conditions and its expression level was actually higher than in the wild-type plants after heat shock. Heat shock protein (HSP) genes, such as *HSP70*, *HSP 22.0*, *HSP 17.6*, *AtHSP23.6-mito* and *Chloroplast-localized small HSP*, were also expressed under normal conditions in the 35S:ZmDREB2A-a plants, whereas in the wild-type plants their expression was undetectable (Figure 6c). The enhanced thermotolerance in transgenic plants was relevant to *HSP* gene expression. However, expression levels of the *HSP* genes under non-stressed conditions remained apparently lower than those detected after heat shock treatment. These

data imply that the production of HSP prior to heat stress may be important to the increased thermotolerance of transgenic plants. Additionally, we could not observe inductions of these HSP genes under NaCl (5 h) or dehydration (5 h) treatment in the wild-type plants (Figure 6c), implying that although the ZmDREB2A protein could affect expression of HSP genes, a heat shock signal was required for highly activating the expression of HSP genes.

Discussion

Unlike DREB1/CBF-type transcription factors, the functional regulation of DREB2-type transcription factors seems to be more variable and complicated. Expression of intact Arabidopsis DREB2A does not activate downstream genes under normal growth conditions. The DREB2A protein, unlike DREB1A, was considered to require post-translational modification in order for it to become activated (Liu *et al.*, 1998; Sakuma *et al.*, 2006a,b). Similar to the results from Arabidopsis, overexpression of the rice ortholog *OsDREB2A* is also not sufficient to activate downstream gene expression (Dubouzet *et al.*, 2003). As a matter of fact, there has not been any clear evidence directly demonstrating to date that intact DREB2-type proteins function in plant stress responses. In this study, we described the isolation and characterization of a DREB2-type transcription factor from maize, named ZmDREB2A. We identified two kinds of transcripts for this gene which were designated as *ZmDREB2A-L* and *ZmDREB2A-S*. Overexpression of *ZmDREB2A-S* showed an enhanced drought stress tolerance and microarray analyses revealed that many stress-inducible genes were upregulated in the transgenic plants under non-stressed control conditions. These data clearly indicate that intact ZmDREB2A can function as a transcriptional activator and does not require post-translational modification. They contrast with the previously characterized DREB2A proteins both in Arabidopsis (DREB2A) and in rice (OsDREB2A).

ZmDREB2A-L contained a 53-bp insertion in contrast with *ZmDREB2A-S*. This insertion resulted in a frame shift and a premature stop in translation and only encoded 89 amino acids. Barley *HvDRF1* and wheat *TaDRF1* have a similar alternative splicing pattern and produce three kinds of transcription forms, two of which could be functionally translated into a DREB2-type transcription factor (Xue and Loveridge, 2004). In the case of maize, *ZmDREB2A* possesses two kinds of transcription forms, with only one functional form. We searched the maize database. All EST or mRNA sequences sharing homology with *ZmDREB2A* were analyzed, but none of them presented a new kind of transcript. Only *ZmDREB2A-L* could be found, and prior to this report the *ZmDREB2A-S* transcript had never been previously identified. Interestingly, the position and structure of this 53-bp fragment in *ZmDREB2A-L* resembled E2 of *HvDRF1.2.*

When *ZmDREB2A-S* was overexpressed in plants, the transgenic plants showed dwarfism and improved tolerance to drought stress. However, transgenic plants overexpressing Arabidopsis *DREB2A* did not show any obvious phenotypic changes or improvement of tolerance to dehydration stress (Liu *et al.*, 1998). Recent results showed DREB2A to be unstable under non-stressed conditions, and modification of this protein may be necessary to stabilize it (Sakuma *et al.*, 2006a,b). Using GENETYX 6.0 software, we searched for a region rich in proline, glutamic acid, serine and threonine residue (PEST) sequence but we were unable to clearly detect a similar sequence in the ZmDREB2A protein (Rechsteiner and Rogers, 1996). Moreover, transactivation domain analysis did not find any negative regulation domain in ZmDREB2A. Unlike Arabidopsis DREB2A protein, ZmDREB2A may be stable without modification in cells even under unstressed conditions. Stress-induced splicing of the *ZmDREB2A* mRNA implies that regulation of *ZmDREB2*A activity may happen before translation. Overexpression of *ZmDRE2A-L* in plants might be helpful for exploring the mechanism of how a stress signal generates the functional form of *ZmDREB2A-S*.

Microarray analyses revealed that nine genes encoding LEA proteins were upregulated more than sevenfold in two independent transgenic lines. Seven of these genes contained the DRE sequence(s) in their promoters and four of them were identified to be stress inducible. Late embryogenesis abundant proteins are produced in abundance during the late stages of embryo development (Hughes and Galau, 1989) and many of them are induced by cold, osmotic stress and exogenous applications of ABA in vegetative tissues (Welin *et al.*, 1994). Although the precise function of these hydrophilic LEA proteins is still unknown, several reports have suggested that LEA proteins play a role in counteracting the crystallization of cellular components or the irreversibly damaging effects of increasing ionic strength which is induced by water deficit (Ingram and Bartels, 1996; Thomashow, 1999; Zhu, 2001). Overexpression of *DREB1A, DREB2A-CA* or the activated form of *AREB1* (Fujita *et al.*, 2005; Maruyama *et al.*, 2004; Sakuma *et al.*, 2006a) in Arabidopsis resulted in upregulation of some LEA gene expression and all these transgenic plants displayed an improved tolerance to drought stress. These observations imply that LEA proteins do function in tolerance of water deficit.

Freezing tolerance tests showed that neither *RD29A:ZmDREB2A* plants (Figure 5c) nor *35S:ZmDREB2A* plants showed an improved tolerance to freezing stress (data not shown). According to microarray analyses, this may be due to low induction of several cold-inducible genes such as *KIN1*, *KIN2*, *COR15A* and *COR15B* in the transgenic plants. Likewise, overexpression of Arabidopsis DREB2A-CA also failed to improve freezing tolerance in transgenic plants (Sakuma *et al.*, 2006a,b). We have shown that DREB1A and

DREB2A exhibit different DNA-binding specificities by using promoter analysis of the DREB1A- and DREB2A-regulated genes and gel mobility shift assay of both recombinant proteins. DREB1A has the highest affinity to A/GCCGACNT, whereas DREB2A preferentially binds ACCGAC. It is likely that this difference controls the induction of different downstream genes between DREB1A and DREB2A (Sakuma *et al.*, 2006a,b). Although the DREB2A-regulated genes have important roles in tolerance of drought stress, they are not sufficient to withstand freezing stress. This kind of DNA-binding preference was also observed for the ZmDREB2A protein. Among 44 upregulated genes, 24 genes contained at least one DRE sequence in their promoters. Eighteen out of these 24 genes (75.0%) contain ACCGAC sequence(s) in the promoters, whereas 10 genes (41.7%) have GCCGAC sequence(s). Moreover, we also analyzed the promoter region for the top 100 upregulated genes and found that the ACCGAC sequence was the most significant.

Expression analysis of *ZmDREB2A* showed that this gene was transiently and significantly induced by heat stress. Microarray analysis revealed that four heat shock-related genes were upregulated more than sevenfold (Table 1) in the *35S:ZmDREB2A* plants. Among the upregulated genes, a gene encoding a heat shock transcription factor, *AtHsfA3* (*At5g03720*) was undetectable under normal conditions and was induced by heat stress treatment in the wild-type plants (Figure 6c). In contrast, in the *35S:ZmDREB2A* plants, *AtHsfA3* was constitutively expressed under normal conditions (Figure 6c). Promoter analysis indicated that this gene contains two DRE sequences in its promoter region, suggesting that *AtHsfA3* is a direct target of ZmDREB2A. The altered expression of *AtHsfA3* in the *35S:ZmDREB2A* plants may result in some *HSP* gene expression even under non-stressed condition (Figure 6c). However, the enhanced expression level of the *HSP* genes under non-stressed conditions was comparably lower than that after heat shock treatment. Similar results have been reported using transgenic plants overexpressing other *HSF* genes such as *HSF1* and *HSF3*. These data suggest that specific activation mechanisms of HSF proteins may exist under heat stress conditions. AtHsfA3 is class A HSF, based on the presence of the conserved DNA-binding domain and the adjacent oligomerization domain HR-A/B (characterized by the heptad pattern of hydrophobic residues; Nover *et al.*, 2001). In Arabidopsis, 21 HSF have been identified, which exceeds the number in vertebrates (four HSFs), *Drosophila* (one HSF) and yeast (one HSF and three HSF-related proteins). This observation implies that a more complex HSF regulation system exists in plants. Heat shock transcription factors are the essential transcription factors for the heat induction of many *HSP* genes, but their regulation of the heat shock response is complicated and not yet understood. Recently, it was reported that *DREB2A-CA* overexpression also enhanced thermotolerance in transgenic Arabidopsis (Sakuma *et al.*, 2006a,b), supporting the notion that DREB2-type transcription factors function in the heat stress response.

Ectopic expression of *ZmDREB2A* also upregulated some genes that function in detoxification and seed maturation, but none of them contained a DRE sequence in their promoter (Table 1). These data indicate that the expression of these genes might be indirectly affected by the ZmDREB2A protein. Detoxification enzymes are thought to play roles in the protection of cells from reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2). H_2O_2 is generated in response to various stimuli, such as dehydration, cold, wounding, UV irradiation and challenge with a elicitor or pathogen (Neill *et al.*, 2002a,b). Recently, there is increasing evidence for considerable interlinking between the responses to heat stress and oxidative stress (Davletova *et al.*, 2005; Panchuk *et al.*, 2002). It is possible that the genes related to detoxification may play important roles in the acquisition of stress tolerance not only to drought but also to heat shock in the *ZmDREB2A* transgenic plants.

In conclusion, the activity of a maize DREB2-type transcription factor ZmDREB2A is regulated by a stress-induced splicing of its mRNA. Constitutive or stress-inducible expression of *ZmDREB2A* resulted in an improved stress tolerance not only to drought but also to heat shock in plants. Microarray analyses of transgenic Arabidopsis plants overexpressing *ZmDREB2A* revealed that in addition to genes encoding LEA proteins, some genes related to heat shock, detoxification and seed maturation were upregulated. We conclude that ZmDREB2A may have a dual function in mediating the expression of genes responsive to both water stress and heat stress.

Experimental procedures

Plant materials

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium (GM) agar plates for 3 weeks, as previously described (Yamaguchi-Shinozaki and Shinozaki, 1994). Stress treatments for RNA gel blot analysis and stress tolerance tests were also performed as previously described (Liu *et al.*, 1998). Arabidopsis T87 suspension-cultured cells were maintained as described previously (Takahashi *et al.*, 2001).

Cloning and primers sequences

We performed a TBLASTN search within the GenBank nucleotide database. Maize EST and mRNA sequences were obtained from the UniGene database. Primers for ZmDREB2A cloning: AY108198-ATG-for, ATGAGGGGAAAAGGGGGGCCTG; AY108198-Long-rev (cover 3′-UTR), AAAAGCAAGCACTCTTTTTA; 16320845-Long-for (cover 5′-UTR), GGTCTTATCGACTCCAAGAAGAAC. Primers for RT-PCR: forward, TTTGGATCCGGTCTATCGACTCC; reverse, GATGACAGCTGCCACTGACGAT. Primers for quantitative real-time RT-PCR: ZmDREB2A-L-156F, GTGCTGTGGTGCATGGT; ZmDREB2A-L-128R, CGTAGGCCCATCTCGTGATC; ZmDREB2A-S-24F, GCAGCCCGGAAGGAAGAA; ZmDREB2A-S-90R, GATGACAGCTGCCACTGACGAT.

Primers for maize 18s rRNA, forward, AAACGGCTACCACATCCAAG; reverse, CCTCCAATGGATCCTCGTTA.

Transient expression experiments

Effector and reporter plasmids used in the transient transactivation experiment examining C-terminal and internal deletion mutants of ZmDREB2A were constructed as described previously (Liu *et al.*, 1998). Isolation of Arabidopsis T87 cell protoplasts and polyethylene glycol-mediated DNA transfection were performed as previously described (Satoh *et al.*, 2004).

Plant transformation

Plasmids used for the transformation of Arabidopsis were constructed with the coding region of the *ZmDREB2A* cDNA. The coding region fragment was cloned into a multi-cloning site of the pBI121 vector (Liu *et al.*, 1998) or the pBI29AP-Not vector (Kasuga *et al.*, 1999) and the plasmids were introduced into *Agrobacterium tumefaciens* C58. Plants were transformed as described previously (Liu *et al.*, 1998).

Microarray analysis

Total RNA was isolated with TRIzol reagent (Invitrogen, http://www.invitrogen.com/) from 3-week-old plants having pBI121 or overexpressing ZmDREB2A. Preparations of fluorescent probes, microarray hybridization and scanning have been described previously (Fujita *et al.*, 2005; Seki *et al.*, 2002).

RNA gel blot analysis

Total RNA was extracted with TRIzol reagent and procedures for RNA gel blot analysis were performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

Quantitative real-time RT-PCR analysis

For quantitative real-time RT-PCR, cDNA was synthesized from total RNA by using Revertra Ace (Toyobo, http:www.toyobo.co.jp/e/) with random primers according to the manufacturer's instructions. One microgram of total RNAs was used for the reverse transcription reaction in 20 μl and 0.2 μl cDNAs was applied for PCR analysis. Real-time PCR was performed on a Light Cycler (Roche Diagnostics, http://www.roche-diagnostics.com/) by using an SYBR Premix Ex Taq kit (Takara, http://www.takara-bio.com/) according to the manufacturer's instructions. Known concentrations of pBluescript SK plasmids carrying *ZmDREB2A-L* or *ZmDREB2A-S* was used as standards in real-time RT-PCR to quantify the actual amount of amplified *ZmDREB2A-L* and *ZmDREB2A-S* transcripts. The amounts of template cDNA that were used in each PCR reaction were corrected by the results of quantification of 18S rRNA. Three replicate PCR amplifications were performed for each sample.

Drought stress tolerance of transgenic plants

Plants were grown in Petri dishes containing GM selective agar medium for 3 weeks and were then transferred to 8-cm pots filled with vermiculite. Plants were subsequently grown for one more week before exposure to drought stress. Drought stress was imposed by withholding water for 10 days in a growth chamber (22°C, 50–60% relative humidity, continuous light) until the lethal effect of dehydration was observed for the majority of wild-type plants. After rewatering for 5 days, the number of plants that survived and continued to grow was counted.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Thirty-one heat stress inducible genes were 3-time upregulated in 35S:ZmDREB2A plants.

A complete data set is available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-819.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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