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# Characterization of a DRE-binding transcription factor from a halophyte Atriplex hortensis

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**Abstract** Environmental stresses, such as salinity, drought and cold, can induce the expression of a large amount of genes. Among these are many transcription factors that regulate the expression of downstream genes by specifically binding to *cis*-elements or forming transcriptional complexes with other proteins. In the present study, a DREB-like transcription factor gene, named *AhDREB1*, was isolated from a halophyte *Atriplex hortensis*. *AhDREB1* encoded a protein containing a conserved EREBP/AP2 domain featuring the DREB family. In yeast one-hybrid analysis AhDREB1 protein was specifically bound to DRE elements and activated the expression of the reporter genes of *HIS3* and *LacZ*. The *AhDREB1* gene was expressed in roots, stems and leaves of *A. hortensis*. Salinity induced its expression in roots, but not in other organs. Overexpression of *AhDREB1* in transgenic tobacco led to the accumulation of its putative downstream genes. The performance of the transgenic lines was also tested under stressed conditions and two lines were found to be stress-tolerant. These results suggest that the AhDREB1 protein functions as a DRE-binding transcription factor and play roles in the stress-tolerant response of *A. hortensis*.

**Keywords** *Atriplex hortensis* · DRE-binding protein · Transcription factor · Salt stress · PEG

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## Introduction

Salt and water stresses induced the expression of many genes in plants. Functional analysis of the promoter regions of some of stress-inducible genes has led to identification of the *cis*-element DRE (Dehydration-responsive element), which is responsible for dehydration-inducible transcription (Yamaguchi-Shinozaki and Shinozaki 1994; Jiang et al. 1996; Ouellet et al. 1998). Using yeast, one-hybrid screening technique involving genes encoding DRE binding proteins (DREB) have been cloned from *Arabidopsis* (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998; Medina et al. 1999). These DRE binding proteins belong to a large family of transcription factors containing a conserved EREBP/AP2 (an ethylene-responsive element binding protein/APETALA2) domain and functioned in plant development, defense responses or hormone responses (Riechmann and Meyerowitz 1998). Expression of the *CBF/DREB1* genes are strongly induced by low-temperature stress, whereas expression of the *DREB2* genes are induced by dehydration. These results indicate that two independent families of DRE-binding proteins function as *trans*-acting factors in two separate signal-transduction pathways under low-temperature and dehydration conditions, respectively (Liu et al. 1998; Shinozaki and Yamaguchi-Shinozaki 2000).

*Atriplex hortensis* is a halophyte that can endure an adverse salinity environment. In previous reports we characterized some effector molecules and the corresponding promoter responsible for Glycine Betaine biosynthesis in *A. hortensis* (Xiao et al. 1995; Shu et al. 1998; Shen et al. 2002). In order to characterize the putative regulatory molecules, we isolated a full-length *AhDREB1* gene from *A. hortensis*. Its expression pattern and transcription activation ability were investigated. The performance of the *AhDREB1* transgenic plants under stress was also evaluated.

## Materials and methods

### Plant materials, growth conditions and stress treatments

Seeds of *A. hortensis* were germinated hydroponically at 25 °C for 2 days, and then moved to soil tanks and grown in the greenhouse at 22 °C under natural daylight. Eight-week-old plants were irrigated with solutions containing 400 mM of NaCl, and mRNA from these plant leaves was used for cDNA library construction. Four-week-old plants were irrigated daily with a solution containing 200 mM of NaCl for 4 days as salt treatment. The leaves, stems and roots were harvested, frozen in liquid nitrogen and stored at –70 °C for DNA and RNA extraction.

### Construction and screening of a cDNA library

Using a poly($A^+$) mRNA purification kit (Promega), poly($A^+$) RNA was isolated from leaves of *A. hortensis* after being stressed with 400 mM of NaCl for 4 days. Two micrograms of poly($A^+$) RNA were used for cDNA library construction as described previously (Zhang et al. 2001). Approximately 500,000 plaques were screened with Arabidopsis DREB2A cDNA as probes (Liu et al. 1998). Three positive plaques were obtained from the third-round screening. The inserts in the recombinant phages were excised in vivo into pExCell plasmids (Amersham) following the instructions, and the plasmids were subjected to sequencing analysis.

### In vivo DRE-binding and the transactivation experiment by using the yeast one-hybrid system

To analyze DRE-binding activity and transactivation activity of the isolated cDNA clone, the full-length cDNA insert was cloned into the yeast expression vector YepGAP under the promoter of the glyceraldehyde 3-phosphate dehydrogenase gene and the terminator of the alcohol dehydrogenase 1 gene. This plasmid was transformed into two yeast strains following the manufacturer's instructions (Clontech). One strain carries the reporter genes *HIS3* and *LacZ* that had been fused to three tandemly repeated 71-bp DNA fragments of the *rd29A* promoter containing the DRE sequence (TACCGACAT). Another strain carries the same construct but with mutated DRE sequences (mDRE), i.e. four base-substitutions in the DRE sequence (TATTTTCAT) (see Fig. 2A). After both yeast transformants grew up on selective medium without Ura and Tyr, these transformants were analyzed on selective medium without His, Ura and Tyr but containing 10-, 20-, 30- or 60-mM of 3-AT (a competitive inhibitor of the *HIS3* gene product) to test the expression of the *HIS3* gene. The $\beta$-galactosidase activities of these colonies were analyzed according to the manufacturer's instruction (Clontech). The yeast expression vector YepGAP and the DRE and mDRE yeast strains, were described previously (Liu et al. 1998).

### DNA sequencing and data analysis

DNA sequences were determined using the *Taq* Dye Primer Cycle Sequencing Kit (Amersham) and the ABI 373A automatic sequencer. The nucleotide and amino-acid sequences were compared with those released in GenBank databases by using the GAPPED BLAST analysis program. The full-length sequence of *AhDREB1* has been deposited in GenBank databases under the accession number AF274033. The alignment report was produced by software DNASTAR. The DOS program designed according to the principles and models described previously (Jones et al. 1992; Okamuro et al. 1997) was used for prediction of the amphipathic $\alpha$-helix domain.

### Genomic Southern-blot analysis

DNA extraction and Southern-blot analysis were carried out as described previously (Li and Chen 2000). High-stringency hybridization was carried out for 16 h at 65 °C with the $\alpha$-$^{32}$P-dCTP-labeled *AhDREB1* gene as a probe. The membrane was washed with 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS and 0.5 × SSC, 0.1% SDS, for 15 min at 65 °C, respectively.

### RNA gel-blot analysis

Total RNA was extracted using the guanidinium isocyanate/acidic phenol method and 30 $\mu$g of total RNA was transferred to the nylon membrane (Hybond $N^+$, Amersham) for analysis as described previously (Li and Chen 2000). Northern hybridization was carried out overnight at 65 °C by using a $\alpha$-$^{32}$P-dCTP-labeled *AhDREB1* gene as a probe. Membranes were washed with 2 × SSC, 0.1% SDS and 1 × SSC, 0.1% SDS for 15 min at 42 °C, respectively, then washed with 1 × SSC, 0.1% SDS for 15 min at 55 °C. After stripping the probes, the membranes were re-hybridized with a positive marker gene *CMO* (choline monooxygenase) from *A. hortensis* (Shen et al. 2002) or a 18s rDNA gene.

### Tobacco transformation and analysis of stress tolerance

A *Sma*I/*Sac*I fragment of the *AhDREB1* gene in sense orientation was ligated into the *Sma*I/*Sac*I site of pBI121 (Clontech) under the control of the 35S promoter and transformed into tobacco (*Nictiana tabacum* var. SR1) by *Agrobacterium*-mediated transformation. The transgenic plants were selected on MS media containing 50 mg/l of kanamycin and confirmed by PCR. Leaves of unstressed *AhDREB1* transgenic plants and control tobacco plants were harvested for RNA analysis. The DNA fragments corresponding to the *rd17* and *rd29A* gene were amplified from Arabidopsis genomic DNA and used as probes for homologous detection of the same genes in tobacco. The expression of the *LHCP* gene from tobacco was also examined as a control. The control and transgenic plants were grown in medium containing different concentrations of NaCl and PEG. Their phenotype was photographed and their dry weight was measured.

## Results

### Isolation and structure analysis of *AhDREB1* cDNA

To isolate the genes encoding DRE-binding proteins from *A. hortensis*, we used 400 mM of NaCl to treat plants for 24 h and constructed a salt-induced cDNA library. Three clones were isolated from 500,000 plaques by using DREB2A cDNA as a probe. Sequence analysis showed that the three cDNAs were the same but of different length, and all included an open reading frame of 723 bp with a long 3′-UTR of 654 bp. The corresponding gene was named *AhDREB1*. *AhDREB1* encodes a polypeptide of 240 amino acids with a calculated molecular mass of 26.1 kDa. Analysis of the deduced amino-acid sequence revealed a conserved DNA-binding domain, which showed high homology to the EREBP/AP2 domain of a large family of plant DNA-binding proteins (Riechmann and Meyerowitz 1998). This domain exhibited 52.9% and 61.8% identities to the EREBP/AP2 domain of DREB1A/CBF1 and DREB2A from *Arabidopsis thaliana*, respectively (Fig. 1). However, in the over-all amino-acid level,

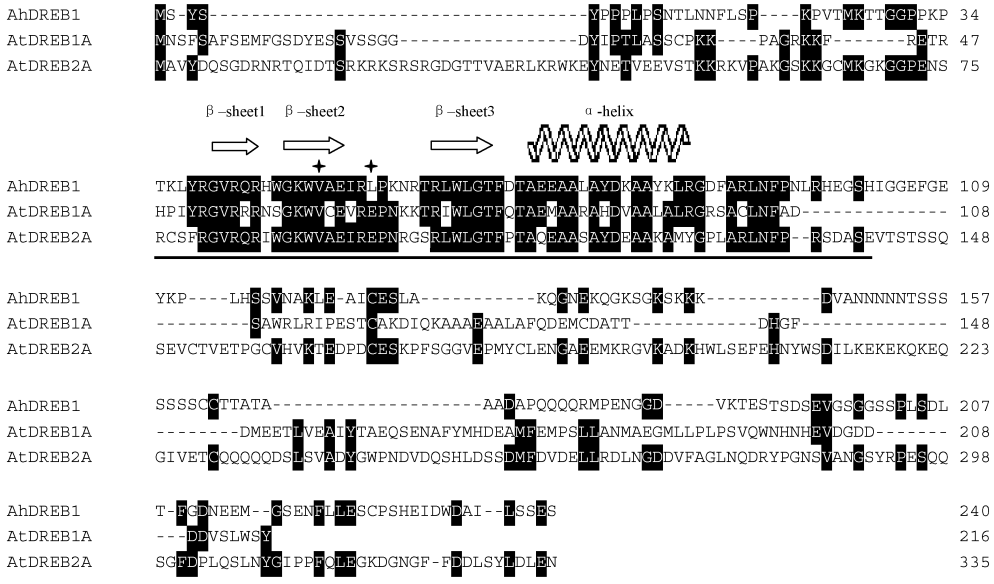


**Fig. 1** Comparison of the deduced amino-acid sequence of AhDREB1 with AtDREB1A and AtDREB2A from *Arabidopsis*. Gaps are introduced to maximize the alignment. *Shaded residues* indicate the identity. The conserved EREBP/AP2 domains are *underlined*. Predicted $\alpha$-helixes and $\beta$-sheets were also indicated. The *asterisks* mark the two residues "V" and "E" which has been especially identified in *Arabidopsis* (Liu et al. 1998; Cao et al. 2001). *Numbers* on the right indicate positions of the amino acid

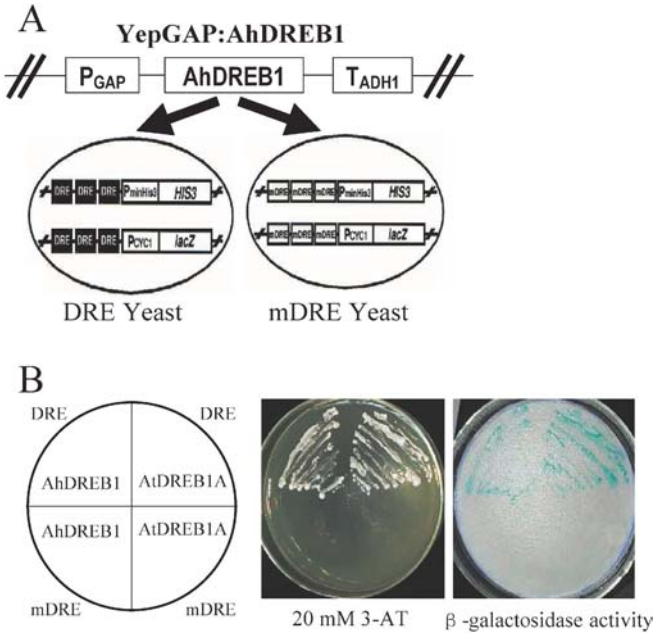


**Fig. 2A, B** AhDREB1 transactivated the dual reporter genes in the yeast one-hybrid system. **A** The AhDREB1 cDNA was cloned into the yeast expression vector YepGAP and used for transformation into DRE yeast and mDRE yeast as described in Materials and methods. **B** Heterogenous expression of the AhDREB1 gene in yeast strains carrying the dual reporter genes under the control of the DRE motifs or mDRE motifs. The expression of AtDREB1A in yeast was used as a control. The transformants were examined for growth in the presence of 3-AT and tested for $\beta$-galactosidase activity

AhDREB1 has much less identity when compared with DREB1A/CBF1 and DREB2A. In the EREBP/AP2 domain, a 18-amino acid region has been predicted to form an amphipathic $\alpha$-helix that may be important for interaction with other proteins (Okamuro et al. 1997). Three $\beta$-sheets were also found in the EREBP/AP2 domain of AhDREB1 and may be responsible for DNA-binding activity (Allen et al. 1998). In $\beta$-sheets, some residues have been identified as key residues for DNA-binding activity. In addition, mutation of another conserved residue V in $\beta$-sheet 2 of DREB1A caused an obvious decrease in DRE-binding activity, whereas mutation of E at the end of $\beta$-sheet 2 had little effect on the binding activity (Fig. 1 and Cao et al. 2001). It was thus proposed that the DRE motif may distinguish the binding protein from others through some particular conserved residues (Liu et al. 1998).

## Heterogenous expression, DRE-binding and transactivation activity of *AhDREB1*

To analyze the in vivo function of this putative DRE binding protein from *A. hortensis*, the cDNA insert was ligated into the yeast expression vector YepGAP and transformed into two yeast strains carrying wild-type and mutant DRE, respectively (Fig. 2A). In wild-type DRE yeast, transformants carrying the plasmid could grow on medium lacking histidine in the presence of 10-, 20-, 30- or 60-mM 3-AT, whereas the transformants of mutant DRE yeast could not grow on the same medium. Together with results of the $\beta$-galactose activity assay shown in Fig. 2B, it can be seen that heterogeneous expression of *AhDREB1* promoted expression of the *HIS3* and *LacZ* genes in wild-type DRE yeast but not in mutant DRE yeast, indicating that the *AhDREB1* gene of *A. hortensis* encodes a transcription factor that can specifically bind to the DRE sequence in the promoter region and activate the transcription of the downstream genes in vivo.

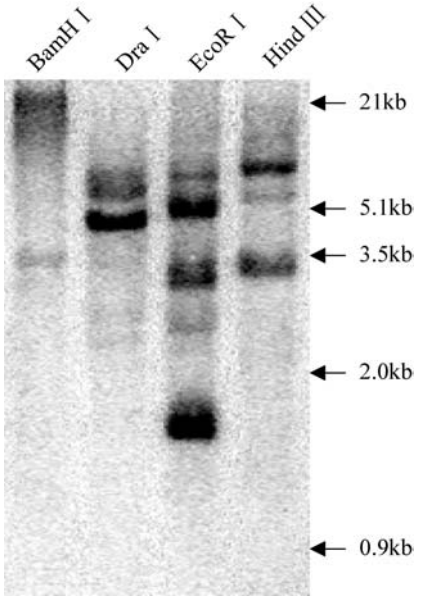


**Fig. 3** Southern-blot analysis of *AhDREB1*. The genomic DNA was completely digested with *Bam*HI, *Dra*I, *Eco*RI and *Hin*dIII, and subjected to hybridization using the full-length sequence of *AhDREB1* as a probe

## Genomic DNA analysis and organ-specific expression pattern

Genomic DNA gel-blot analysis was carried out to investigate the genomic organization of the *AhDREB1* gene. It was observed that two to five apparent hybridization bands were identified in each lane under high-stringency conditions (Fig. 3). Considering the presence of one *Hin*dIII site and one *Eco*RI site in the *AhDREB1* cDNA, it is likely that one to two copies of the *AhDREB1* gene were present in the genome of *A. hortensis*, although interpretation of such data is not simple.

Northern analysis was performed to investigate the expression of the *AhDREB1* gene (Fig. 4). It was observed that, upon salt stress, the *AhDREB1* expression was substantially induced in roots of *A. hortensis*. However, in stems and leaves, its expression was not significantly affected. The salt-inducible expression of the choline mono-oxygenase gene (*CMO*) encoding an enzyme in glycine betaine biosynthesis was also examined and compared as a positive control (Shen et al. 2002).

## Analysis of gene expression and stress tolerance of the transgenic tobacco plants

To characterize the function of the *AhDREB1* gene in plants, the *AhDREB1* gene was inserted into the binary vector pBI121 under the driving of the CaMV 35S promoter and was transformed into tobacco plants using the *Agrobacterium*-mediated transformation method. Nine primary transformants were obtained after selection on kanamycin media and confirmed by PCR analysis (data not shown). The expression of the *AhDREB1* gene in these primary transgenic tobacco lines was investigated and is shown in Fig. 5 that *AhDREB1* was expressed at various levels, with line No.5 and No.8 having relatively higher expression levels and other lines having lower expression levels. In the control tobacco plants, there was no detectable *AhDREB1* expression (Fig. 5). The expression of the DREB-regulated downstream genes, *rd17* and *rd29A*, was also examined by using the *Arabidopsis rd17* and *rd29A* genes as probes. These genes are typical effector molecules and have DRE sequences in their promoter region. The results in Fig. 5 showed that the two genes were expressed in all nine transgenic lines but at various levels. For *rd17*, its expression was almost identical in the nine lines, whereas for *rd29A*, its expression was relatively higher in line Nos. 5, 7, 8 and 9, but lower in other lines. In the control tobacco plants, there was no expression of the two genes. A *LHCP* (light

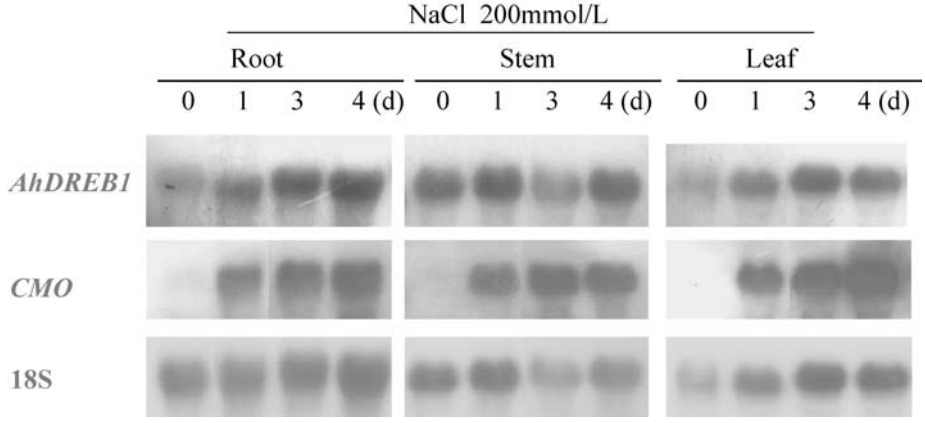


**Fig. 4** *AhDREB1* expression in roots, stems and leaves of *A. hortensis* in response to salinity. Young plants were stressed with 200 mM of NaCl for 4 days and 30 $\mu$g of RNA was used for Northern analysis. A choline mono-oxygenase gene from *A. hortensis*, *AhCMO* (AF270651), was used as a salt-induced marker gene (Shen et al. 2002). A 18S rDNA hybridization was also performed to normalize the loading

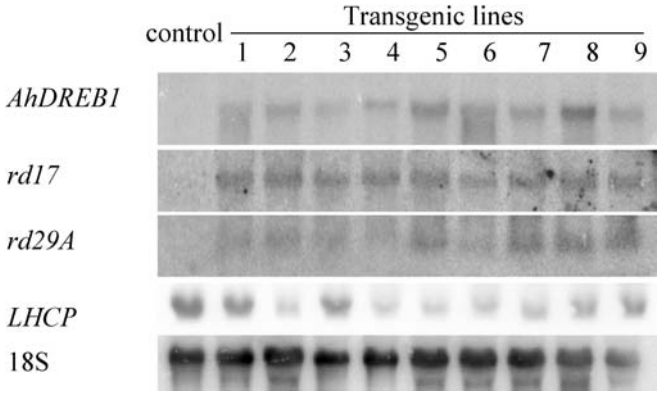


**Fig. 5** Expression of *AhDREB1* and its downstream genes in transgenic lines. Thirty micrograms of total RNA from control and nine transgenic lines were used in Northern analysis and probed with the *AhDREB1* gene. Two putative downstream marker genes, *rd17* (AB004872) and *rd29A* (D13044), were generated by PCR from Arabidopsis genomic DNA and used as homologous probes. These marker genes were all dehydration-induced and had DRE motifs in the promoters. The expression of an unrelated gene *LHCP* was examined for comparisons. A 18S rDNA hybridization was also performed to normalize the loading

harvesting chlorophyll protein) gene, which does not have DRE sequences in its promoter region, was found to be expressed in all the nine transgenic lines and the control plants.

To test the function of the *AhDREB1* gene in transgenic plants, we treated the transgenic lines and the control plant with NaCl and PEG, and observed their performance under these treatments. Two lines (No. 4 and No. 9) were found to be stress-tolerant, and the result from one line (No. 9) was presented. The result in Fig. 6A, B showed that, under normal condition, both the control and the transgenic line grew very well. Their dry weights were at similar levels. When exposed to NaCl stress, the growth of control plants was inhibited whereas the transgenic line remained green and healthy at 1.5% NaCl, with a dry weight two-fold higher than that of the control. At 2.0% NaCl, both the control and the transgenic line were strongly inhibited although the transgenic line survived better. The plants were also subjected to PEG treatment to mimic the drought stress (Fig. 6A). It can be seen that the transgenic line grew taller and healthy, and had more dry weight at both PEG concentrations when compared with the control (Fig. 6A, B). These results indicated that the *AhDREB1* gene conferred salt and drought tolerance in the transgenic tobacco plants. It should be noted that salt and drought stresses resulted in different phenotypes. Under salt stress, the transgenic plant had large and green leaves but with short stems. Whereas under PEG treatment, the transgenic plant had relatively small leaves but with long stems. It is possible that salt stress inhibited the shoot meristem and thus led to the stem retardation, whereas drought stress does not have such an effect.

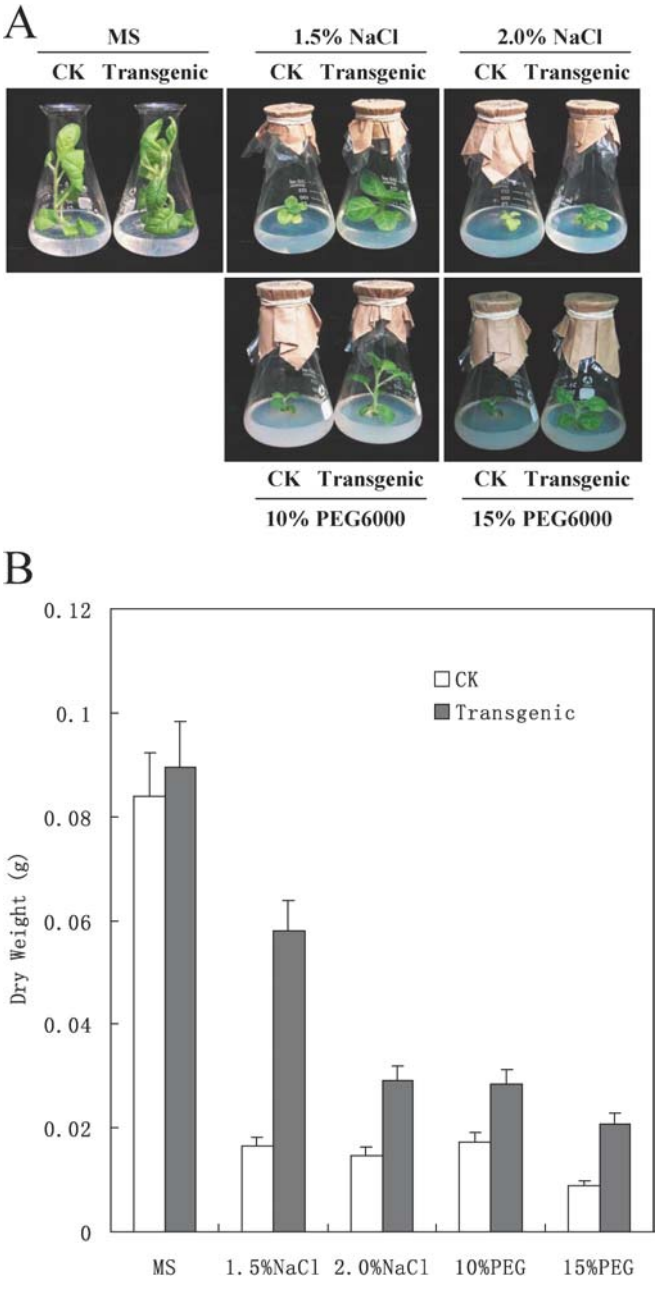


**Fig. 6A, B** Performance of *AhDREB1* transgenic tobacco upon NaCl and PEG treatments. **A** Phenotype of the transgenic plants under stressed conditions. Kanamycin-selected T1 seedlings from transgenic line No.9 were transferred into flasks containing MS, MS plus NaCl and MS plus PEG. The plants were photographed after culture for 3 weeks. The cover of the flask in the first panel was removed for better observation. **B** The plants in **A** were harvested after 1 more week and their dry weights were measured. Each value represented the average dry weight of 8–10 plants

## Discussion

The EREBP/AP2 domain is a conserved DNA-binding domain in a super family of plant transcription factors, in which some proteins such as AtERF, Pti, APETALA2 and AtDREB have important regulatory functions in ethylene

response, defense response, plant development and environment stress responses, respectively (Jofuku et al. 1994; Ohme-Takagi and Shinshi 1995; Zhou et al. 1997; Liu et al. 1998; Park et al. 2001). Previously, we have identified a DREB-like transcription factor gene from wheat. This gene was cold-responsive and was also expressed upon other stresses (Shen et al. 2003). In this study, we isolated and characterized a *AhDREB1* gene from a halophyte, *A. hortensis*. The in vivo binding assay in yeast showed that AhDREB1 could specifically bind the DRE motif in the promoter and activate the transcription of the fused reporter genes. Thus AhDREB1 is a DRE-binding transcription factor in *A. hortensis*.

*AhDREB1* expression in *A. hortensis* can be detected in the root, stem and leaf under normal conditions. This expression pattern indicated that AhDREB1 functioned in the normal program of plant growth and development. When exposed to salt stress, *AhDREB1* expression was induced in the root, but not in the stem and leaf. The inducible expression in the root implied that the root needed more AhDREB1 product to re-establish the disturbed endogenous environment caused by salt stress, and thus maintained normal growth and development of the plant. In *Arabidopsis*, *DREB2A* transcription can also be detected in root, stem and leaf while *DREB1A* was not expressed under normal conditions. Cold and salt stresses induced transcription of *DREB1A* and *DREB2A*, respectively (Liu et al. 1998). Comparison of these expression patterns indicated that AhDREB1 was probably more similar to DREB2A.

The *AhDREB1* gene was transformed into tobacco and its expression was investigated in each individual plant. We found that *AhDREB1* and its downstream gene *rd17* and *rd29A* were expressed at various levels. The performance of these lines under salt and drought stresses was examined and two lines (No. 4 and No. 9) were found to survive better under stressed conditions (Fig. 6 and data not shown). However, the better performance of transgenic plants does not correlate with the expression levels of the *AhDREB1* gene or its putative downstream genes *rd17* and *rd29A*. The two lines that showed stress-tolerance only expressed *AhDREB1* in a middle level, whereas the two lines (No. 5, No. 8) of higher *AhDREB1* expression did not show tolerance to stresses (data not shown). This inconsistency may reflect the complexity and subtleness of the functions of the transgene in transgenic plants. In *Arabidopsis*, overexpression of the *DREB1A* and *DREB2A* gene caused a dwarf phenotype in transgenic plants (Liu et al. 1998). In a previous study, we have isolated a DREB-like gene (*TaDREB1*) from wheat (Shen et al. 2003). Overexpression of *TaDREB1* in rice led to the dwarf phenotype, whereas overexpression in *Arabidopsis* resulted in a normal phenotype. In the present study, the *AhDREB1* transgenic tobacco plants have a normal phenotype under normal conditions. These facts suggested that different DREB members may play slightly different roles in various plant systems and hence resulted in different effects.

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