Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 353 (2007) 299–305
BBRC
www.elsevier.com/locate/ybbrc

GmDREB2, a soybean DRE-binding transcription factor, conferred drought and high-salt tolerance in transgenic plants

Ming Chen [a], Qiao-Yan Wang [a], Xian-Guo Cheng [b], Zhao-Shi Xu [a], Lian-Cheng Li [a], Xing-Guo Ye [a], Lan-Qin Xia [a], You-Zhi Ma [a,*]

[a] *National Key Facility for Crop Gene Resources and Genetic Improvement (NFCRI), Key Laboratory of Crop Genetics and Breeding, Ministry of Agriculture, Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing 100081, China*
[b] *Institute of Natural Resources and Regional Planning, CAAS, Beijing 100081, China*

Received 24 November 2006
Available online 13 December 2006

Abstract

A novel DREB (dehydration responsive element binding protein) homologous gene, *GmDREB2*, was isolated from soybean. Based on its similarity with AP2 domains, *GmDREB2* was classified into A-5 subgroup in DREB subfamily in AP2/EREBP family. Expression of *GmDREB2* gene was induced by drought, high salt, and low temperature stresses and abscisic acid treatment. The GmDREB2 bound specifically to DRE element *in vitro*. Furthermore, the overexpression of *GmDREB2* activated expression of downstream genes in transgenic *Arabidopsis*, resulting in enhanced tolerance to drought and high-salt stresses and did not cause growth retardation. Analysis of free proline contents in transgenic tobacco indicated that the overexpression of *GmDREB2* accumulated higher level of free proline compared to the wild type plants under drought condition. The results from this study indicate that this novel soybean *GmDREB2* gene functions as an important transcriptional activator and may be useful in improving of plant tolerance to abiotic stresses in plants.

Keywords: Abiotic stress; DREB transcription factor; *Glycine max* L.; Drought tolerance; High-salt tolerance; Free proline

Drought and high-salt stresses cause yield losses on as much as half of the world's irrigated lands. Molecular and genomic analyses have shown that there are different transcriptional regulatory pathways involved in stress-responsive gene expression. The *cis*-acting elements and *trans*-acting factors were involved in the transcriptional regulation of a dynamic network of genes controlling various biological processes, including abiotic and biotic stress response [1]. Dehydration responsive element (DRE) with the core sequence A/GCCGAC and similar elements CRT (C-repeat) were identified as an important *cis*-acting element in regulating gene expression under drought, high salt, and cold stresses in *Arabidopsis* [2]. Three DRE-binding protein including *AtDREB1A*, *AtDREB2A*, and C-repeat-binding factor 1 (*CBF1*) were isolated from *Arabidopsis* [3].

In *Arabidopsis*, 145 AP2/EREBP-related transcription factors reported so far were classified into five subfamilies, i.e., DREB, APETALA2, RAV (related to ABI3/VP1), ERF (ethylene-responsive transcription factor) and a very specific gene AL079349, based on the similarities of their DNA-binding domain (AP2 domain). Among them, the DREB subfamily was further divided into 6 subgroups (A-1 to A-6) [4]. Subgroups A-1 and A-2, harbouring the DREB1-type and DREB2-type genes, respectively, are the largest ones that are involved in two ABA-independent pathways [3,5]. The DREB1-type genes (*AtDREB1A*, *AtDREB1B*, and *AtDREB1C*) were thought to be involved in cold-responsive pathway, whereas DREB2-type genes (*AtDREB2A* and *AtDREB2B*) were mainly involved in osmotic-responsive pathway [1]. DNA-binding specificities

* Corresponding author. Fax: +86 10 6891 8789.
E-mail address: mayzh@mail.caas.net.cn (Y.-Z. Ma).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.027

and expression pattern of the genes, which belong to A-5 subgroups under stress condition, remain unclear.

Overexpression of DREB homologous gene isolated from various plants, such as *Arabidopsis*, rice (*Oryza sativa* L.), and maize (*Zea mays* L.), could enhance tolerance transgenic plants to abiotic stresses [3,6,7]. Soybean (*Glycine max* L.) is one of the most economically important crops in the world. Expressed sequence tags (EST) analyses of transcription factors in soybean indicated that the AP2/EREBP family contained 290 members [8]. However, few studies on AP2/EREBP transcription factor gene in soybean have been reported. Recently, three DREB homologous genes, *GmDREBa*, *GmDREBb*, and *GmDREBc*, were isolated from soybean and the expression characteristics of these genes were further investigated, but their functions remain unclear [9].

In this paper, we report a novel DREB homologous gene, *GmDREB2*, which was isolated from soybean and classified into A-5 subgroup in DREB subfamily. Our objectives were (i) to characterize the expression pattern, DNA-binding ability of GmDREB2 protein, and transcriptional activation activity; and (ii) to investigate the function through expression of *GmDREB2* in transgenic *Arabidopsis* and tobacco plants.

Materials and methods

Plant materials and growth conditions. *Arabidopsis* plants (ecotype Colombia) used for transformation were grown in soil at 22 °C and approximately 70% humidity under 14 h light and 10 h dark. T_2 seeds of transformed *Arabidopsis* were surface-sterilized and planted on MS medium supplemented with 50 μg ml^{-1} of kanamycin for selecting transgenic plants. After seedlings emergence, transgenic plants were transferred into pots for further functional analyses. The seedlings of tobacco (*Nicotiana tabacum* L. cv. W38) grown on MS medium agar plates were used for transformation. Transgenic tobacco plants were identified by PCR, and seedlings were transferred into pots, which were placed in 12 h light and 12 h dark at 25 °C for further functional analyses.

Expression characterization of GmDREB2 gene under various stress treatments. Soybean cv. Tiefeng 8 is salt tolerant and was used for gene isolation and expression pattern analyses. Initially, soybean seeds were grown in pots (12 h light and 12 h dark at 25 °C) irrigated with water. To determine expression pattern of *GmDREB2* gene under high-salt stress, 2-week-old soybean plants were removed from soil and soaked in a solution containing 250 mM NaCl for various periods. Dehydration was induced by removing plants from the plots and placing them on filter paper at 25 °C under dim light for various periods. For abscisic acid (ABA) treatment, leaf tissues of the soybean plants were sprayed with 200 μM ABA solution and then harvested at different time points. For cold treatment, soybean plants were transferred into refrigerator at 4 °C under dim light. After exposure to these stresses, soybean plants were immediately frozen in liquid nitrogen for analyzing expression pattern. Total RNA was extracted from plant samples harvested at different time points. Hybridization was performed as described in the Instruction Manual of Hybond-N^+ nylon membrane filter (Amersham Biosciences, Piscataway, USA). The *GmDREB2* probe was labeled with [α-^{32}P]dCTP and Random Primer DNA Labeling Kit (TaKaRa Biotech, Dalian, China).

Glutathione S-transferase (GST) fusion proteins preparation and gel mobility shift assay. The 480 bp fragment of gene *GmDREB2* containing DNA-binding domain was amplified using the primer pair: GmDREB2-1: 5′-GGGGAATTCATGGAAGAAGCGGGTTTA-3′ (forward); GmDREB2-2: 5′-GGGCTCGAGATCTTCAGGTTTGGGATA-3′ (reverse). This fragment was cloned into the *Eco*RI–*Xho*I sites of the pGEX4T-1 vector (Amersham Biosciences, Piscataway, USA) and transformed into *Escherichia coli* BL21 cells (Amersham Biosciences, Piscataway, USA) to produce the GST-fusion protein. The GST-fusion protein was purified using a glutathione Sepharose 4B column according to the manufacturer's instruction (Amersham Biosciences, Piscataway, USA). The DNA probe preparation and gel mobility shift assay was conducted as described previously [3]. The 37 bp DNA fragments containing two copies of the wild type or the mutant DRE elements were synthesized. The probe sequence of the wild type and the mutant DRE element is shown in Fig. 2A.

Plant transformation and stresses tolerance analyses of transgenic Arabidopsis plants. *GmDREB2* gene was cloned into polylinker site of two binary vectors, p35S and pRd29A, through the restriction sites of *Sma*I and *Sac*I to product p35S-GmD2 and pRd29A-GmD2, respectively. Gene *GmDREB2* was driven by the constitute expressing promoter CaMV35S (cauliflower mosaic virus 35S) and stress-inducible promoter Rd29A in vector p35S-GmD2 and pRd29A-GmD2, respectively. These plasmids were introduced into *Agrobacterium tumefaciens* strain C58C1. *Arabidopsis* plants used for transformation were grown in 8-cm pots at 25 °C for 5 weeks and then transformed by vacuum infiltration as described by Bechtold and Pelletier [10]. T_2 transgenic *Arabidopsis* lines were identified by kanamycin resistant selection and further used for functional analyses. For drought tolerance analysis, the wild type, 35S::GmDREB2, and Rd29A::GmDREB2 transgenic *Arabidopsis* plants grown in pots were treated without watering for 19 days before resuming watering. At the eighth day after rewatering, survival rate was determined. For high-salt tolerance analysis, the wild type and 35S::GmDREB2 transgenic *Arabidopsis* plants grew on MS medium in the presence of 200 mM NaCl for 22 days at 22 °C. Then, the growth status of the wild type and 35S::GmDREB2 transgenic plants was evaluated. The p35S-GmD2 vector was also transformed into tobacco mediated by *A. tumefaciens* strain EH105. Transgenic tobaccos were identified using PCR amplification with specific primers of GmDREB2-1 and GmDREB2-2.

Content analysis of free proline as osmolyte in GmDREB2 transgenic tobacco plants. For measuring of free proline content, the wild type and transgenic tobacco plants were grown in pots under 12 h light and 12 h dark at 25 °C. Then, 8-week-old plants were prevented from watering for various durations and leaves were harvested at different time points. Free proline contents of plants were measured as described previously by Zhang et al. [11]. Data of free proline content were analyzed by SAS (Statistical Analysis System) software (SAS Corporation, Cory, NC, USA) by the *t*-test to assess significant differences among means.

Results

Isolation and phylogenetic analysis of the GmDREB2 gene

To isolate the genes encoding the DRE-binding protein from soybean, an AP2 consensus sequence was used as a query to search the EST database of soybean in GenBank (http://www.ncbi.nlm.nih.gov/), and four partial sequences were obtained. The full-length cDNA sequence of a DREB homologue, *GmDREB2* gene (GenBank Accession No. DQ208968), was further isolated from soybean total RNA via RACE method. It is 480 bp in length with a complete open reading frame of 159 amino acids. Further analysis of its deduced amino acids sequence using SMART program (http://smart.embl-heidelberg.de) revealed that this protein contained a conserved DNA-binding domain (AP2 domain) of 58 amino acids and putative nuclear localization signal sequence (NLS) (Fig. 1A in supplementary files). To investigate the relationship between *GmDREB2* and other DREB genes, the deduced amino acid sequence of *GmDREB2* gene was compared with other

six DREB proteins from soybean and *Arabidopsis*, respectively. GmDREB2 had the same 14th valine and the 19th glutamic acid in AP2 domain as other DREB gene, suggesting that *GmDREB2* is a member of the DREB subfamily (Fig. 1A in supplementary files). Among them, GmDREB2 is closely related to soybean GmDREB1 (AF514908), with 64% identity at the amino acid level, and has less similarity with other DREB proteins from soybean (i.e., GmDREBa, GmDREBb, and GmDREBc) and the DREB proteins from *Arabidopsis* (i.e., AtDREB1A and AtDREB2A) (Fig. 1B in supplementary files).

To further determine the evolutionary distance among the AP2/EREBP proteins, systematic phylogenetic analysis was carried out based the similarities of AP2 domains in AP2/EREBP proteins isolated from soybean, *Arabidopsis*, maize, rice, tomato (*Lycopersicon esculentum* L.), barley (*Hordeum vulgare* L.), cotton (*Gossypium hirutum* L.), tobacco, and *Brassica napus* L. using the CLUSTAL W software [4]. GmDREB2 was classified into the A-5 subgroup of DREB subfamily (Fig. 2 in supplementary files). Besides, soybean GmDREB1, *Arabidopsis* RAP2.1, and RAP2.10 also belong to the A-5 subgroup. However, the characteristics and function of *GmDREB1*, *RAP2.1*, and *RAP2.10* remain unclear.

Expression characteristics of GmDREB2 gene in soybean

Northern blot analysis showed that the transcription of *GmDREB2* gene was responsive to drought, high salt, and cold stresses and ABA treatment (Fig. 1). Under drought treatment, *GmDREB2* mRNA began to accumulate at 1 h and reached its maximum at 6 h after treatment. At 24 h, no mRNA was detected. Under high-salt condition, the expression pattern of *GmDREB2* was similar to that of drought treatment. For cold treatment, the transcription of *GmDREB2* was induced later than that of the drought treatment, and reached its maximum at 17 h. Interestingly, the expression of *GmDREB2* was also induced by ABA treatment at 0.5 h, reached its maximum at 3 h, and then began to reduce at 17 h. These results suggest that unlike DREB1-type and DREB2-type transcription factors, *GmDREB2* was responsive to ABA phytohormone signal and involved in ABA-dependent signal pathways.

DNA-binding activity analysis of GmDREB2 using gel mobility shift assay

Gel mobility shift assay revealed that the DRE element could interact with GmDREB2-GST fusion protein and retarded on SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis), while the DRE core sequence with two-base substitution at 5′ end or three-base substitution at 3′ end or completely mutant in DRE core sequence inhibited its interaction with GmDREB2-GST fusion protein severely (Fig. 2B). These results suggest that GmDREB2 protein could specifically bind to DRE element containing -CCGAC- core sequence *in vitro*.

Overexpression of GmDREB2 gene in Arabidopsis activated expression of downstream genes containing DRE element

Using three genes *Rd29A*, *Rd17A*, and *cor15a*, which contain DRE element in their promoter regions and already have been identified as downstream genes of *AtDREB1A* in *Arabidopsis*, semi-quantitative RT-PCR analysis was performed with the wild type and GmDREB2 transgenic plants [12]. Constitutive high-level expression of *GmDREB2*, *Rd29A* and *cor15a* was clearly observed in T_2 generation of 35S::GmDREB2 transgenic *Arabidopsis* plants, and the expression of these genes was increased in Rd29A::GmDREB2 transgenic *Arabidopsis* upon drought treatment with the expression level of *Rd29A* and *cor15a* correlated with that of the *GmDREB2* (Fig. 2C). Thus, it is most likely that *GmDREB2* could probably activate expression of stress-related genes containing DRE element in promoter regions, which, in turn, exhibit causal effect in improving stress tolerance of transgenic *Arabidopsis* plants. However, expression of *Rd17A* gene remained unchanged in transgenic plants. This might indicate that GmDREB2 could activate the expression of *Rd29A* and *cor15a* gene

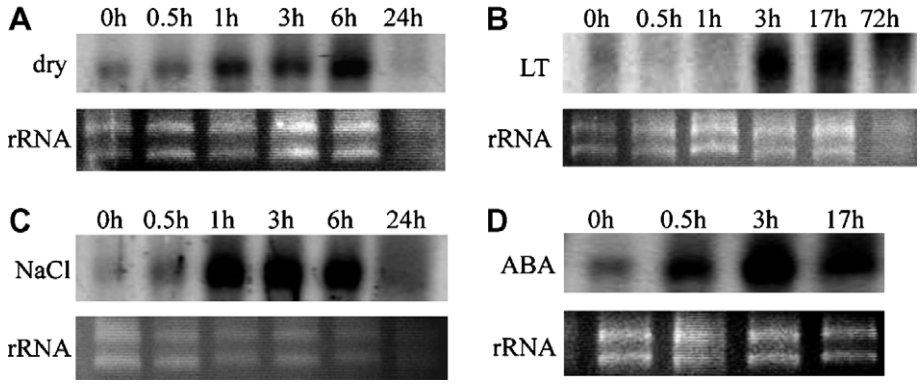

Fig. 1. Expression pattern of *GmDREB2* gene in soybean under different abiotic stresses condition including drought (A), low temperature (B), 200 mM NaCl (C), and 200 μM ABA (D). The rRNA was used as the RNA control.

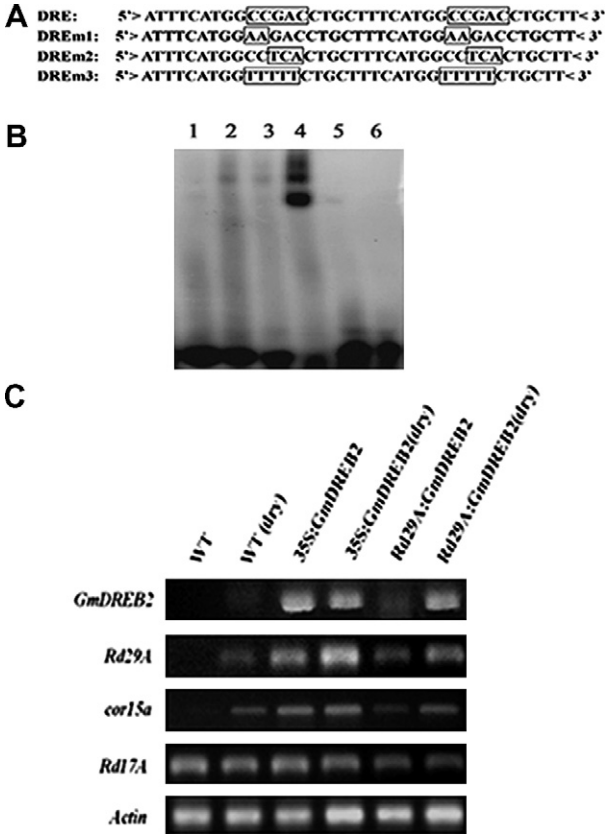

Fig. 2. Analyses of DNA-binding activity and transcriptional activation activity of GmDREB2 protein. (A) The nucleotide sequences of DRE (DRE) and mutated DRE (DREm1-m3) probes used in gel mobility shift assay of GmDREB2. The nucleotide mutation in DRE core motif of each probe is shown in box. (B) Gel mobility shift assay shows GmDREB2 binding to DRE probes. Lanes 1–3, fusion GST-GmDREB2 proteins plus labeled the mutant DRE element (DREm1-m3); lane 4, fusion GST-GmDREB2 proteins plus labeled the wild type DRE element; lane 5, GST proteins control; and lane 6, free labeled DRE probes. (C) To investigate the transcriptional activation of downstream genes controlled by GmDREB2 in plants, total RNA were isolated from two-week-old wild type (WT) and the transgenic *Arabidopsis* (35S::GmDREB2 and Rd29A::GmDREB2) plants that had been dehydrated for 3 h (dry) or untreated. Semi-quantitated RT-PCR analysis was performed using genes *Rd29A*, *cor15a*, and *Rd17A* which contain DRE element in their promoter regions and already have been identified as downstream genes of *AtDREB1A* in *Arabidopsis*. The house-keeping gene (*Actin* gene) was shown as equal controls.

in transgenic *Arabidopsis* plants but not that of *Rd17A* gene.

Overexpression of GmDREB2 gene improved stress tolerance of transgenic plants

Drought tolerance analysis indicated that after drought treatment and rewatering, all the wild type *Arabidopsis* plants died (0/58), whereas 45.9% (28/61) and 21% (8/38) of the 35S::GmDREB2 and the Rd29A::GmDREB2 transgenic plants survived, respectively, although their survival rate under normal growth condition was 100% (Fig. 3). These results indicated that the expression of *GmDREB2* gene improved drought tolerance of the transgenic plants.

For high-salt tolerance analysis, seeds of the wild type, 35S::GmDREB2 and Rd29A::GmDREB2 transgenic *Arabidopsis* were germinated and maintained on MS medium containing 0 and 200 mM NaCl for 22 days at 22 °C, respectively. The growth status of these plants is shown in Fig. 4. From the seedling stage on normal MS medium (Fig. 4A,a) to the mature stage in soil (Fig. 4A,b), there was no morphological difference between the transgenic and the wild type *Arabidopsis* plants. However, when supplemented with 200 mM NaCl, the phenotypes of the wild type and the transgenic plants were different and the 35S::GmDREB2 plants displayed strong tolerance against high-salt stress with surviving rate higher than that of the wild type plants (Fig. 4B).

To evaluate physiological changes of transgenic plants, contents of free proline as osmolyte in the wild type and 35S::GmDREB2 transgenic tobaccos were measured following drought treatment. After 16 d, free proline content in the wild type tobacco under drought stress condition increased markedly and was approximately 4.5-fold higher than that under normal condition. However, even under control condition, 35S::GmDREB2 transgenic lines D26-37 and D26-39 accumulated 2.8- and 1.8-fold higher levels of free proline, respectively, compared with the wild type tobacco ($P < 0.05$). After 12 d and 16 d of drought treatments, the transgenic lines D26-37 and D26-39 accumulated higher level of free proline than the wild type plants (Fig. 4C).

Discussion

The expression of GmDREB2 was induced by drought, high salt, cold stresses, and ABA treatments and might be involved in both ABA-independent and ABA-dependent regulatory pathways

Many abiotic-stress-inducible genes are controlled by ABA, but some are not, which indicates that both ABA-dependent and ABA-independent regulatory pathways are involved in stress-responsive gene expression [13]. Recent research on gene regulatory pathway in response to drought, high salt, and cold stresses shows that there are at least five regulatory pathways involved [13]. Two regulatory pathways are ABA-dependent, and the other three are ABA-independent [1]. The regulatory elements are important molecular switches involved in the transcriptional regulation of a dynamic network of gene activities controlling various biological processes and two major *cis*-acting elements, ABRE and DRE, are involved in ABA-dependent and ABA-independent regulatory pathways, respectively. Interactions among different *cis*-acting elements function in cross-talk between different regulatory pathways [1]. For example, the interaction of *cis*-acting elements was observed between ABRE and DRE in the Rd29A promoter, and expression of *Rd29A* gene was induced by drought, high salt, and cold stresses and ABA treatment [14]. In this study, one copy of CRT/DRE element and ABRE element

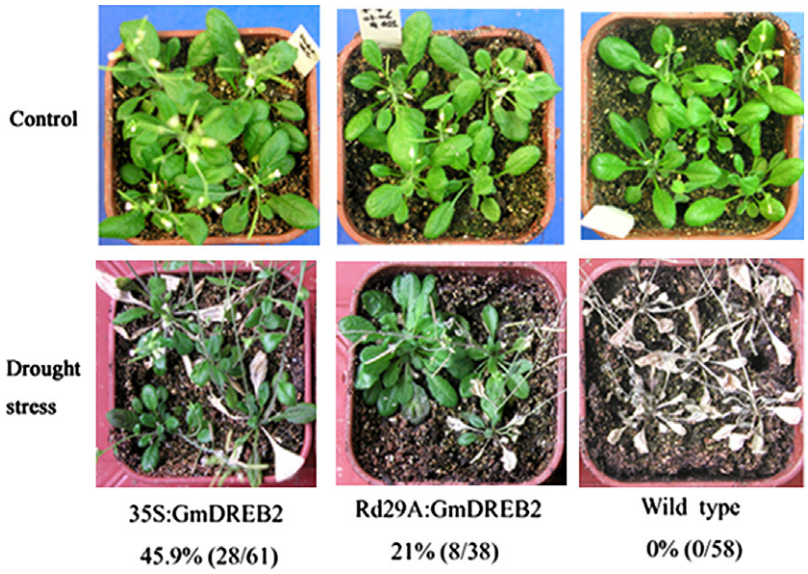

Fig. 3. Drought tolerance analysis of 35S::GmDREB2 and Rd29A::GmDREB2 transgenic *Arabidopsis* plants. Control, 3-week-old plants grew under normal conditions; Drought stress, the wild type and the transgenic *Arabidopsis* plants had been treated without watering for 19 d, followed by rewatering. At the eighth day after rewatering, the growth status of the wild type and the transgenic *Arabidopsis* plants are shown. Percentages of the survival plants and numbers of survival plants and total number of tested plants are shown under the photographs.

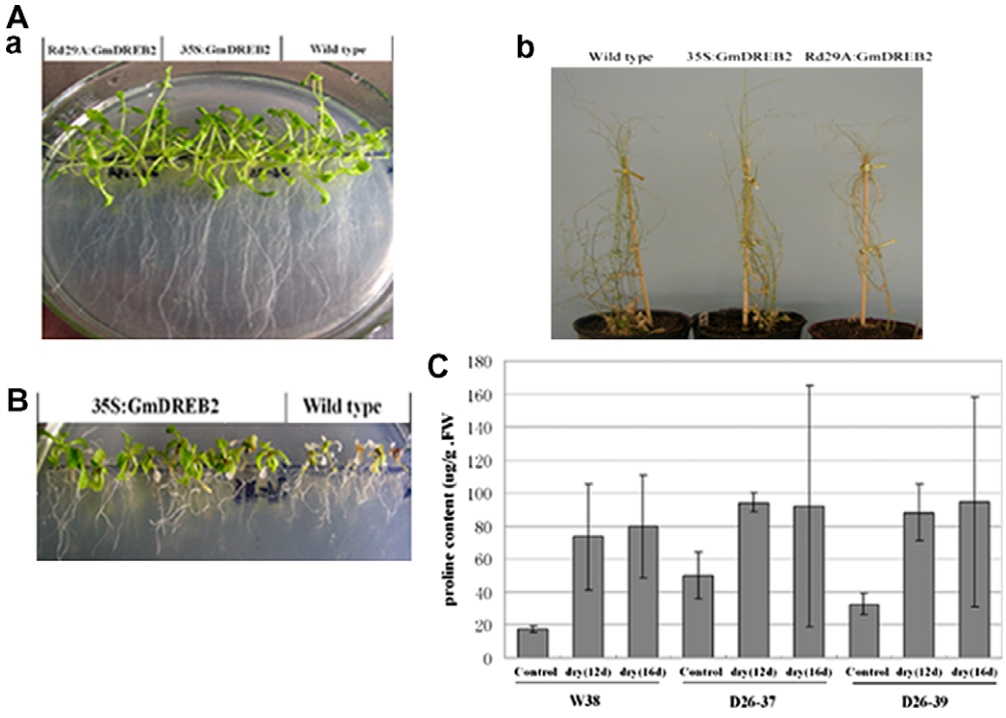

Fig. 4. High salt tolerance and free proline contents analyses of transgenic plants. (A) Wild type, Rd29A::GmDREB2 and 35S::GmDREB2 plants grew on normal MS medium for 22 d (a) and in soil for 80 d (b) at 22 °C. (B) Wild type and transgenic *Arabidopsis* plants grew on MS medium in the presence of 200 mM NaCl for 22 d at 22 °C. (C) The wild type (W38) and transgenic tobacco lines (D26-37 and D26-39) were grown in pots under normal condition for 8 weeks and then leaves of plants were harvested as control. Then, the wild type and transgenic plants were treated without watering 16 d (dry), while leaves were harvested at 12th and 16th days for free proline content analysis, respectively.

were detected in the promoter region of *GmDREB2* gene by isolating promoter sequence and searching *cis*-acting element against the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Fig. 1C in supplementary files).

Most of DREB genes isolated from various plants belong to A-1 and A-2 subgroup in DREB subfamily. These genes were not activated by ABA treatment and were involved in the ABA-independent pathways [3,6]. In this study, the expression pattern analysis showed that

GmDREB2 was induced by drought, high salt, and cold stresses and ABA treatment (Fig. 1). In addition, the expression pattern of *GmDREB2* gene was similar to *Rd29A* gene and markedly different from *AtDREB1* and *AtDREB2* genes [3]. These results suggest that unlike DREB gene in A-1 and A-2 subgroups, which are involved only in ABA-independent pathway, *GmDREB2* acts as an overlap point and might take part in both ABA-independent and ABA-dependent pathways, simultaneously.

Expression of GmDREB2 gene in plants conferred tolerance against drought and high-salt stresses and caused no growth retardation

Expression of *GmDREB2* gene induced the expression of *Rd29A* and *cor15a* genes, that already have been identified as downstream genes of *AtDREB1A* in *Arabidopsis*, and therefore enhanced their tolerance to drought and high-salt stresses. In the present study, we observed that 35S::GmDREB2 transgenic tobaccos accumulated higher levels of free proline that function as osmolyte in the stress tolerance of plants, than the wild type plants under unstressed and drought stress conditions (Fig. 4C) [15]. These results suggest that overexpression of *GmDREB2* gene activated the expression of some downstream genes involving free proline biosynthesis, which, in turn, enhanced tolerance to drought stresses in transgenic plants.

Overexpression of *OsDREB1A* and *AtDREB1A* genes caused mild growth retardation of transgenic plants, whereas overexpression of the *OsDREB2A* and *AtDREB2A* genes resulted in little phenotypic change [6]. Maruyama et al. [12] studied the mechanism of growth retardation of the 35S::DREB1A transgenic plants and found that some transcriptional factors were up-regulated by AtDREB1A, such as *STZ* and At5g04340, whose expression represses photosynthesis and carbohydrate metabolism in transgenic plants [12]. Thus, using the stress-inducible Rd29A promoter to controlling expression of *AtDREB1A* gene, the growth retardation could be minimized in transgenic plants [16]. In this study, both Rd29A and CaMV35S promoters were employed to control expression of *GmDREB2* gene in transgenic plants, and the wild type, 35S::GmDREB2, and Rd29A::GmDREB2 transgenic plants had no obvious phenotypic changes during either the seedling stage on media (Fig. 4A,a) or the mature stage in soil (Fig. 4A,b). Moreover, we found that the surviving rate of 35S::GmDREB2 plants was higher than that of Rd29A::GmDREB2 plants. It was postulated that because GmDREB2 could not activate expression of downstream gene repressing growth of plants, phenotypic changes arisen from these downstream genes were not observed.

Acknowledgments

The authors are grateful to Dr. Gerard Branlard of INRA, France and Dr. Hongjie Li from CAAS, China for their critical review of this manuscript. This work was funded by National 863 high-tech Project (2002AA 224081) and National Key Project for Researches on Transgenic Plant (JY03-A-18-01).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.027.

References

[1] K. Yamaguchi-Shinozaki, K. Shinozaki, Organization of *cis*-acting regulatory elements in osmotic- and cold-stress responsive promoters, Trends Plant Sci. 10 (2005) 88–94.
[2] K. Yamaguchi-Shinozaki, K. Shinozaki, A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress, Plant Cell 6 (1994) 251–264.
[3] Q. Liu, M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinazaki, K. Shinozaki, Two transcription factors, DREB1 and DREB2, with an AP2/EREBP DNA-binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression in *Arabidopsis*, Plant Cell 10 (1998) 1391–1406.
[4] Y. Sakuma, Q. Liu, J.G. Dubouzet, H. Abe, K. Shinozaki, K. Yamaguchi-Shinozaki, DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold- inducible gene expression, Biochem. Biophys. Res. Commun. 290 (2002) 998–1009.
[5] Z.K. Shinwari, K. Nakashima, S. Miura, M. Kasuga, M. Seki, K. Yamaguchi-Shinozaki, K. Shinozaki, An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression, Biochem. Biophys. Res. Commun. 250 (1998) 161–170.
[6] J.G. Dubouzet, Y. Sakuma, Y. Ito, M. Kasuga, E.G. Dubouzet, S. Miura, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high- salt- and cold-responsive gene expression, Plant J. 33 (2003) 1–13.
[7] F. Qin, Y. Sakuma, J. Li, Q. Liu, Y.Q. Liu, K. Shinozaki, K. Yamaguchi-Shinozaki, Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L., Plant Cell Physiol. 45 (2004) 1042–1052.
[8] A.G. Tian, J. Wang, P. Cui, Y.J. Han, H. Xu, L.J. Cong, X.G. Huang, X.L. Wang, Y.Z. Jiao, B.J. Wang, Y.J. Wang, J.S. Zhang, S.Y. Chen, Characterization of soybean genomic features by analysis of its expressed sequence tags, Theor. Appl. Genet. 108 (2004) 903–913.
[9] X.P. Li, A.G. Tian, G.Z. Luo, Z.Z. Gong, J.S. Zhang, S.Y. Chen, Soybean DRE-binding transcription factors that are responsive to abiotic stress, Theor. Appl. Genet. 110 (2005) 1355–1362.
[10] N. Bechtold, G. Pelletier, In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration, Methods Mol. Biol. 82 (1998) 259–266.
[11] D.Z. Zhang, P.H. Wang, H.X. Zhao, Determination of the content of free proline in wheat leaves, Plant Physiol. Commun. 4 (1990) 62–65 (in Chinese).
[12] K. Maruyama, Y. Sakuma, M. Kasuga, Y. Ito, M. Seki, H. Goda, Y. Shimada, S. Yoshida, K. Shinozaki, K. Yamaguchi-Shinozaki, Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems, Plant J. 38 (2004) 982–993.

[13] K. Shinozaki, K. Yamaguchi-Shinozaki, M. Seki, Regulatory network of gene expression in the drought and cold stress responses, Curr. Opin. Plant Biol. 6 (2003) 410–417.

[14] Y. Narusaka, K. Nakashima, Z.K. Shinwari, Y. Sakuma, T. Furihata, H. Abe, M. Narusaka, K. Shinozaki, K. Yamaguchi-Shinozaki, Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of *Arabidopsis rd29A* gene in response to dehydration and high-salinity stresses, Plant J. 34 (2003) 137–148.

[15] T. Taji, C. Ohsumi, S. Iuchi, M. Seki, M. Kasuga, M. Kobayashi, K. Yamaguchi-Shinozaki, K. Shinozaki, Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*, Plant J. 29 (2002) 417–426.

[16] M. Kasuga, S. Miura, K. Shinozaki, K. Yaimaguchi-Shinozaki, A combination of the *Arabidopsis DREB1A* gene and stress-inducible rd29A promoter improved drought- and low temperature stress tolerance in tobacco by gene transfer, Plant Cell Physiol. 45 (2004) 346–350.