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REGULAR PAPER

Molecular cloning, expression profiling and trans-activation property studies of a *DREB2*-like gene from chrysanthemum (*Dendranthema vestitum*)

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Abstract Dehydration responsive element binding (DREB) proteins are important transcription factors in plant stress response and signal transduction. In this study, a *DREB* homolog gene, *DvDREB2A*, was isolated from chrysanthemum (*Dendranthema vestitum*) by reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) methods. It contained an open reading frame (ORF) of 1,471 bp encoding 366 amino acid residues and was classified as a DREB2 subfamily member based on multiple sequence alignment. The predicted protein sequence contained a typical AP2/EREBP DNA-binding domain near the N-terminal region. In yeast one-hybrid analysis DvDREB2A protein was specifically bound to DRE elements (core sequence, A/GCCGAC) and activated the expression of the reporter *HIS3* and *LacZ*. Transient expression experiment suggested that DvDREB2A protein was localized to the nucleus of onion epidermis cells. Quantitative real-time PCR (QRT-PCR) experiments showed that expression level of *DvDREB2A* was significantly affected by heat, low temperature, drought, abscisic acid (ABA) and high salinity treatments. These results indicated that the *DvDREB2A* gene is a new member of the DREB transcription factors, which may play an important role in providing tolerance to environmental stresses.

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Introduction

Environmental stresses such as drought, high salt and low temperature affect plant growth and productivity extremely. These stress conditions induce the expression of many genes in plants (Thomashow 1999; Bray et al. 2000; Shinozaki et al. 2003). In stress-responsive gene expression, both abscisic acid (ABA)-dependent and ABA-independent regulatory systems are involved (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2005). For example, the expression of the *rd29A* gene is ABA inducible but is controlled by an ABA-independent pathway (Nordin et al. 1991, 1993; Kurkela and Borg-Franck 1992; Yamaguchi-Shinozaki and Shinozaki 1993, 1994; Iwasaki et al. 1997). A novel *cis*-acting dehydration-responsive element (DRE) with a 9-bp conserved sequence, TACCGACAT, has been identified in the promoter region of the *rd29A* gene and is responsible for dehydration- and cold-induced expression (Yamaguchi-Shinozaki and Shinozaki 1994; Jiang et al. 1996; Ouellet et al. 1998). Similar *cis*-acting elements, named C-repeat (CRT; TGGCCGAC) and low-temperature-responsive element (LTRE; CCGAC) have been demonstrated in cold-inducible genes (Baker et al. 1994; Jiang et al. 1996; Thomashow 1999). The DRE and DRE-like *cis*-acting elements are essential in regulating gene expression in response to cold, drought and high-salt stresses (Yamaguchi-Shinozaki and Shinozaki 1994; Liu et al. 1998).

The DRE-binding (DREB) proteins, belonging to the transcription factors family AP2/EREBP (APETALA2/an ethylene-responsive element binding protein), are able to

bind specifically to the DRE/CRT sequence and activate the transcription of genes driven by the DRE/CRT sequence, so the DREB proteins play an important role in regulating abiotic stress-related genes and thereby imparting stress tolerance to the plant system (Liu et al. 1998; Dubouzet et al. 2003). Many *DREB* genes have been found. The first isolated cDNAs encoding DREB proteins by the yeast one-hybrid screening technique were *CBF1*, *DREB1A* and *DREB2A* from *Arabidopsis* (Stockinger et al. 1997; Liu et al. 1998). Then, two cDNA clones homologous to *DREB1A* (*DREB1B* and *DREB1C*) and one cDNA homologous to *DREB2A* (*DREB2B*) were isolated (Liu et al. 1998; Shinwari et al. 1998). *CBF1* is identical to *DREB1B*, and its two homologs, *CBF2* and *CBF3*, are identical to *DREB1C* and *DREB1A*, respectively (Gilmour et al. 1998). The *DREB1/CBF* genes play a critical role in cold-responsive gene expression, whereas *DREB2* genes show expression under dehydration and high-salt stresses (Liu et al. 1998; Nakashima et al. 2000). Later, three novel *DREB1/CBF*-related genes and six novel *DREB2*-related genes in the *Arabidopsis* genome were reported, but their expression levels were low under the stress conditions (Sakuma et al. 2002). One of them, *DREB1D/CBF4*, is weakly induced by osmotic stress (Haake et al. 2002; Sakuma et al. 2002), and the other two, *DREB1F/DDF1* and *DREB1E/DDF2*, are induced by high-salinity stress (Sakuma et al. 2002; Magome et al. 2004). The expression of *DREB2E* was slightly induced only by ABA treatment in roots (Sakuma et al. 2002). Furthermore, the expression of *DREB* genes can be altered by members of the same DREB protein family. Novillo et al. (2004) reported that *Arabidopsis DREB1C/CBF2* was a negative regulator of *DREB1A/CBF3* and *DREB1B/CBF1* expression, although all three genes are responsive to abiotic stresses, indicating that their expressions are tightly controlled and thus guarantee the proper inductions of the downstream genes. These observations suggest the complexity of the mechanism of DREBs.

Transgenic *Arabidopsis* plants with *DREB1* genes driven by the 35S promoter of cauliflower mosaic virus (CaMV) exhibited growth retardation under normal growth conditions and showed strong tolerance to drought, high-salt and freezing stresses (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000). It was demonstrated that overexpression of the *Arabidopsis DREB1/CBF* genes in transgenic *Brassica napus*, tobacco plants, and *Triticum aestivum* cv. induced expression of orthologs of *Arabidopsis DREB1/CBF*-targeted genes and increased the cold tolerance of the transgenic plants (Jaglo et al. 2001; Kasuga et al. 2004; Liu et al. 1998). However, overexpression of *DREB2A* in transgenic plants neither caused growth retardation nor improved stress tolerance (Liu et al. 1998). Recently, Sakuma et al. (2006a) have shown that overexpression of the active form of *DREB2A* (*DREB2A-CA*) increased expression of many stress-responsive genes and improved drought stress tolerance of *Arabidopsis*. All the above observations suggested that the DRE/DREB regulon can be used to improve the tolerance of various kinds of agriculturally important crop plants to drought, high-salt and cold stresses by gene transfer. Lately, more *DREB* homologs have been cloned from some crops, both monocotyledons and dicotyledons, such as the *PgDREB2A* gene from *Pennisetum glaucum*, *HvDREB* gene from barley (*Hordeum vulgare*), *ZmDREB* gene from maize (*Zea mays*), *OsDREB* gene from rice (*Oryza sativa*), and the *TaDREB* gene from common wheat (*Triticum aestivum*) (Agarwal et al. 2007; Choi et al. 2002; Qin et al. 2007; Dubouzet et al. 2003; Shen et al. 2003).

Chrysanthemum is one of the most famous flowers in China and ornamental species in the world. However, the chrysanthemum industry is one of the environmentally susceptible industries. Improvement of the resistance of chrysanthemum plants to abiotic stresses such as heat, low temperatures, drought and salt is a pressing challenge. Isolation and characterization of *DREB* genes from this plant may facilitate the generation of stress-tolerant chrysanthemum cultivars by a genetic engineering approach. In this study, we isolated one *DREB2*-like gene from chrysanthemum (*Dendranthema vestitum*), studied expression patterns of this gene under different stress conditions, and examined the transcriptional activation abilities of this new gene.

Materials and methods

Plant materials, growth conditions and stress treatments

Chrysanthemum (*D. vestitum*) plants were grown in plastic pots under natural conditions. We performed cold and heat shock treatment by maintaining the plants at 4°C and 40°C, respectively. We stressed the plants with salinity, ABA or drought treatments by submerging their roots in a solution containing 1 mol/l NaCl, 100 μmol/l ABA and 20% polyethylene glycol (PEG) 6000. All excised leaf samples of control and stress-treated plants were collected at intervals of 0.5 h, 2 h, 4 h, 6 h, 12 h, and 24 h, frozen immediately in liquid nitrogen and stored at −80°C for DNA and RNA extraction.

Amplification of *DvDREB2A* cDNA

Based on the AP2 DNA-binding domain of DREB2 proteins, a pair of degenerated primers (P1, 5′-TGA AAC

ACT GGC AAA ATG GAW NGA RTA YA-3′ and P2, 5′-CCA TAC ATA GCC ATA GCA GCT TCR TCR TAN GC-3′) (degenerate bases N = A/G/T/C; R = A/G; Y = C/T; M = A/C; W = A/T) was designed. The extracted genomic DNA was used as a template to amplify the partial fragment. Genomic DNA was extracted from tender leaves of chrysanthemum by the cetyltrimethyl ammonium bromide (CTAB) method described by Weising et al. (1995) with minor modifications. The polymerase chain reaction (PCR) amplification was performed as follows: one cycle at 95°C for 5 min; 29 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; one cycle at 72°C for 10 min. Specific primers were then designed for rapid amplification of cDNA ends (RACE), according to the sequence information of the partial fragment to obtain the full length sequence of the gene. For 3′ RACE, two gene-specific primers (GSP) were designed (GSP1, 5′-AGA TGA AAA AGC AAA GCC AGC CCG AAA A-3′, and GSP2, 5′-GAC AAC GGA CTT GGG GAA AAT GGG TGG C-3′). Primers for 5′ RACE were: GSP3, 5′-AAG CAC TAC CAT TCG GCA CAA CCA TCT G-3′, and GSP4, 5′-CCA AAG CAG CCT CAA CAG CAG AAC CAA AAG-3′. The RACE reactions were performed according to the manufacturer's protocol (BD SMART RACE cDNA amplification kit, Clontech). The nested PCR was performed as follows: pre-denaturation at 95°C for 5 min, 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and a final extension of 8 min at 72°C. WE obtained a single, full-length, cDNA sequence by combining the 5′-RACE fragment, cDNA fragment and the 3′-RACE fragment. Finally, a pair of primers (F1, 5′-CCA CTA TTT GAA GGA AAT ACG A-3′ and F2, 5′-ACT CTC TCG GTA GAT AGC CTA A-3′) was then designed from the putative 3′ and 5′ untranslated region (UTR) of the full-length cDNA sequence.

The resultant DNA fragments and RACE products were gel purified and cloned into pGEM-T (Promega). They were then transformed into the competent *E. coli* strain DH5α. The positive transformants were screened by PCR and sequenced (Invitrogen).

Quantitative real-time PCR analysis

Total RNA was prepared from treated plants with an RNAultra plant kit (Tiangen), according to the manufacturer's instructions, and was then treated with 10 units of RNase-free DNase I (Takara). First-strand cDNA was synthesized in a total volume of 20 μl, in which there were 4 μl of 5× buffer, 1 μg of total RNA, 1 μl of 2.5 μmol/l oligo-(dT)$_{18}$ primer, 5 units of avian myeloblastosis virus (AMV) reverse transcriptase, 2 μl of 10 mmol/l dNTPs, and 20 units of RNase inhibitor (Takara).

Real-time PCR analysis of transcript copy number was conducted with the Evagreen qPCR Master Mix (Biotium). The PCR system contained 50 ng of first-strand cDNAs, 0.5 μl of 10 μmol/l gene-specific primer (Q1 and Q2) and 10 μl of mix, and the amplification condition was 95°C for 15 s, followed by 55 cycles at 95°C for 15 s, at 58°C for 30 s and at 72°C for 50 s, with a final extension at 72°C for 10 min (the Light Cycler Instrument, Bio-Rad iQ5). Elongation factor 1-alpha (*EF1α*) amplification was added as a control with primers E1and E2. Two replicates of each reaction were performed, and data were analyzed by Bio-Rad iQ5 Optical System Software version 1.0 (graph error ± SD = 0.1, normalized expression $\Delta\Delta C_T = (C_{T,\mathrm{Target}} - C_{T,\mathrm{EF1}\alpha})_{\mathrm{Time}\ x} - (C_{T,\mathrm{Target}} - C_{T,\mathrm{EF1}\alpha})_{\mathrm{Time}\ 0}$. The primers used were as follows: Q1 5′-GAC AAC GGA CTT GGG GAA AAT GGG TGG C-3′, Q2 5′-AAG CAC TAC CAT TCG GCA CAA CCA TCT G-3′, E1 5′-AGA CCA CCA AGT ACT ACT GCA C-3′ and E2 5′-CCA CCA ATC TTG TAC ACA TCC-3′.

Subcellular localization of DvDREB2A protein

The *DvDREB2A* gene was fused to the 5′ end of the fluorescent protein [green fluorescent protein (GFP)] reporter gene using the pBI121 plant expression vector without a stop codon. Recombinant DNA constructs encoding the DvDREB2A–GFP fusion protein downstream of the cauliflower mosaic virus (CaMV) *35S* promoter were introduced into onion epidermal cells by the *Agrobacterium tumefaciens*, EHA105. Onion cells were also transformed with pBI121-GFP vector as a control. Transformed cells were placed on Murashige Skoog (MS) solid medium at 28°C and incubated for approximately 48 h (16 h light/8 h dark) before being examined. The subcellular localization of GFP fusion proteins was visualized with a fluorescence microscope (Leica TCS SP2).

DRE-binding activities of the DvDREB2A protein

To analyze DRE-binding activity we subcloned the open reading frame (ORF) of DvDREB2A into the *Bam*HI–*Pst*I sites in the multicloning sites (MCSs) downstream of the GAL4 activation-domain (AD) in the pAD-GAL4 vector. The combined plasmids and pAD vector (as a negative control) were transformed into the yeast strain YM4271 carrying the reporter genes *His3* and *LacZ* under the control of the 71-bp promoter region of *rd29A* containing three tandemly repeated DRE sequence (TACCGACAT) or mutated DRE (mDRE) sequence (TATTTTCAT), respectively (Liu et al. 1998). The transformed yeast cells were streaked on selective medium of synthetic dextrose (SD)

medium without His plus 10 mmol/l 3-aminotriazole (SD/−His + 10 mM 3-AT). The recombinant yeast with two reporter genes grew in the selective medium plate, and its growth was completely inhibited by 10 mM 3-AT. The β-galactosidase activities were analyzed as described by Chen et al. (2003).

Transcriptional activation abilities of DvDREB2A protein

The coding region of *DvDREB2A* was inserted between the *Sal*I and *Pst*I restriction sites of the yeast expression vector (pBD) containing the binding domain (BD) of GAL4. The plasmids were introduced into the yeast strain YRG-2 with the reporter gene *His3* by the same method as described for the DRE-binding analysis. If the encoded proteins possessed activation ability, it would work together with the BD of GAL4 to promote the expression of the reporter gene *His3*, resulting in the growth of the transformed yeast cells on the SD/−His + 10 mM 3-AT medium. Yeast cells containing pGAL4 and pBD were used as positive and negative controls, respectively.

Results

Isolation and sequence analysis of the *DvDREB2A* gene from *D. vestitum*

A 292-bp DNA fragment was isolated from leaves of chrysanthemum (*D. vestitum*). To obtain a full length cDNA, we employed 5′ and 3′ RACE to extend to both ends of the putative *DREB2*-like homolog. A 1,471 bp full-length cDNA sequence with 29 bp polyA was obtained. This cDNA contained an ORF of 366 amino acid residues (Fig. 1). Genomic PCR using primers designed from the 5′ and 3′ UTR and the subsequent sequencing of the PCR product revealed that the gene had no introns (data not show). The corresponding gene was named *DvDREB2A*, and it has been deposited into the GenBank (EF633987). The predicted molecular mass was 40.98 kDa and theoretical isoelectric point (pI) was 5.29.

As shown in Fig. 2, analysis of the deduced amino acid sequence revealed a conserved AP2 domain. In the AP2 domain, a 16-amino acid region has been predicted to form an amphipathic α-helix that may be important for interaction with other proteins (Okamuro et al. 1997; Shen et al.

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1     acgcgggggctccaagctagcaacgttgatttggtgtggccattttaaaccactatttgaaggaaatacga
73    gaatgactaattcggatcaaccttcaaccacaccttcgtcatcaatggattcccctagaaagcgtaaatcaa
        M  T  N  S  D  Q  P  S  T  T  P  S  S  S  M  D  S  P  R  K  R  K  S  R
145   gaagcagacgtgatgggccaaaaggggtagctgaaacactcgcaaaatggaaagaatacaacaataaaatcg
        S  R  R  D  G  P  K  G  V  A  E  T  L  A  K  W  K  E  Y  N  N  K  I  D
217   actcactagatgaaaaagcaaagccagcccgaaaagtacccgcgaaagggtctaaaaagggttgtatgaaag
        S  L  D  E  K  A  K  P  A  R  K  V  P  A  K  G  S  K  K  G  C  M  K  G
289   gtaaaggtgggcccgagaattcgcgatgcaagtttcgtggcgtgagacaacggacttggggaaagtgggtgg
        K  G  G  P  E  N  S  R  C  K  F  R  G  V  R  Q  R  T  W  G  K  W  V  A
361   ccgagattcgagaaccgaatagagggagtcgattgtggttagggactttggttctgctgttgaggctgctt
        E  I  R  E  P  N  R  G  S  R  L  W  L  G  T  F  G  S  A  V  E  A  A  L
433   tggcttatgatgaagccgctagagttatgtatgggtcttgtgctaggcttaatttacctaattgtaggtcga
        A  Y  D  E  A  A  R  V  M  Y  G  S  C  A  R  L  N  L  P  N  C  R  S  M
505   tgaatgagtatagtcagatggttgtgccgaatggtagtgcttctagttgtgactcgactacgacctgtagtc
        N  E  Y  S  Q  M  V  V  P  N  G  S  A  S  S  C  D  S  T  T  T  C  S  H
577   attctttggaagattctaaagcttcttcgagtcggcaaatgattaaacaagatgaaggtgactcgaaacgtg
        S  L  E  D  S  K  A  S  S  S  R  Q  M  I  K  Q  D  E  G  D  S  K  R  E
649   aagatactatagttaaacatgaagttaaggaagagcctgttgaggaatttaaaagtattagaatcaaggatg
        D  T  I  V  K  H  E  V  K  E  E  P  V  E  E  F  K  S  I  R  I  K  D  E
721   aaccggtagagaccaacgaggacatcagtttgccacatgttaatgagatgtttgatatggaggagttacttg
        P  V  E  T  N  E  D  I  S  L  P  H  V  N  E  M  F  D  M  E  E  L  L  E
793   aaatgatgggccagagaaagtcccaaaatacgggaaacagaatgggtcaaaacgggtttgatgagcctagtg
        M  M  G  Q  R  K  S  Q  N  T  G  N  R  M  G  Q  N  G  F  D  E  P  S  G
865   gcgtgtctcagccttggataaaaattgagccacaaagcgaatatcagcaagatacatatgggacaggaggtg
        V  S  Q  P  W  I  K  I  E  P  Q  S  E  Y  Q  Q  D  T  Y  G  T  G  G  G
937   gttttgggtttgatttttcgatgccaaagcagcctgcgatggatcagaagctcggtgacatgggttggttcg
        F  G  F  D  F  S  M  P  K  Q  P  A  M  D  Q  K  L  G  D  M  G  W  F  D
1009  atcaggagccaatgaacccatatactagacccaagtgggaagataaatgcgatgttggtgctgttgctgatt
        Q  E  P  M  N  P  Y  T  R  P  K  W  E  D  K  C  D  V  G  A  V  A  D  Y
1081  atggttttgatttttaatgccgggacggcctgaagatggtaacttcacattacaagagttggggtttgatt
        G  F  D  F  L  M  P  G  R  P  E  D  G  N  F  T  L  Q  E  L  G  F  D  L
1153  tgggagctgatttggcttgtgagataactgagatataataagcctgttacttttatttacaggagctaaga
        G  A  D  F  G  L  *
1225  gcctgagattgctggtccttgttcaatatgggattaagaaaacgccaatttgtaaatgttcttagtgatata
1297  gtggtagtcacatgttttgctagtctattttcgactatagtagttataaaccaaattataattaggctatct
1369  accgagagagttattatgtatatgtcccttattaggaacttccaatggtgaatgtaaataatgacttatgtg
1441  acaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 1 Nucleotide acid sequence and deduced amino acid sequence of *DvDREB2A*. *Shaded* residues indicate the location of the pair of degenerate primers. The *single lines* indicate the primers for 3′ RACE, and the *double horizontal lines* indicate the primers for 5′ RACE. The pair of primers for full length is *boxed*. An *asterisk* marks the stop code

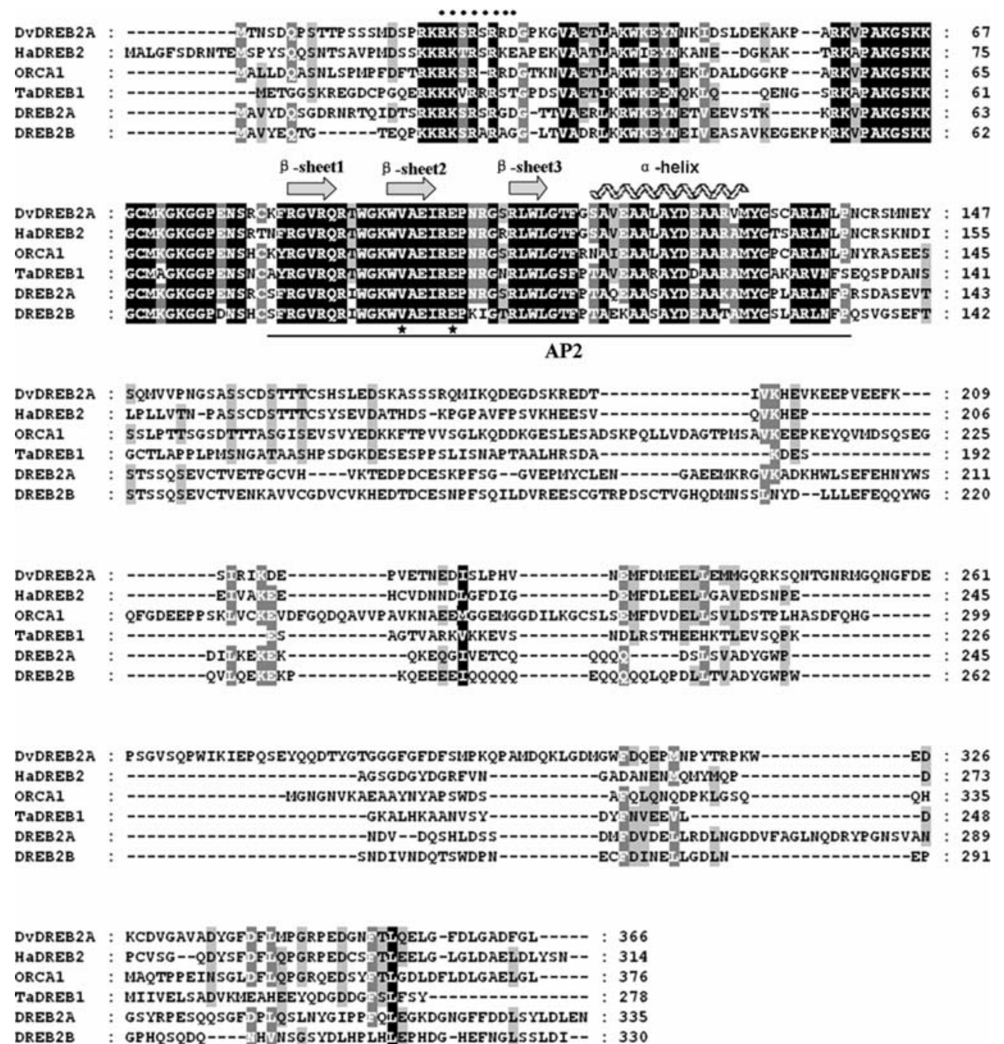

Fig. 2 Comparison of the deduced amino-acid sequences of DvDREB2A with other DREB2-type proteins. The *shading* indicate identities; the highly conserved AP2/EREBP domain is *underlined*. The putative nuclear localization signals (RKSRSRRD) are shown by *dots*. The *stars* indicate the conserved valine (*V*) and glutamic acid (*E*) at the 14th and the 19th position in the ERF/AP2 domain, respectively. Predicted α-helix and β-sheets are also indicated. The deduced amino-acid sequences are DREB2A (O82132), DREB2B (O82133) of *Arabidopsis thaliana*, HaDREB2 (AAS82861) of *Helianthus annuus*, ORCA1 (CAB93939) of *Catharanthus roseus*, TaDREB1 (AF303376) of *Triticum aestivum*, DvDREB2A (EF633987) of *Dendranthema vestitum*

2003). Three β-sheets were also found that may be responsible for DNA-binding activity (Allen et al. 1998). In addition, valine and glutamic acids in β-sheets were the crucial sites responsible for DNA-binding activity (Sakuma et al. 2002; Cao et al. 2001; Qin et al. 2004).

Phylogenic analysis among currently known DREB2-related proteins has shown that the DvDREB2A belongs to a DREB2-type transcription factor (Sakuma et al. 2002), sharing a high homology with the *Helianthus annuus* HaDREB2 (Fig. 3).

Quantitative real-time PCR analysis

The organ-specific expression of *DvDREB2A* in chrysanthemum plants was examined by quantitative real-time (QRT)-PCR analysis. As shown in Fig. 4a, the *DvDREB2A* was expressed in all organs under the natural conditions. Its transcripts were mainly expressed in flowers, while less accumulation was detected in roots, stems and young leaves.

We also quantified the expression patterns of *DvDREB2A* under different stresses for different lengths of time. The *DvDREB2A* transcript was up-regulated strongly in response to ABA treatment as compared to cool, heat, salt or drought treatments (Fig. 4b–e). Induction of the *DvDREB2A* gene under ABA was transient, and significant reduction was observed in the transcript level after 0.5 h, which was 300-times higher than that of untreated control plants (Fig. 4e). The low temperature treatment also transiently activated the *DvDREB2A* expression, similar to that under ABA stress. It reach a maximum after 6 h and decreased rapidly (Fig. 4d). The level of *DvDREB2A* transcript increased within 0.5 h after the heat shock treatment; after 4 h it decreased gradually and then came down to the initial level after 24 h (Fig. 4f). Because *DREB2* is involved in osmotic stress response, we also explored the changes in transcript levels of *DvDREB2A* under salt and drought treatments. There were some fluctuations in the case of salinity stress or dehydration stresses, and the results showed that expression patterns of *DvDREB2A* were more complex than previously thought.

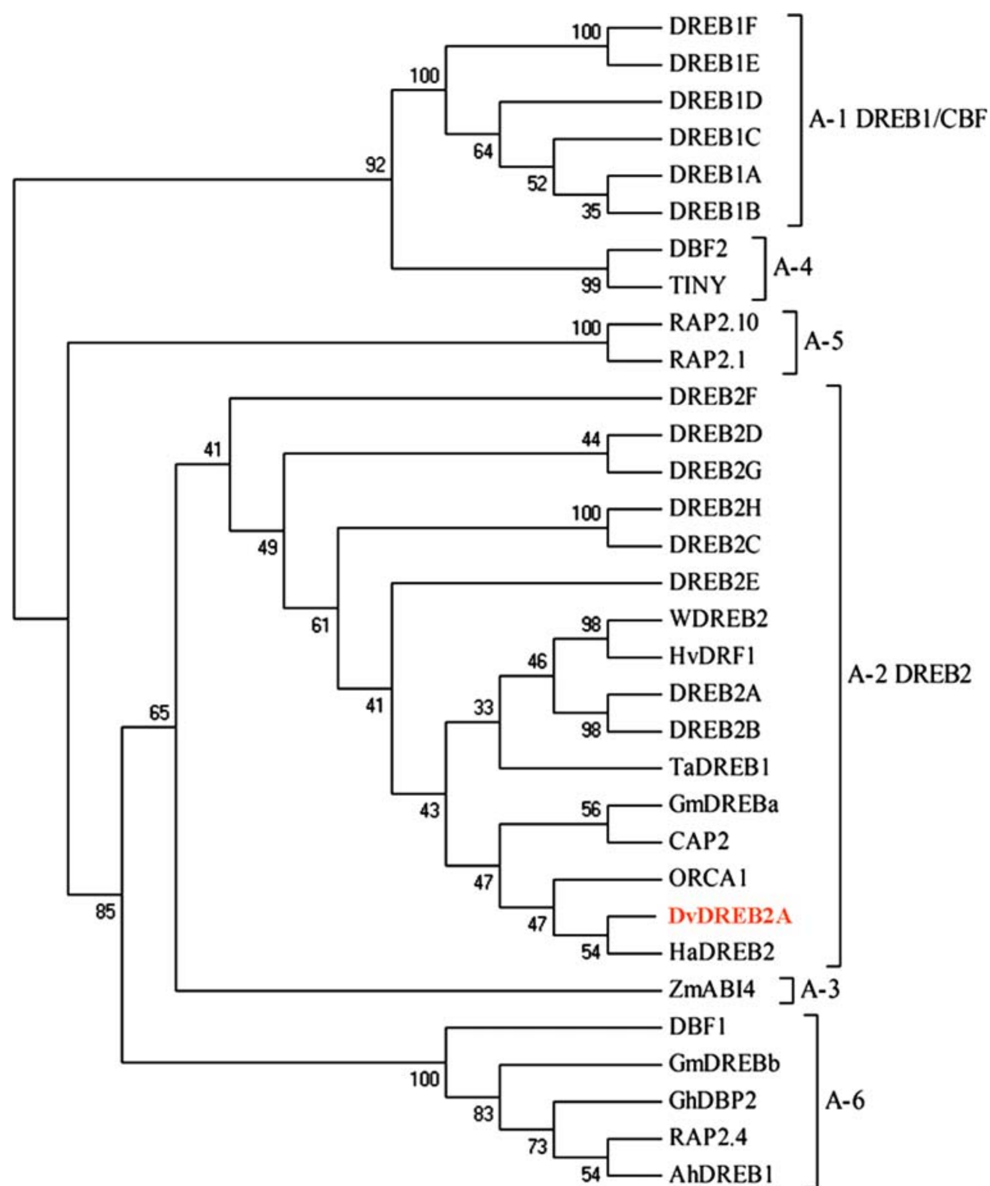

Fig. 3 Relationships of the isolated DREB proteins from different species is represented by MEGA3.1 produced by ClustalX1.83. The phylogenetic tree shows the position of DvDREB2A in the DREB2 subfamily. The neighbor-joining tree is based on an alignment of the complete protein sequences. The sequences used are from *Arabidopsis* DREB1A (Q9M0L0), DREB1B (P93835), DREB1C (Q9SYS6), DREB1D (Q9FJ93), DREB1E (Q9SGJ6), DREB1F (Q9LN86), DREB2A (O82132), DREB2B (O82133), DREB2C (Q8LFR2), DREB2D (Q9LQZ2), DREB2E (O80917), DREB2F (Q9SVX5), DREB2G (P61827), DREB2H (Q9SIZ0); TINY (CCA64359); RAP2.1 (NP_564496), RAP2.4 (NP_177931), RAP2.10 (NP_195408); *Atriplex hortensis* AhDREB1 (AF274033); *Catharanthus roseus* ORCA1 (CAB93939), *Glycine max* GmDREBa (AY542886), GmDREBb (AY296651); *Hordeum vulgare* HvDRF1 (AAO38209); *Triticum aestivum* WDREB2 (BAD97369); TaDREB1 (AF303376); *Zea mays* ZmABI4 (AAM95247), DBF1 (AF493800), DBF2 (AAM80485); *Helianthus annuus* HaDREB2 (AAS82861); *Cicer arietinum* CAP2 (ABC49682); *Gossypium hirsutum* GhDBP2 (AAT39542); *Dendranthema vestitum* DvDREB2A (EF633987)

Transcript copy number changed rapidly over 24 h, and this change possessed periodicity (Fig. 4b, c).

Intracellular localization of the DvDREB2A protein

Inspection of the amino acid sequences of DvDREB2A showed that it contains a short stretch of basic amino acids near the N-terminal (Fig. 2). This region (RKSRSRRD) is characterized by a core peptide enriched in arginine (R) and lysine (K) and might function as a potential nuclear localization signal (NLS) (Dingwall and Laskey 1991), so the protein is expected to be localized in the nucleus. We examined the transient expression to confirm the subcellular localization of DvDREB2A protein. A DvDREB2A-GFP

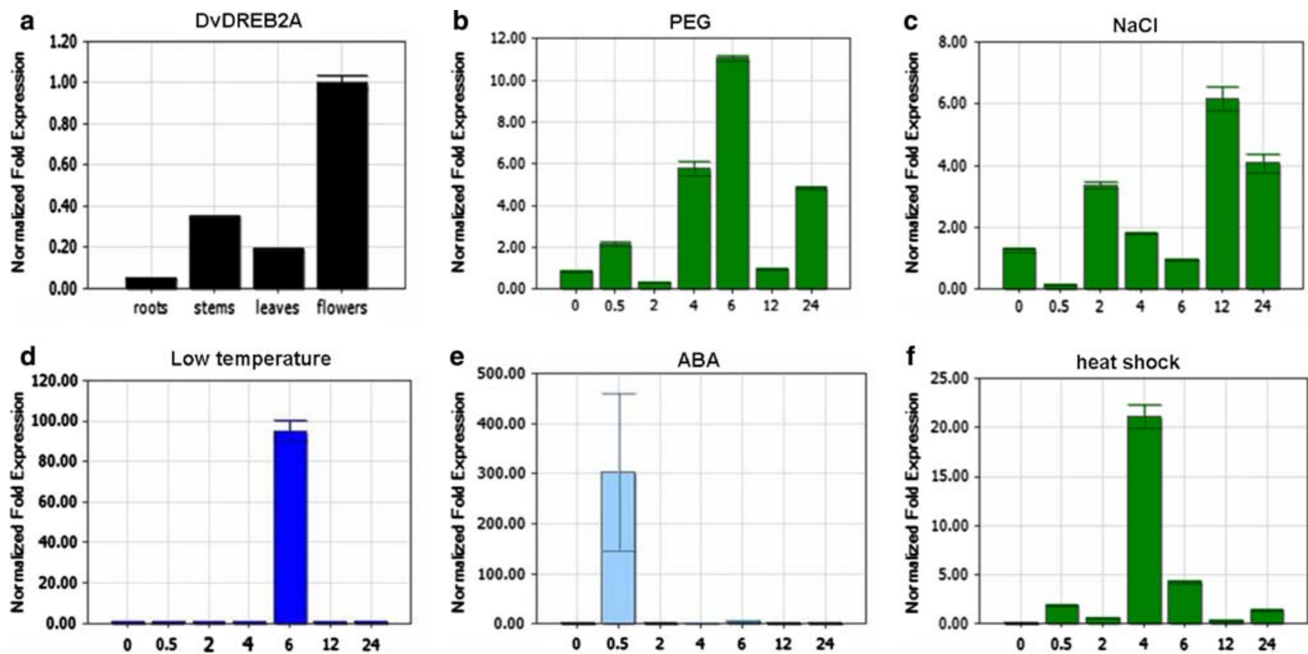

Fig. 4 Expression patterns of *DvDREB2A* in different organs and in response to various treatments. **a** Expression patterns of *DvDREB2A* in roots, stems, leaves, and flowers under natural conditions. **b** Low temperature (4°C). **c** Heat shock (40°C). **d** 1 mol/l NaCl. **e** 20% PEG (6,000). **f** 100 µmol/l ABA. The elongation factor EF1α was amplified as a control. The time course of each treatment was 0.5 h, 2 h, 4 h, 6 h, 12 h, and 24 h, respectively

construct was introduced into the epidermal cells of onion by *Agrobacterium tumefaciens.* Confocal microscopic observation demonstrated that GFP fluorescence was distributed in the whole cell with the control plasmid 35S-GFP (Fig. 5c). By contrast, the DvDREB2A–GFP fusion protein was targeted to the nucleus (Fig. 5f). Thus, the results clearly indicated that DvDREB2A is a nuclear-localized protein.

DRE-binding activities of the DvDREB2A protein

To confirm the DRE-binding activity of DvDREB2A, we ligated the entire coding region of the gene into the yeast expression vector pAD with the GAL4 activation domain, and the plasmids obtained were transformed into two yeast strains carrying the dual reporter genes *His3* and *LacZ* under the control of DRE or mDRE motif, respectively (Fig. 6a). Hybrid proteins that recognize the binding site act as transcriptional activators of the reporter genes, allow the cells to grow in the presence of 3-AT (a competitive inhibitor of the *His3* gene product), and turn the cells blue in a β-galactosidase assay (Liu et al. 1998). The results indicated that in DRE yeast, transformants carrying the plasmid could grow well on both plates, whether containing a selective medium of SD/−His + 10 mM 3-AT or not, and colonies turned blue when *LacZ* activity was examined with X-gal (Fig. 6b). However, the transformant yeast cells harboring the DvDREB2A and the mDRE-controlled reporter genes did not show 3-AT resistance (Fig. 6b). These results indicated that DvDREB2A can specifically bind to the DRE element but not the mDRE element in the promoter region and activate the transcription of the downstream genes in vivo.

Analysis of the transcriptional activation ability of the DvDREB2A

We studied the transcriptional activation ability of the DvDREB2A. The coding regions of DvDREB2A were introduced into pBD containing the DNA-BD of GAL4. The plasmids were introduced into the yeast YRG-2 strain, which contained the *His3* reporter gene in its genome (Fig. 7a). The transformed yeast cells harboring pBD-DvDREB2A, pGAL4 (positive control), and pBD (negative control) all could grow on SD medium. Moreover, the yeast cells carrying the pGAL4 and pBD-DvDREB2A constructs survived on the selective medium of SD/−His + 10 mM 3-AT. These results indicated that the *DvDREB2A* gene has transcriptional activation ability in yeast (Fig. 7b).

Discussion

DREB genes have been isolated from a wide variety of plants; however, few DREB proteins have been isolated

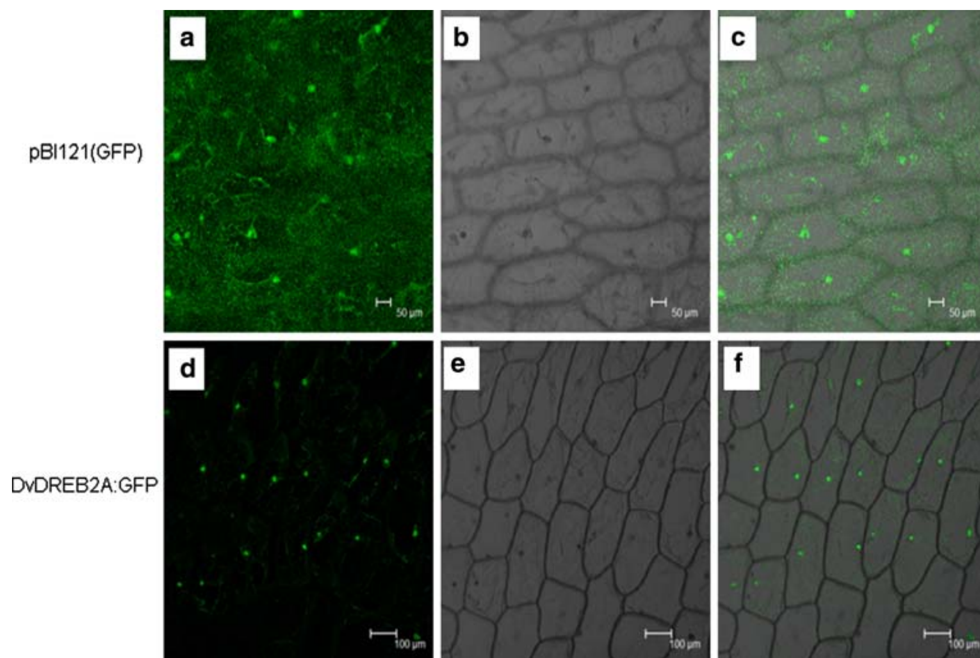

Fig. 5 Intracellular localization of DvDREB2A protein in the onion epidermis cells. Onion epidermis cells were transformed with 35S-GFP (**a**–**c**) and DvDREB2A-GFP (**d**–**f**). After incubation for 20 h, the transformed cells were observed under a confocal microscope. The photographs were taken in the dark field for green fluorescence (**a**, **d**), in the bright light for the morphology of the cells (**b**, **e**) and in combination (**c**, **f**)

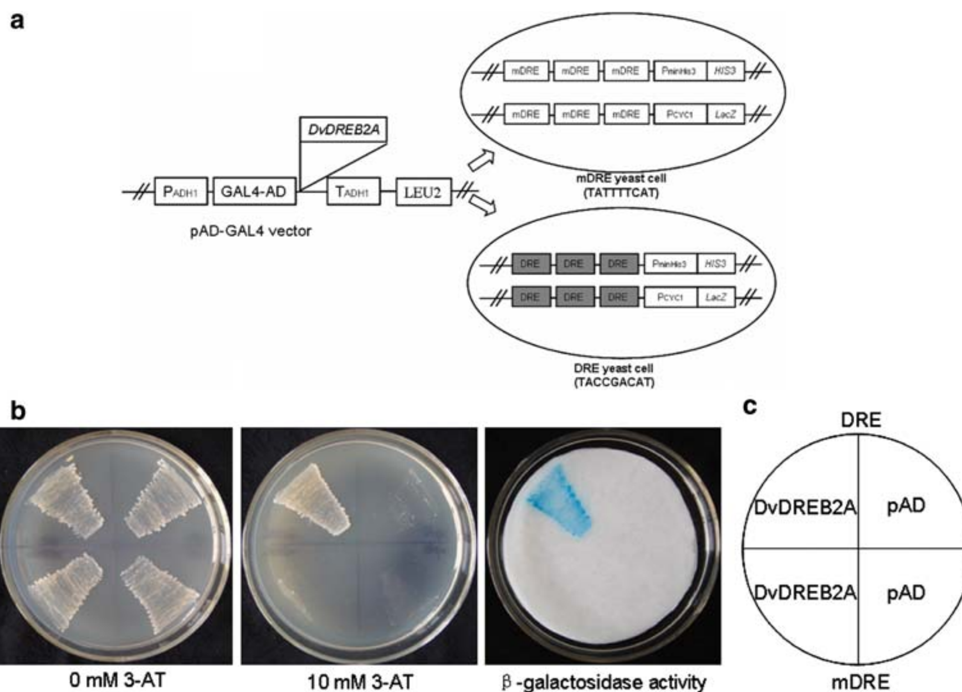

Fig. 6 The dehydration-responsive element (DRE)-binding (DREB) specificity analysis. **a** Construction of pAD-*DvDREB2A* plasmids. The entire coding region of *DvDREB2A* was cloned into the activation domain of GAL4, and then the plasmids were transformed into yeast strains carrying the dual reporter genes under the control of the promoter region containing the DRE (TACCGACAT) or a mutated DRE (mDRE) (TATTTTCAT), respectively. P_{ADH1} and T_{ADH1} are the promoter and terminator of the *ADH1* gene, respectively. **b** The transformed yeast cells were examined for growth on synthetic dextrose (SD) medium in the presence of 3-AT without His (SD/−his + 10 mM 3-AT) at 30°C (*middle panel*) and tested for *β*-galactosidase activity (*right panel*). **c** A *sketch panel* indicates the position of each yeast strain. The *upper part* indicates yeast cells harboring DvDREB2A protein and DRE-controlled reporter genes, and the *lower part* indicates yeast cells harboring DvDREB2A protein and mDRE-controlled reporter genes

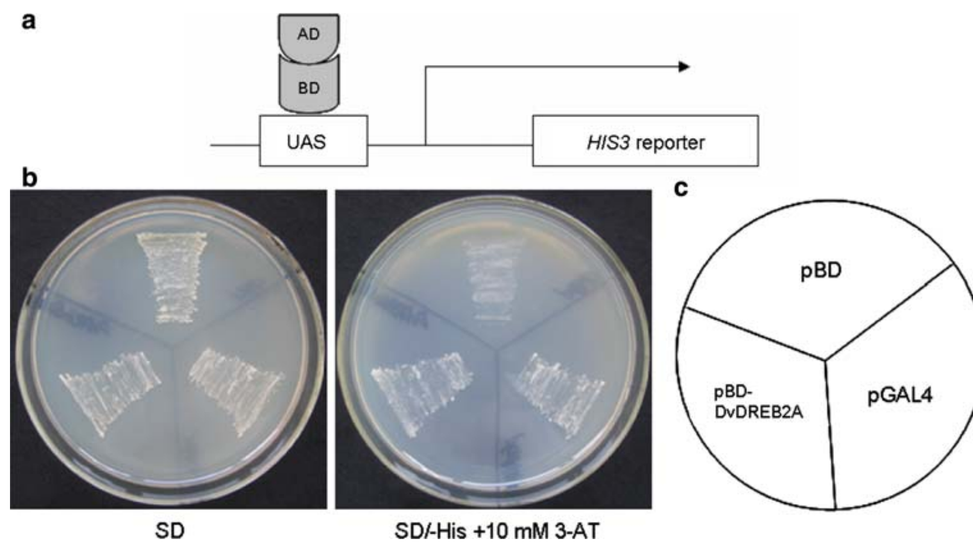

Fig. 7 Analysis of the transcriptional activation ability. **a** A sketch map of *His* reporter gene expression. *UAS* indicates upstream activating sequences, *AD* indicates the activation domain of the DvDREB2A, and *BD* indicates the binding domain of GAL4 encoded in yeast expression vector (pBD) vector. *Arrowhead* indicates the direction of *His* gene expression. **b** Plasmids of pBD-*DvDREB2A*, pGAL4 (positive control), and pBD (negative control) were transformed into yeast strain YRG-2, respectively. The transformed yeast cells were incubated on SD medium containing no 3-AT (*left panel*) or on SD/−His + 10 mM 3-AT (*right panel*) at 30°C for 3–4 days for examination of their growth status. **c** A sketch panel indicates the position of each yeast strain

from ornamental plants. Though the DREB1 class of transcription factors has been studied in some detail, information on DREB2 is limited. Therefore, we isolated and characterized an important transcription factor, DvDREB2A, from chrysanthemum (*D. vestitum*).

In this study, DvDREB2A transcription factor had the typical characters of DREB proteins. It had one conserved AP2/EREBP domain and was localized to the nucleus. It also could specifically bind to the DRE motif and activate the transcription of the dual reporter genes in yeast.

DvDREB2A expression in chrysanthemum can be detected in roots, stems, leaves and flowers under normal conditions. This expression pattern indicates that *DvDREB2A* may function in the normal program of plant growth and development. In *Arabidopsis*, *DREB2* transcription can also be detected in roots, stems and leaves, while the *DREB1* is not expressed under normal growth conditions (Liu et al. 1998). Comparison of this expression pattern indicated that *DvDREB2A* was more similar to *DREB2*. The most accumulation detected in flowers indicated that *DvDREB2A* may play a more important role during the procreation.

The phylogenetic analysis justifies the position of DvDREB2A in the DREB2 class of transcription factors, and expression of *DvDREB2A* was induced slightly by drought and high-salinity treatments, showing that it may function under dehydration conditions just like the other *DREB2* genes.

ABA plays an important role in the signal transduction of osmotic stress in plants. In the case of *Arabidopsis* the *DREB2E* gene was up-regulated only during ABA treatment in roots (Sakuma et al. 2002). We found that the expression of *DvDREB2A* was also regulated by ABA. The earlier study showed that *DREB1/CBF* and *DREB2* distinguished two different ABA-independent pathways in response to cold and drought stresses, respectively (Liu et al. 1998), and many of the genes are not induced by the accumulation of endogenous ABA but respond to ABA (Shinozaki and Yamaguchi-Shinozaki 2000). However, the ABA-dependent pathway can also involve in the CRT/DRE elements and AP2-type transcription factors. Haake et al. (2002) found that the drought induction of *CBF4* (*DREB1D*) expression is dramatically reduced in the ABA-deficient mutant aba1-1, indicating that ABA biosynthesis is required for the proper drought-induced induction of *CBF4* expression. Therefore, ABA-independent and ABA-dependent pathways are not completely independent. The *CBF4* gene may have a unique role, distinct from that of *CBF1-3*, so there is the possibility that a similar mechanism also exists in the *DREB2* genes. Furthermore, ABRE (ABRE: ACGTGG/TC) is a major *cis*-acting element that regulates ABA-responsive gene expression. Precise analysis of DRE/CRT and ABRE *cis*-acting elements have revealed that DRE/CRT functions cooperatively with ABRE as a coupling element in ABA-responsive gene expression, indicating that there are interactions between the DREB and the AREB/ABF regulons (Narusaka et al. 2003). In addition, in *Zea mays*, *ZmDBF1* was induced by ABA and was shown to function as a transcriptional activator of the responsive to *ABA17* (*rab17*) promoter by

ABA (Kizis and Pages 2002). This also suggests that the DRE/CRT is involved in an ABA-dependent pathway for the regulation of stress-inducible genes in some plants. In our research, the maximum induction was seen after 0.5 h of ABA treatment and earlier than drought and high-salinity treatments. These results indicated that *DvDREB2A* might require an accumulation of endogenous ABA for its response to drought and high salinity, or it might interact with *AREB* genes in an ABA-dependent pathway. Therefore, further investigations of the promoter region are necessary.

DvDREB2A also can be induced by low temperature. When exposed to cold stress, *DvDREB2A* was highly expressed. It is generally known that genes of the *DREB1* type are mainly induced by cold stress (Liu et al. 1998). Since some *DREB1* genes are induced by both osmotic stress and high-salinity stress, Sakuma et al. (2006a) considered the existence of crosstalk between the *DREB1/CBF* and *DREB2* genes. Our data also showed that *DvDREB2A* was involved in drought and low-temperature stress response and demonstrated that the networks of transcription factors were very complex.

Temperature is an important environmental factor that limits plant distributions, survival, and crop yields worldwide. In plants, as in other eukaryotes, heat stress proteins (HSPs) are expressed not only during the heat shock (HS) response but also during development in the absence of exogenous stresses (Sun et al. 2002). Heat stress transcription factors (HSFs) may be the central regulators in the modulation of HSP-encoding gene expression in the three phases of the HS response (triggering, maintenance and recovery) (Baniwal et al. 2004). Sakuma et al. (2006b) found that the expression of *DREB2A* was rapidly and transiently induced by HS stress, and thermotolerance was significantly increased in plants overexpressing *DREB2A-CA*. Qin et al. (2007) reported the cloning of a *DREB2* homolog from maize, *ZmDREB2A*, whose transcripts were accumulated by HS stress in maize seedlings. In our study we found a similar transient induction of *DvDREB2A* in response to HS stress, but the peak value occurred 4 h after the treatment. Díaz-Martín et al. (2005) reported that the binding of a DREB2-type transcription factor HaDREB2 (*Helianthus annuus*) and an HSF HaHSFA9 synergistically *trans*-activated the *Hahsp17.6G1*, which is the promoter of a small HSP from sunflower (*H. annuus*). Phylogenetic analysis showed that DvDREB2A was very similar to HaDREB2. We suppose that DvDREB2A might also be a functional transcription factor that activates the HSP promoter like HaDREB2 in sunflower embryos. Further research will help us to understand the regulatory mechanism of DvDREB2A in detail.

Generally, plants in the field are not subjected to only a single stress at a time, but they face numerous stresses collectively. Many results have indicated that transgenic methods are effective in improving the stress tolerance of crops and economic plants, especially that of genes encoding transcription factors. Earlier studies have suggested that DREB2A proteins require post-translational activation for their activation (Liu et al. 1998; Dubouzet et al. 2003). Recently, however, a report of a new DREB2 homolog from maize, *ZmDREB2A*, has suggested that protein modification is not necessary for ZmDREB2A to be active (Qin et al. 2007). Overexpression of *DREB2A-CA* and *ZmDREB2A* in *Arabidopsis* both resulted in enhanced tolerance to dehydration and in enhanced thermotolerance (Sakuma et al. 2006b; Qin et al. 2007). In addition, several unique genes were up-regulated exclusively in DREB2A-CA but not in DREB1A overexpression in *Arabidopsis* plants. These results indicated that DREB2 was also an important transcription factor regulating the expressions of stress-responsive genes through DRE *cis*-elements and that it might play a crucial role in providing tolerance to multiple stresses. In our study, *DvDREB2A* showed up-regulation at transcript level by different abiotic stresses, such as cold, heat, salt, dehydration and ABA, suggesting that DvDREB2A might be an important transcription factor and that it could be used to produce transgenic plants with higher tolerance to environmental stresses. Transformation of the *DvDREB2A* gene into plants, and further analysis, should reveal its possible functions in plant abiotic stress tolerance.

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