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RESEARCH PAPER

Cold-induced modulation and functional analyses of the DRE-binding transcription factor gene, *GmDREB3*, in soybean (*Glycine max* L.)

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Abstract

DREB (dehydration-responsive element-binding protein) transcription factors have important roles in the stress-related regulation network in plants. A DREB orthologue, *GmDREB3*, belonging to the A-5 subgroup of the DREB subfamily, was isolated from soybean using the RACE (rapid amplification of cDNA ends) method. Northern blot analysis showed that expression of *GmDREB3* in soybean seedlings was induced following cold stress treatment for 0.5 h and was not detected after 3 h. However, it was not induced by drought and high salt stresses or by abscisic acid (ABA) treatment. This response was similar to those of members in the A-1 subgroup and different from those of other members in the A-5 subgroup, suggesting that the *GmDREB3* gene was involved in an ABA-independent cold stress-responsive signal pathway. Furthermore, analysis of the *GmDREB3* promoter elucidated its cold-induced modulation. A promoter fragment containing bases −1058 to −664 was involved in response to cold stress, and its effect was detected for 1 h after treatment, but a transcriptional repressor appeared to impair this response by binding to a *cis*-element in the region −1403 to −1058 at 24 h after the beginning of cold stress. Moreover, the GmDREB3 protein could specifically bind to the DRE element *in vitro*, and activated expression of downstream reporter genes in yeast cells. In addition, overexpression of *GmDREB3* enhanced tolerance to cold, drought, and high salt stresses in transgenic *Arabidopsis*. Physiological analyses indicated that the fresh weight and osmolality of *GmDREB3* transgenic *Arabidopsis* under cold stress were higher than those of wild-type controls. *GmDREB3* transgenic tobacco accumulated higher levels of free proline under drought stress and retained higher leaf chlorophyll levels under high salt stress than wild-type tobacco. In addition, constitutive expression of *GmDREB3* in transgenic *Arabidopsis* caused growth retardation, whereas its expression under control of the stress-inducible *Rd29A* promoter minimized negative effects on plant growth under normal growth conditions, indicating that a combination of the *Rd29A* promoter and *GmDREB3* might be useful for improving tolerance to environmental stresses in crop plants.

Key words: Abiotic stress, DREB transcription factor, drought tolerance, gene function, soybean.

Introduction

Plants encounter variable environmental stresses such as cold and osmotic stresses that may affect normal growth and productivity. Investigations on physiological, biochemical, and molecular aspects of plant stress tolerance have unravelled aspects of the intrinsic mechanisms developed during evolution to mitigate against stresses (Vij and Tyagi, 2007).

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So far, 299 drought-inducible genes, 213 high-salt stress-inducible genes, and 54 cold-inducible genes have been identified in *Arabidopsis* (Seki *et al.*, 2002; Shinozaki and Yamaguchi-Shinozaki, 2003). Among these genes, many are transcription factors involved in responses to drought, high salt, and cold stresses, and regulate the expression of downstream target genes through specific binding to *cis*-acting elements in the promoters of down-regulated genes (Liu *et al.*, 1998; Bartels and Sunkar, 2005; Vinocur and Altman, 2005). These genes were classified into several large families, such as AP2/EREBP, bZIP, NAC, MYB, MYC, Cys2His2 zinc-finger, and WRKY (Umezawa *et al.*, 2006).

AP2/EREBP is an important transcription factor family. In *Arabidopsis*, 145 AP2/EREBP transcription factors were classified into five subfamilies, including DREB(dehydration-responsive element-binding protein), ERF (ethylene-responsive transcription factor), AP2 (APETALA 2), RAV (related to ABI3/VP1), and one very specific gene, AL079349, based on the similarities of their DNA-binding domain (AP2 domain). Genes belonging to the DREB subfamily were thought to be important switches to regulate expression of many stress-inducible genes. The group was further divided into six subgroups (A-1–A-6), among which *DREB1*/*CBF* (*C-repeat binding factor*)-like genes, belonging to the A-1 subgroup, are induced by low temperature and activate the expression of many cold stress-responsive genes, whereas *DREB2*-like genes, belonging to the A-2 subgroup, are mainly involved in osmotic stress-responsive gene expression (Sakuma *et al.*, 2002; Nakashima and Yamaguchi-Shinozaki, 2006). Currently, homologous *DREB1*/*CBF* genes have been identified in a variety of plants, such as *Arabidopsis*, common wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), rye (*Secale cereale* L.), and maize (*Zea mays* L.), and overexpression of these genes in transgenic plants increases tolerance to drought, high salt, and freezing stresses (Dubouzet *et al.*, 2003; Shen *et al.*, 2003; Qin *et al.*, 2004; Zhang *et al.*, 2004; Hong and Kim, 2005; Benedict *et al.*, 2006; Zhao *et al.*, 2006). Thus, it is apparent that the DREB1/CBF regulon is conserved and plays a key role in regulating stress responses of higher plants, and that *DREB1*/*CBF*-like genes may be useful for improving the stress tolerance of crops (Yamaguchi-Shinozaki and Shinozaki, 2006). Except for *DREB1*/*CBF*-like and *DREB2*-like genes, the characteristics and functions of members of the other subgroups in the DREB subfamily remain to be studied. Novel members belonging to the A-5 subgroup, such as *PpDBF1*, *GmDREB2*, and *GhDBF1*, have been identified (Huang and Liu, 2006; Chen *et al.*, 2007; Liu *et al.*, 2007). For example, *PpDBF1* from the moss *Physcomitrella patens* was induced by drought, high salt, cold stresses, and abscisic acid (ABA) treatment, and overexpression of this gene enhanced tolerance of transgenic plants to drought, high salt, and cold stresses, but did not cause growth retardation (Liu *et al.*, 2007). A soybean *GmDREB2* gene, whose expression and function under various abiotic stresses were similar to those of *PpDBF1* (Chen *et al.*, 2007), were also isolated, suggesting that members of the A-5 subgroup, like those in A-1, are important genetic resources for improving stress tolerance in crop plants. However, more members of this subgroup need to be identified in order to elucidate their function and regulation mechanisms in plants tolerant to various stresses.

The DREB pathway plays an important role in the stress-responsive regulation network of plants (Cook *et al.*, 2004), and important insights about the regulatory mechanisms are beginning to emerge. A transcription factor gene, *Inducer of CBF Expression 1* (*ICE1*), encoding a MYC-like basic helix–loop–helix (bHLH) protein, binds specifically to MYC sites in the *DREB1A*/*CBF3* promoter region and increases the expression of *DREB1A*/*CBF3*, which in turn activates expression of many downstream genes, leading to a significantly enhanced tolerance to chilling and freezing (Chinnusamy *et al.*, 2003). However, an *ice1* mutation had little effect on cold-induced accumulation of *DREB1C*/*CBF2* transcripts, and two sequences, designated as ICEr1 and ICEr2 (induction of CBF expression region 1 or 2), in the promoter of *DREB1C*/*CBF2* stimulated transcription of *DREB1C*/*CBF2* in response to cold stress (Zarka *et al.*, 2003). In addition to MYC-like bHLH proteins, other proteins affecting the expression of *DREB1*/*CBF*-like genes were also identified. For example, the gene *LOS4* encoding a DEAD-box RNA helicase had a positive role in regulating *DREB1*/*CBF* expression (Gong *et al.*, 2002). In contrast, *FRY2* (Xiong *et al.*, 2002) and *HOS1* (Lee *et al.*, 2001) appeared to down-regulate *DREB1*/*CBF* expression. *FRY2*, encoding a transcriptional repressor, showed limited homologies to the genes of yeast and human C-terminal domain (CTD) phosphatases that were recently found to be involved in gene transcription and pre-mRNA processing, and *HOS1*, encoding a RING finger protein, might be an E3 ligase involved in ubiquitination and protein degradation of DREB1/CBF proteins (Lee *et al.*, 2001; Xiong *et al.*, 2002). Moreover, DREB1C/CBF2 negatively regulated the expression of *DREB1B*/*CBF1* and *DREB1A*/*CBF3* (Novillo *et al.*, 2004). These results indicate that expression of *DREB* genes is tightly controlled by a complex gene network, which guarantees the correct induction of downstream genes and precise development of tolerance to freezing and other stresses. However, the regulatory mechanisms of most of the *DREB* genes remain unclear, probably due to the lack of focus on the promoters.

In this study, a *DREB* gene, *GmDREB3*, belonging to the A-5 subgroup, was isolated from soybean. Northern blot analysis showed that *GmDREB3* was induced only by cold stress, and its overexpression in transgenic plants increased their tolerance to cold, drought, and high salt stresses, similar to *DREB1*/*CBF*-like genes, but obviously different from other members in the A-5 subgroup. In addition, promoter analysis of *GmDREB3* showed that a promoter segment, from bp −1058 to −664, was sufficient to activate cold-responsive expression, but elements in region −1403 to −1058 might work with a transcriptional repressor to impair this activity, suggesting that both transcriptional activators and repressors are involved in fine-tuning expression of *GmDREB3* in response to cold stress.

Materials and methods

Plant materials and growth conditions

Arabidopsis plants (genotype Colombia) used for transformation were grown in soil at 22 °C and ~70% humidity under 14 h light and 10 h darkness. T_1 seeds were surface-sterilized and planted on MS medium supplemented with 50 μg ml^{-1} kanamycin for the selection of transgenic plants. After emergence, seedlings of transgenic *Arabidopsis* plants were transferred to pots for further functional analyses. Tobacco (*Nicotiana tabacum* L., genotype W38) seedlings grown on MS medium were used for transformation. After transgenic plants were identified by PCR, seedlings were transferred to pots and grown under a 12 h light:12 h darkn:25 °C regime for further functional analyses.

Isolation of the GmDREB3 *gene*

In order to isolate the genes encoding DREB from soybean, an AP2 consensus peptide sequence was used as a query to search the expressed sequence tag (EST) database of soybean (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=soybean). A total of 103 EST sequences containing AP2 domains were obtained and further systematic phylogenetic analyses of those sequences were carried out on the basis of homology of AP2 domains. In order to study the characteristics and functions of a member belonging to the A-5 subgroup of the DREB subfamily, an EST sequence belonging to the A-5 subgroup was chosen for further analyses (EST code in GenBank: BQ629398). Using the RACE (rapid amplification of cDNA ends) method, the full-length cDNA sequence, designated *GmDREB3* (GenBank accession no. DQ208969), was isolated from total RNA of soybean cv. Tiefeng 8 (a salt-tolerant soybean cultivar). RACE was carried out as described in the Instruction Manual (Rapid Amplification of cDNA Ends System, Gibco-BRL, Rockville, MD, USA).

Application of abiotic stresses and northern blot analysis

Soybean cv. Tiefeng 8 plants were used for gene isolation and expression pattern analyses. Soybean seeds were planted in pots, irrigated with water, and subjected to a 12 h light:12 h dark:25 °C regime. To determine the expression pattern of *GmDREB3* under high salt stress, some 2-week-old soybean plants were removed from the soil. An initial sample represented an untreated control (or high salt treatment at 0 h). Other soybean plants were soaked in a solution containing 250 mM NaCl for various time periods prior to sampling. For ABA treatment, leaf tissues of soybean plants were sprayed with 200 μM ABA solution and then sampled after different time intervals. For drought treatment, soybean plants were sampled at 0 h; other soybean plants were sampled after being placed on filter paper for various time periods. For cold treatment, soybean plants were placed in a refrigerator at 4 °C under dim light. After exposure to stresses, samples were immediately frozen in liquid nitrogen for later analysis of expression patterns. Total RNA was extracted from plant samples harvested after different time points as described by Zhang *et al.* (1996). About 30 μg of total RNA was fractionated in a 1% (w/v) agarose gel containing formaldehyde and then transferred onto a Hybond-N^+ nylon membrane in 20× SSC. The *GmDREB3* probe was labelled with [α-^{32}P]dCTP and the Random Primer DNA Labeling Kit (TaKaRa Biotech, Dalian, China). Hybridization was performed as described in the Instruction Manual for the Hybond-N^+ nylon membrane filter (Amersham Biosciences, Piscataway, NJ, USA).

Isolation and activity analysis of the GmDREB3 *promoter*

The promoter fragment of *GmDREB3* was isolated from the soybean genome using the SiteFinding-PCR method (Tan *et al.* 2005). Promoter sequence analysis was performed using the PLACE Signal Scan Search Program (http://www.dna.affrc.go.jp/PLACE/signalscan.html). According to the predicted position of the *cis*-element in the *GmDREB3* promoter, five fragments deleted from the 5′ end of the promoter containing bp −1587 to +41, −1403 to +41, −1058 to +41, −644 to +41, and −226 to +41 were amplified, and separately inserted into binary vector pBI121 (Clontech, Mountain View, CA, USA) to replace the *CaMV 35S* (cauliflower mosaic virus 35S) promoter upstream of the GUS (β-glucuronidase) reporter gene. These vectors and a positive control pBI121 were transferred into calli induced from mature embryos of wheat (cv. Yumai 66) using a biolistic particle acceleration device (PDS 1000/He, Bio-Rad, Hercules, CA, USA) under a chamber pressure of 27 mmHg and 6 cm distance from the rupture disc to the microcarriers (Vasil *et al.*, 1993). After transformation, calli were held on MS medium plus 100 μM ABA, 200 mM NaCl, or low temperature (4 °C) conditions for 1 h and 4 d, respectively. Treated calli were then stained with GUS staining solution (Jefferson *et al.*, 1987) to identify GUS activity, which was quantified using a Lambda 35 UV/VIS Spectrometer (Perkin Elmer, Foster, City, CA, USA) as described previously (Facchini *et al.*, 1996). The relative GUS activity was calculated as the ratio of GUS activities of the deleted *GmDREB3* promoter series to that of the *CaMV 35S* promoter (pBI121) under the same stress treatments. This transient expression assay was repeated three times for each construct within each treatment.

Preparation and electrophoretic mobility shift assays (EMSA) of glutathione S*-transferase (GST) fusion proteins*

The 528 bp fragment of *GmDREB3* containing the DNA-binding domain was amplified using the primer pair: GmDREB3PP-1, 5′-CCCTCTAGAGAATTCATGGCGA-AACCCAGCAGC-3′ (forward); and GmDREB3PP-2, 5′-CCCCTCGAGCGGCATTT CCGGCACATA-3′ (reverse). This fragment was cloned into the *Eco*RI–*Xho*I site of the

pGEX4T-1 vector (Amersham Biosciences) and transferred into *Escherichia coli* BL21 cells (Amersham Biosciences) to produce a GST fusion protein. The GST fusion protein was purified using a glutathione–Sepharose 4B column (Amersham Biosciences) according to the manufacturer's instructions. EMSA was completed as described previously (Liu *et al.*, 1998). The 37 bp DNA fragment containing two copies of the wild-type or mutant DRE elements was synthesized (Fig. 3A). The DNA fragment was labelled by filling in 5′ overhangs with [γ-^{32}P]dCTP (25 μCi μl^{-1}; Amersham Biosciences). The DNA-binding reaction was allowed to proceed for 30 min at 25 °C in 20 μl of binding buffer [25 mM HEPES/KOH, pH 7.9, 50 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA, 5% (w/v) glycerol, 5 μg μl^{-1} bovine serum albumin (BSA)] that contained 20 000 dpm of the ^{32}P-labelled probe, 2 μl of glycerine, and purified fusion protein as described previously (Liu *et al.*, 1998). The resulting DNA–protein complexes were loaded on 0.5× Tris-borate-EDTA, 6% polyacylamide gels. After eletrophoresis, gels were dried and visualized by autoradiography.

Transcriptional activation assay of GmDREB3 protein

The coding region of the *GmDREB3* gene was cloned into YepGAP, a yeast expression vector with the promoter of the glyceraldehyde 3-phosphate dehydrogenase gene and terminator of the *ADH1* gene (Liu *et al.*, 1998). The recombinant plasmid was then transferred into yeast strain YM4271 carrying the reporter genes *His3* and *LacZ* with four copies of a tandemly repeated 71 bp DNA fragment containing the DRE core sequence (TACCGACAT) or a mutated DRE (mDRE) core sequence (TATTTTCAT) upstream of a TATA element (Liu *et al.*, 1998). Both the yeast expression vector YepGAP and yeast strain YM4271 were kindly provided by Professor Qiang Liu, Tsinghua University, Beijing. The yeast strain expressing the *His* gene at the basal level grows on synthetic dextrose (SD) medium lacking histidine, but cannot grow in the presence of 10 mM 3-AT (3-aminotriazole; Sigma, St Louis, MO, USA), a competitive inhibitor of the *His3* gene product, and cannot induce LacZ (β-galactosidase) activity. The growth status of the yeast cells transferred with the recombinant plasmids was compared on SD medium without His plus different concentrations of 3-AT (10, 20, 30, 40, 50, and 60 mM). LacZ activity was assayed as described by Chen *et al.* (2003) and was semi-quantified by comparing fluorescence intensities of stained yeast cells using Quantity One software (Bio-Rad, Hercules, CA, USA).

Transformation and stress tolerance analyses of transgenic Arabidopsis *plants*

The coding region of *GmDREB3* was amplified using the primer pair: GmDREB3FP-1, 5′-TATCCCGGGATGGCGAAACCCAGCAGCGA-3′ (forward); and GmDREB3FP-2, 5′-GCAGAGCTCGCGGCCGCTCAAAAATTCCACAAGAAAGAC-3′ (reverse), and inserted into the MCS (multiple cloning site) of two binary vectors, p35S and pRd29A, derived from pBI121, to produce vectors p35S-GmD3 and pRd29A-GmD3, respectively. For the construction of the pRd29A vector, pBI121 was digested with *Hin*dIII and *Sac*I to delete the *GUS* gene and *CaMV 35S* promoter, and ligated with a fragment containing the Rd29A promoter and MCS of the pBluescript SK vector (Clontech, Mountain View, CA, USA). To construct the p35S vector, the pRd29A vector was digested with *Hin*dIII and *Sma*I to delete the *Rd29A* promoter and ligated with the *CaMV 35S* promoter. To construct the p35S-GmD3 and pRd29A-GmD3 vectors, the amplified fragments of the *GmDREB3* cDNA were inserted into the *Xba*I–*Sac*I site of the p35S and pRd29A vectors, with *GmDREB3* being driven by the constitutive *CaMV 35S* and stress-inducible *Rd29A* promoters in vectors p35S-GmD3 and pRd29A-GmD3, respectively.

Two plasmids, p35S-GmD3 and pRd29A-GmD3, were introduced into *Agrobacterium tumefaciens* strain C58C1. The *Arabidopsis* plants used for transformation were grown in 8 cm pots at 25 °C for 5 weeks and transferred by vacuum infiltration as described by Bechtold and Pelletier (1998). T_2 transgenic *Arabidopsis* plants were identified by selection for kanamycin resistance and used for further functional analysis. For drought tolerance analyses, the wild-type, and *35S:GmDREB3* and *Rd29A:GmDREB3* transgenic *Arabidopsis* plants grown in pots were treated without watering for 19 d, followed by rewatering. Survival rates of the wild-type and transgenic plants were evaluated 8 d later. For high salt tolerance analyses, seeds of wild-type and *35S:GmDREB3* plants were germinated on normal MS medium and then moved onto MS medium in the presence of 160 mM NaCl for 18 d at 22 °C. For cold tolerance analysis, 2-week-old wild-type and *35S:GmDREB3* transgenic plants were removed from the agar plates and put on filter paper saturated with water exposed to −6 °C for 1 h, and allowed to recover at 25 °C for 24 h, prior to evaluation of survival rates. Two plasmids, p35S-GmD3 and pRd29A-GmD3, were transferred into tobacco calli by *Agrobacterium tumefaciens* strain EH105. T_0 transgenic tobacco plants were selected on MS medium containing 100 μg ml^{-1} kanamycin and further identified using PCR with the specific primers GmDREB3FP-1 and GmDREB3FP-2. All transgenic tobacco genotypes were transformed to pots for functional analyses.

Analysis of osmolality, free proline, and chlorophyll in GmDREB3 *transgenic plants*

Before and after cold stress, the fresh weights and osmolalities of wild-type and *35S:GmDREB3* transgenic *Arabidopsis* plants were measured with a vapour pressure osmometer (VAPRO™ 5520, Wescor, Logan, UT, USA) (1–3 replicates per genotype). Average osmolality values for each line were used for statistical analysis. For measurement of free proline content, wild-type and transgenic tobacco plants were grown in pots under a 12 h light:12 h dark:25 °C regime. Eight-weeks-old plants were treated without watering for 16 d and leaves were harvested at 0 d and 16 d. Free

proline contents of harvested leaves were measured as described previously by Zhang *et al.* (1990). To determine the salt tolerance of transgenic tobacco, leaf discs of 1 cm diameter were cut from healthy and fully expanded leaves of wild-type and *GmDREB3* transgenic tobacco plants, and floated on MS liquid medium containing 400 mM NaCl for 5 d (12 h of white light:12 h of darkness:25 °C). Chlorophyll contents were determined following Aono *et al.* (1993). Data for osmolality values, free proline, and chlorophyll contents were analysed by SAS software (SAS Corporation, Cory, NC, USA) using *t*-tests to test the significance of differences between means.

Results

The phylogenetic analysis of the GmDREB3

The full length of the *GmDREB3* gene was 597 bp, encoding a protein of 199 amino acids. Further analysis of its deduced amino acid sequence using the SMART program (http://smart.embl-heidelberg.de) revealed that this protein contained a conserved AP2 domain of 58 amino acids and a putative NLS (nuclear localization signal sequence) (Supplementary Fig. S1A available at *JXB* online). Systematic phylogenetic analysis was carried out on the basis of the similarities of the AP2 domains in AP2/EREBP proteins isolated from soybean *Arabidopsis*, maize, rice, tomato (*Solanum lycopersicum* L.), barley (*Hordeum vulgare* L.), cotton (*Gossypium hirsutum* L.), tobacco (*N. tabacum* L.), and canola (*Brassica napus* L.) using CLUSTAL W software (Sakuma *et al.* 2002). The results showed that the *GmDREB3* gene was classified into the A-5 subgroup of the DREB subfamily (data not shown), which was according to the study of Liu *et al.* (2007). In addition, *GmDREB1* and *GmDREB2* from soybean, *RAP2.1* and *RAP2.10* from *Arabidopsis*, *GhDBF1* from cotton, and *PpDBF1* from moss (*P. patens*) were also classified into the A-5 subgroup. In order to investigate the relationship between *GmDREB3* and the other members in the A-5 subgroup, the deduced full-length amino acid and AP2 domain sequence of GmDREB3 was compared with that of the six DREB proteins from soybean, moss, cotton, and *Arabidopsis* (Okamuro *et al.*, 1997; Huang and Liu, 2006; Chen *et al.*, 2007; Liu *et al.*, 2007) (Supplementary Fig. S1B, S1C). GmDREB3 had high similarity to other members of the A-5 subgroup in the AP2 domain (>68%, Supplementary Fig. S1C) and low similarity outside of the AP2 domain. Homologies of the full-length amino acid sequences were low (<38%, Supplementary Fig. S1B). In the AP2 domain, the GmDREB3 protein had the same 14th valine as other members in the A-5 subgroup, but contained a 19th leucine instead of a glutamic acid (Supplementary Fig. S1A). Wang *et al.* (2005) reported that the C-terminal 98 amino acids of CBF1 function in transactivation. The LWSY domain in the C-terminus is the characteristic of CBF proteins (Jaglo *et al.*, 2001). In the present work, the C-terminus of GmDREB3 shared a similar LWSY domain with PpDBF1, whereas it shared low similarity with GmDREB2 in the C-terminus (Supplementary Fig. S1A), suggesting that the transactivation activity of GmDREB3 might be different from that of GmDREB2.

Expression pattern of the GmDREB3 *gene in soybean*

Northern blots showed that *GmDREB3* was responsive to cold stress, and not to high salt and drought stresses, or to ABA treatment. With cold stress treatment, *GmDREB3* mRNA began to accumulate after 0.5 h and reached a maximum at 1 h after treatment, after which it was not detectable (Supplementary Fig. S2 at *JXB* online). The *GmDREB3* gene was not responsive to ABA treatment or osmotic stresses, suggesting that this gene might be involved in an ABA-independent cold stress-responsive signal pathway.

Isolation and activity analysis of the GmDREB3 *promoter*

The promoter sequence of the *GmDREB3* with a length of 1587 bp was isolated from the soybean genome. Using the PLACE Signal Scan Search Program, several *cis*-elements were predicted to be involved in transcriptional regulation; among them were one typical TATA-box located about −35 upstream of the transcription initiation point, ten MYC transcription factor recognition sites belonging to seven types (MC-1 to MC-7), and seven MYB transcription factor recognition sites belonging to five types (MB-1 to MB-5) (Fig. 1). Among the MYC recognition sites, MC-1, MC-4, and MC-7 shared the same consensus sequence with MYC-2, MYC-3, and MYC-4, respectively, in the *DREB1A/CBF3* promoter (Chinnusamy *et al.*, 2003). Deletion analysis from the 5′ end of the promoter showed that the *GmDREB3* promoter was responsive only to cold stress (4 °C), and not to normal conditions, high salt stress, and ABA treatment (Fig. 2). However, the *GmDREB3* promoter showed different regulation patterns after cold treatments for 1 h and 4 d. After treatment for 1 h, 5′ deletions to −1403 and −1058 did not impair cold responsiveness, whereas deletion to −664 resulted in almost complete elimination of cold responsiveness (Fig. 2B, C), indicating that part of the sequence between −1058 and −664 had an important role in the cold responsiveness of *GmDREB3*. After cold stress for 4 d, the 5′ deletion to −1058 showed a higher level of GUS activity (2.38±0.34) than after 1 h (1.96±0.16), whereas fragments containing −1587 to +41 and −1403 to +41, which include the region from −1403 to –1058, impaired the cold responsiveness (Fig. 2D, E). This indicated that the region from −1403 to −1058 was sufficient to impair the cold responsiveness of the *GmDREB3* promoter and that the region might contain cold-responsive transcriptional repressor elements. The overall results suggested that a transcriptional activator might activate *GUS* expression after cold stress for 1 h by binding to cold-responsive elements in the region −1058 to −664, and that a transcriptional repressor impaired this activity by competitive binding to elements in region −1403 to −1058 after cold stress for 4 d. This probably also explained the greatly

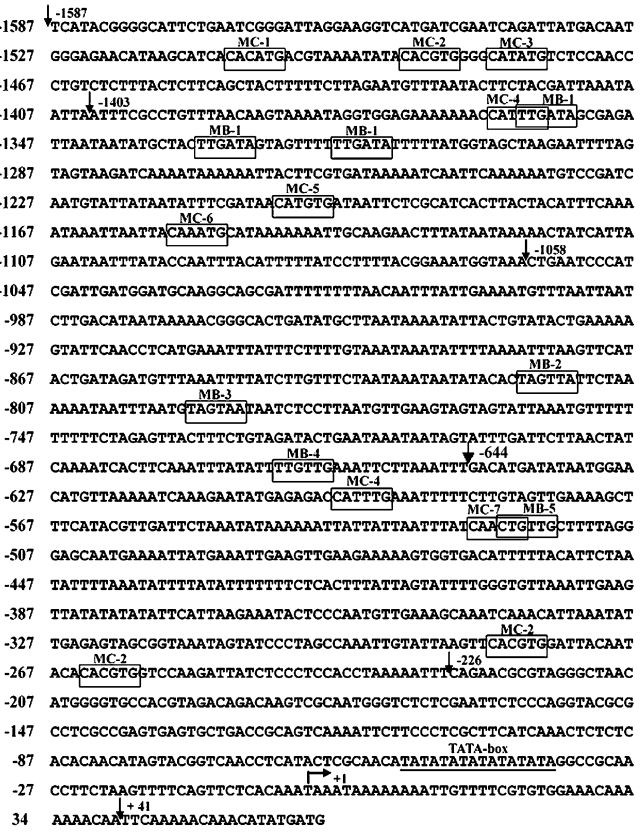

Fig. 1. Nucleotide sequence of the promoter region of *GmDREB3* including some predicted known *cis*-elements. The sequences homologous to MYC (MC) and MYB (MB) recognition sites are highlighted. A putative TATA box is underlined. An arrow shows the initiation point (+1) of transcription. Downward pointing arrows show the 5′ and 3′ ends of five deleted fragments of the *GmDREB3* promoter.

decreased expression of the *GmDREB3* after cold stress for 3 h (Supplementary Fig. S2 at *JXB* online).

DNA-binding activity and transcriptional activation analysis of GmDREB3

The wild-type DRE motif interacted with the GmDREB3–GST fusion protein and was retarded on SDS–PAGE, but the DRE mutation motifs with a two-base substitution at the 5′ end or a three-base substitution at the 3′ end, or a completely mutant DRE core sequence, severely inhibited interaction with the GmDREB3–GST fusion protein (Fig. 3B), suggesting that GmDREB3 protein could bind specifically to the DRE CCGAC core sequence *in vitro*.

Yeast cells with the recombinant plasmid harboring *GmDREB3* grew on media lacking histidine in the presence of 10, 20, 30, and 40 mM 3-AT (Fig. 3C), and stained blue in X-gal solution after 4 h (Fig. 3D). However, when the recombinant plasmid containing *GmDREB3* was transferred into yeast carrying the dual reporter genes fused to a 71 bp DNA fragment with base substitutions in the DRE core sequence, the yeast strain neither grew on media lacking histidine in the presence of all concentrations of 3-AT, nor stained blue (Fig. 3C, D), indicating that GmDREB3 protein bound specifically to the DRE element in order to activate transcription of the dual reporter genes in yeast cells.

Overexpression of GmDREB3 *improved drought, high salt, and cold stress tolerance of transgenic* Arabidopsis *lines*

In drought tolerance analyses, all wild-type plants were dead (0/58), whereas 32% (14/30) of the *35S:GmDREB3* and 85% (26/42) of the *Rd29A:GmDREB3* transgenic plants survived (Fig. 4A). This indicated that overexpression of *GmDREB3* improved the drought tolerance of the transgenic plants.

High salt tolerance analyses showed that the growth of wild-type plants was severely inhibited, whereas the transgenics maintained normal growth (Fig. 4B). The average lengths of roots and aboveground parts of the transgenics were 3.48±0.38 cm and 1.13±0.29 cm, respectively, 1.88-fold (P <0.01) and 2.17-fold (P <0.01) greater than the wild-type controls (1.85±0.38 cm and 0.52±0.06 cm) (Fig. 4C). Thus *GmDREB3* enhanced tolerance to high salt stress in transgenic plants.

Cold tolerance analyses showed that all of the wild-type plants died (0/30), whereas 63% (19/30) of the *35S:GmDREB3* transgenic plants survived (Fig. 5A), indicating that overexpression of *GmDREB3* improved the cold tolerance of transgenic *Arabidopsis* plants.

To evaluate phenotypic changes, wild-type and *35S:GmDREB3* and *Rd29A:GmDREB3* transgenic *Arabidopsis* plants were grown on MS medium during the seedling stage (Fig. 6C) and then transformed to pots (Fig. 6A, B). At maturity, the average height of *Rd29A:GmDREB3* transgenics (26.16±0.76 cm) was similar to that of the wild type (23.16±1.25 cm), but significantly higher (P <0.001) than that of the *35S:GmDREB3* transgenics (15.50±0.50 cm). Likewise, the average heights of the *Rd29A:GmDREB3* transgenics (3.02±0.68 cm) and wild type (3.37±0.56 cm) seedlings were greater than that of the *35S:GmDREB3* transgenics (1.05±0.26 cm) (P <0.025) (Fig. 6D). Thus constitutive expression of *GmDREB3* in *Arabidopsis* induced undesirable dwarfing, whereas the expression of *GmDREB3* controlled by the stress-inducible *Rd29A* promoter minimized negative effects on growth under normal conditions.

Overexpression of GmDREB3 *led to physiological changes in* Arabidopsis *and tobacco transgenic lines under cold, drought, and high salt stresses*

To evaluate physiological changes in transgenic plants after cold stress, plant fresh weights and osmolalities were compared. Before cold stress, the fresh weights of the wild type (0.036667±0.001761 g) and *35S:GmDREB3* transgenics (0.036663±0.002002 g) were similar, whereas after cold stress (−6 °C for 1 h) *35S:GmDREB3* plants (0.02115± 0.002527 g) were 2.2-fold (P <0.005) heavier than the wild type (0.00958±0.001249 g) (Fig. 5B). Similarly, before cold

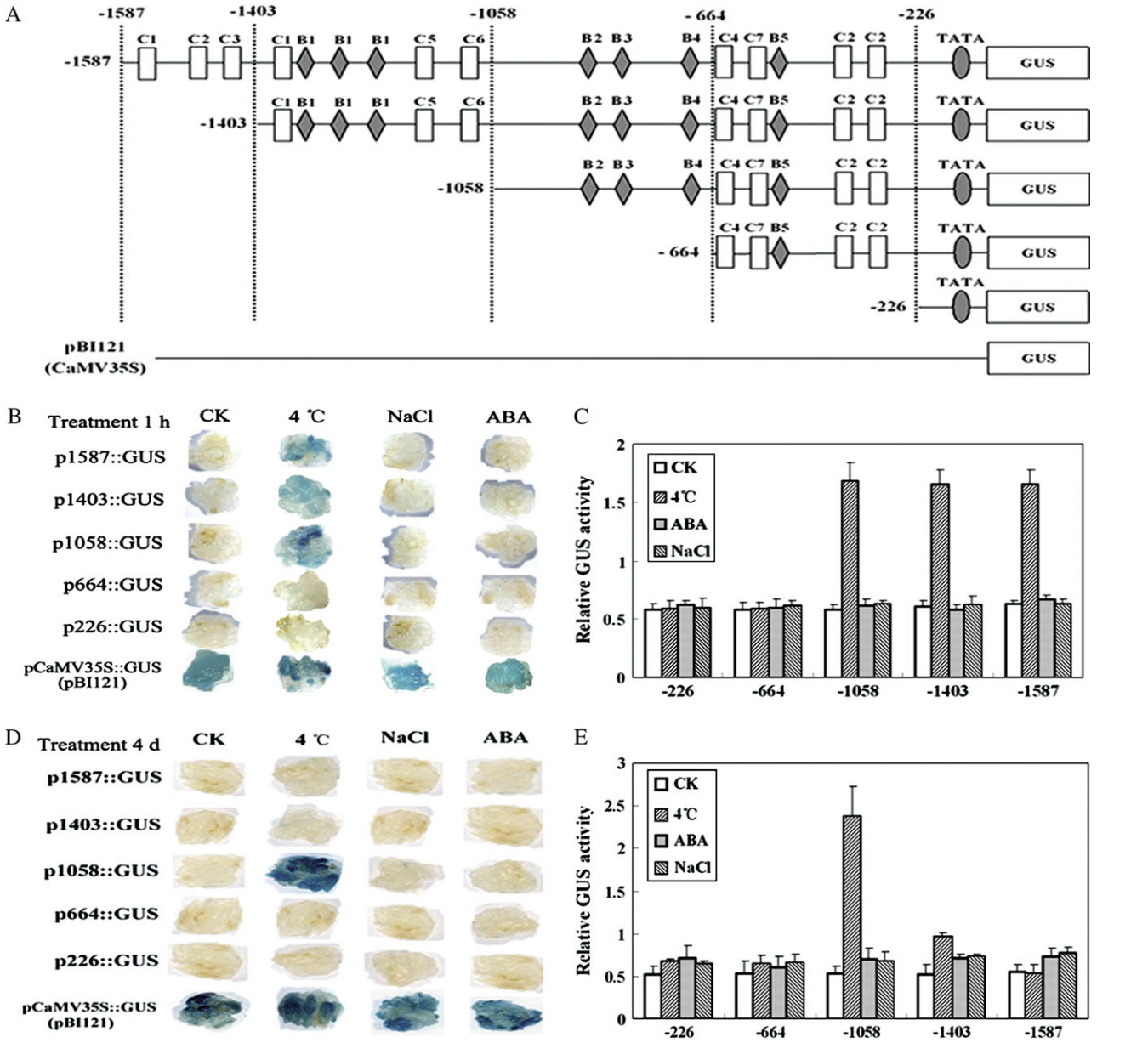

Fig. 2. Activity analysis of the *GmDREB3* promoter. (A) Five fragments that were deleted from the 5′ end of the *GmDREB3* promoter were inserted into a binary vector (pBI121). The locations of the 5′ ends of the five fragments of the *GmDREB3* promoter are indicated; 'C' and 'B' represent MYC and MYB recognition sites, respectively. Binary vector pBI121 was used as the positive control. (B and D) After transformation with vectors containing the five fragments of the *GmDREB3* promoter, calli were treated on MS medium plus 100 μM ABA (ABA), plus 200 mM NaCl (NaCl), and under low temperature (4 °C) stress and normal conditions (CK) for 1 h and 4 d. The histochemical staining results (GUS) are shown. (C and E) Quantitative results of GUS activity after treatments for 1 h and 4 d. The relative GUS activity was calculated as the ratio of GUS activity of a mutant *GmDREB3* promoter to that of the *CaMV 35S* promoter (pBI121) under the same stress treatments.

stress, the osmolality of wild-type (156.4±2.0 mmol kg^{-1}) and transgenic plants (155.5±1.3 mmol kg^{-1}) were similar, whereas after cold stress, the osmolality of transgenic plants (250.5±9.7 mmol kg^{-1}) was higher than that of the wild-type plants (180.3±3.7 mmol kg^{-1}) (P <0.001) (Fig. 5C).

The chlorophyll contents in the leaves of wild-type and transgenic tobacco plants were measured following high salt treatment. Leaf discs from wild-type plants were bleached, whereas most of those from the transgenic plants remained green (Fig. 7A). The chlorophyll contents in the leaves of most of the transgenic tobacco plants were similar to those of wild-type discs floated in water, and higher than those of the wild type floated on 400 mM NaCl solution. Among the transgenic tobacco lines, 35S-3 and 35S-12 (*35S:GmDREB3*) at 449.33±125.65 μg g^{-1} and 494.79±110.72 μg g^{-1} were 7.6-fold (P <0.05) and 8.4-fold (P <0.01), and 29A-5 and 29A-18 (*Rd29A:GmDREB3*) at 343.76±89.11 μg g^{-1} and 364.86±197.78 μg g^{-1} were 5.8-fold (P <0.05) and 6.2-fold (P <0.05) higher in chlorophyll content compared with the wild-type tobacco (58.77±36.90 μg g^{-1}) (Fig. 7A). These

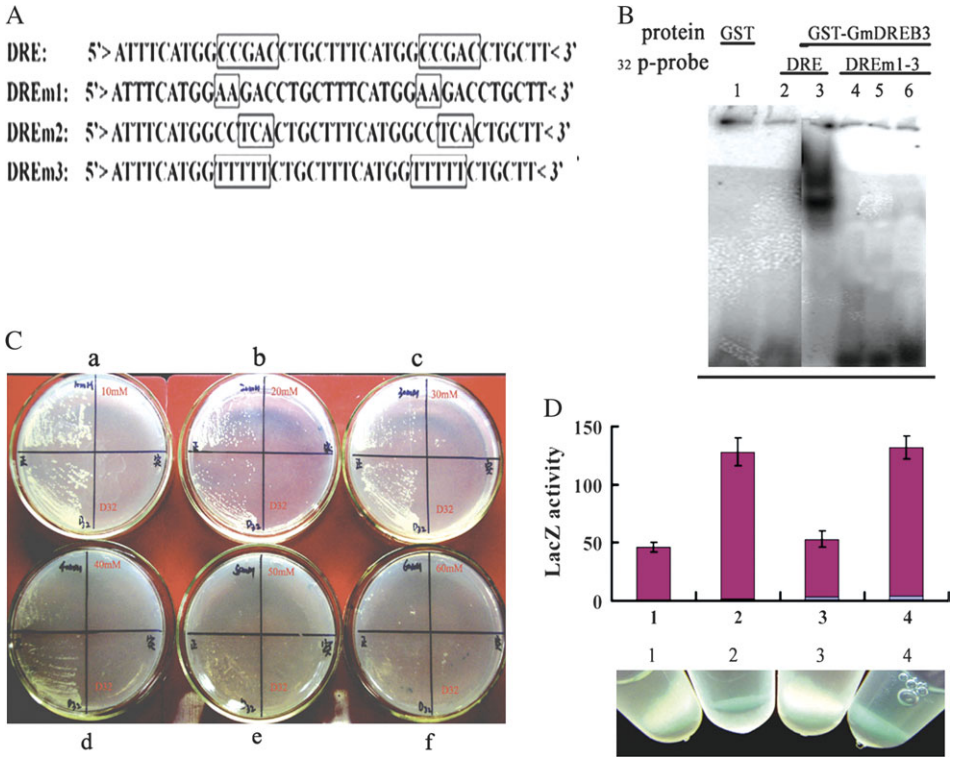

Fig. 3. EMSA and transcriptional activation analyses of GmDREB3 proteins. (A) Nucleotide sequences of DRE (DRE) and mutated DRE (DREm1–m3) probes. The nucleotide mutation in the DRE core motif of each probe is boxed. (B) EMSA was performed using 0.2 μg of GmDREB3 protein, and radiolabelled DRE or mutant DRE probes. Lanes: 1, GST proteins control; 2, free labelled DRE probe; 3, GST–GmDREB3 fusion plus labelled wild-type DRE element; 4–6, GST–GmDREB3 fusion proteins plus labelled mutated DRE (DREm1–m3) probes. (C) Recombinant plasmids containing *GmDREB3* were transferred into yeast cells carrying the dual reporter genes under the control of the 71 bp promoter region containing the wild-type DRE (left half of each plate) or the mutant DRE core sequence (right half of every plate). Transferred yeast cells were examined for growth on the medium in the presence of 10, 20, 30, 40, 50, and 60 mM 3-AT (a–f). (D) LacZ activity of yeast cells transformed with *GmDREB3*. 1 and 3, stain intensities of yeast cells carrying mutant DRE elements; 2 and 4, stain intensities of yeast cells carrying the wild-type DRE element. LacZ activities of the transformed yeast cells were semi-quantitated. The results are shown above the photographs.

results indicated that overexpression of the *GmDREB3* gene reduced the effects of high salt stress on chlorophyll formation and enhanced the salt tolerance of transgenic plants.

The ability of transgenic tobacco to accumulate osmolytes, such as free proline, under normal and drought stress conditions was also investigated. Under normal conditions, free proline contents of transgenic plants were the same as those of wild-type plants (Fig. 7B). After drought treatment for 16 d, the free proline levels of the wild type and all transgenics were increased, and 35S-13 and 35S-14 (*35S:GmDREB3*) (117.79±80.89 μg g^{-1} and 239.73±115.66 μg g^{-1} FW) were 1.5-fold and 3-fold (P <0.2), and 29A-3 and 29A-4 (*Rd29A:GmDREB3*) (169.21±35.21 μg g^{-1} and 111.83±51.89 μg g^{-1} FW) were 2.1-fold (P <0.1) and 1.4-fold higher than wild-type tobacco (79.90±31.32 μg g^{-1} FW) (Fig. 7B). While none of these differences was significant, the consistent trend was that the transgenic tobacco plants accumulated higher levels of free proline than the wild type under drought stress, and that plants transferred with *GmDREB3* are likely to be more tolerant to drought and high salt stresses.

Discussion

GmDREB3 belongs to the A-5 subgroup in the DREB subfamily and is a novel transcriptional activator

Most research on the DREB subfamily has focused on the A-1 and A-2 subgroups, and little is known about the characteristics and functions of members of the other subgroups. Studies on A-5 subgroup members, such as *PpDBF1*, *GmDREB2*, and *GhDBF1* (Huang *et al.*, 2006; Liu *et al.*, 2007; Chen *et al.*, 2007), suggested that the A-5 subgroup, like the A-1 subgroup, are important genetic resources, potentially useful for the improvement of crop stress tolerance. In this study, a novel DREB subfamily A-5 subgroup member, *GmDREB3*, was isolated from soybean. It was hypothesized that a gene transfer event might have introduced an AP2 gene from lower organisms to the common ancestor of the moss and plant, and then the AP2 genes began to spread in the genome by transposition and homing recombination (Magnani *et al.*, 2004). During the course of evolution, AP2 genes diverged and acquired new functions by transposition and duplication events, and the DREB gene subfamily might have evolved from such events

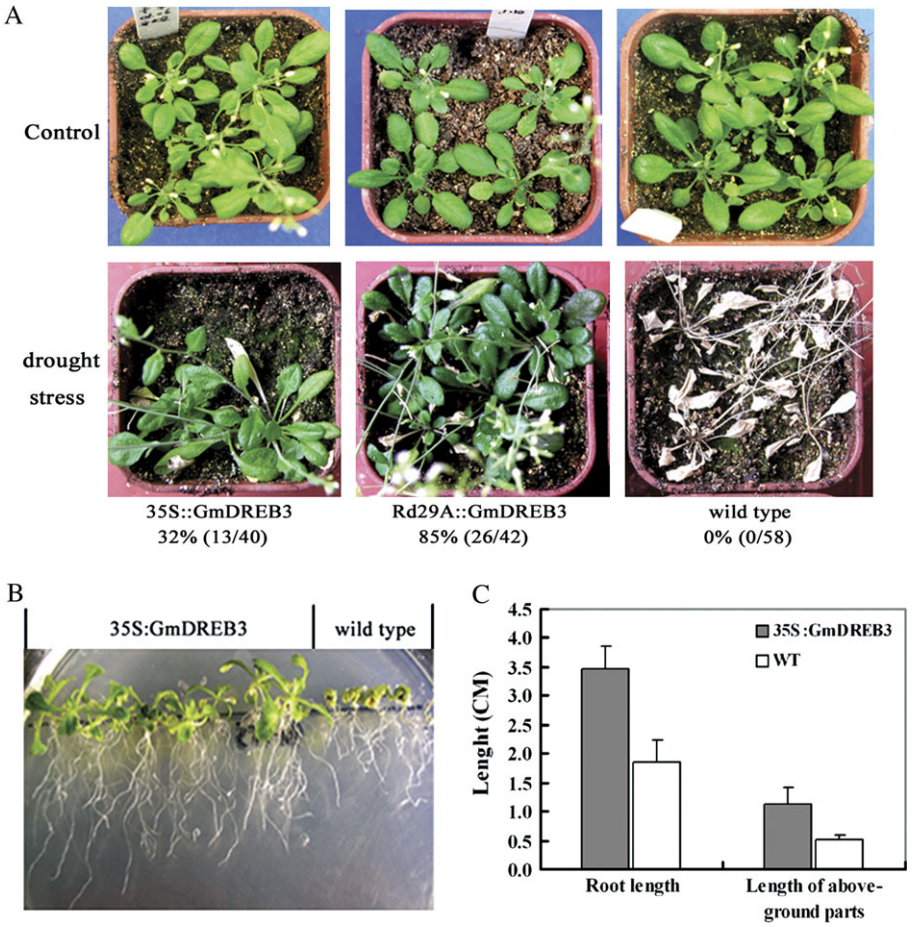

Fig. 4. Drought and high salt stress tolerance analyses of *GmDREB3* transgenic *Arabidopsis* plants. (A) Drought tolerance analysis of *GmDREB3* transgenic *Arabidopsis* plants. Control: 3-week-old plants grown under normal conditions. Drought stress: after 3 weeks wild-type and transgenic plants were treated without watering for 19 d, followed by rewatering. The growth status of treated plants 8 d after watering is shown. Percentage survival is indicated. (B) High salt tolerance analysis of *35S::GmDREB3* transgenic *Arabidopsis* plants. (C) Average lengths of roots and aboveground parts of *35S:GmDREB3* transgenics and controls shown in B.

(Liu *et al.*, 2007). During the course of the evolution of transcription factor genes in higher plants, the DNA-binding domain maintained a substantial selective constraint, whereas the non-DNA-binding region showed a higher level of evolutionary divergence due to relaxed constraint (Chang *et al.*, 2005). In this study, the homology comparisons of DREB genes in the A-5 subgroup showed that *GmDREB3* shared high similarity in the AP2 domain and low similarity in the full-length sequence with six other members, which might be due to the different rates of evolution within the AP2 conserved domain and other parts of the DREB proteins. Similar results for both the MYB family (Dias *et al.*, 2003) and the bHLH family (Atchley *et al.*, 1994) were also reported. In addition, the variation occurring in the promoter regions of different members, including insertions, deletions, transpositions, and substitutions of nucleotides, might result in the changes in expression pattern.

Expression of GmDREB3 *might be co-regulated by different transcription factors*

Among the members in the A-5 subgroup, *PpDBF1* and *GmDREB2* were induced by drought, high salt, cold stresses, and ABA treatment (Chen *et al.*, 2007; Liu *et al.*, 2007), and *GhDBF1* was mainly induced by drought and high salt stress (Huang *et al.*, 2006), whereas *GmDREB3* was only responsive to cold stress (Supplementary Fig. S2 at *JXB* online). During the cold response, the transcription of *GmDREB2* was induced after 3 h of treatment, and reached its maximum at 17 h (Chen *et al.*, 2007), whereas the induction of *GmDREB3* occurred much earlier than that of *GmDREB2*. Its expression had been restrained before 3 h, suggesting that *GmDREB3* might be involved in the early cold response compared with *GmDREB2* in soybean. In addition, the promoter sequence of *GmDREB2* was obtained by searching Scaffold 26 in the Soybean genome project (www.phytozome.net/soybean.php). Sequence alignment indicated that the promoter sequence of *GmDREB3* shared low identity (43.5%) with the promoter sequence of *GmDREB2* (Supplementary Fig. S3 at *JXB* online), suggesting that although the two genes belonged to the same subgroup, they were involved in different stress-related signal pathways due to the different promoters controlling their expression.

Promoter activity analysis showed that a cold-responsive element located in region −1058 to −664 of the *GmDREB3*

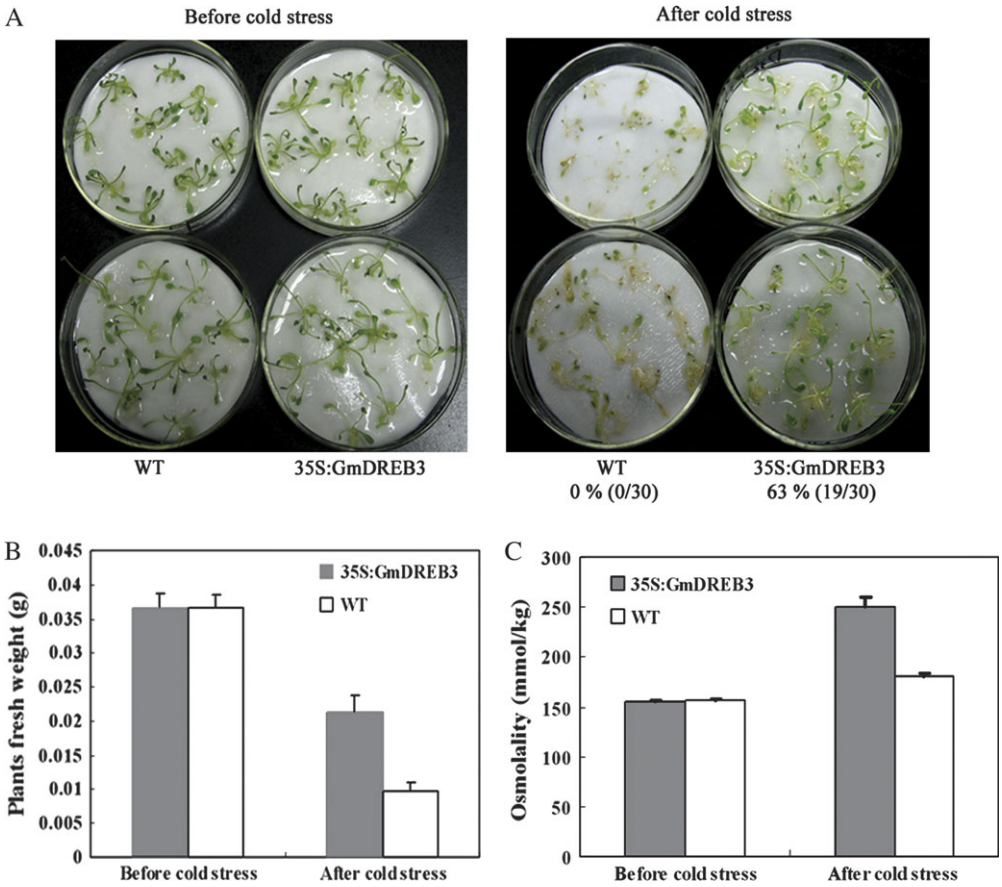

Fig. 5. Evaluations of phenotypic and physiological effects of cold stresses on *GmDREB3* transgenic *Arabidopsis*. (A) Cold stress tolerance analysis of *GmDREB3* transgenic *Arabidopsis* plants. Two-week-old wild-type (WT) and transgenic (*35S:GmDREB3*) plants were removed from agar plates and exposed to –6° C for 1 h and then allowed to recover at 25 °C for 24 h, prior to scoring. Survival rates are shown below the photographs. (B and C) Average fresh weights and the osmolalities of transgenic (*35S:GmDREB3*) and wild-type (WT) plants before and after cold stress.

promoter induced *GUS* expression after cold stress for 1 h. Following prolonged cold stress, it seemed that another transcriptional repressor could impair the activity of the *GmDREB3* promoter by binding to an element in the region −1403 to −1058 (Fig. 2 and Supplementary Fig. S2 at *JXB* online). *ICE1*, a MYC-type bHLH transcription factor, regulates the expression of *DREB1A*, and there are five potential MYC sites (CANNTG) in the promoter region of *DREB1A* (Shinwari *et al.*, 1998; Chinnusamy *et al.*, 2003; Nakashima *et al.*, 2006). Zarka *et al.* (2003) reported that a region in the *DREB1C*/*CBF2* promoter, ICEr1, played a key role in cold-induced expression, and a MYC site (CACGTG) was detected in this ICEr1 region. However, in the present study, motif analysis indicated that there were no typical MYC recognition site in region −1058 to −664 of the *GmDREB3* promoter, whereas three MYB recognition sites were in this region (MB2, MB3, and MB4) (Fig. 1). Overexpression of a rice MYB gene, *Osmyb4*, increased chilling and freezing tolerance of *Arabidopsis* plants, and its recognition sequence was mAC-II (AAGAAGGAAACC) (Vannini *et al.*, 2004). Transcription factors belonging to the MYB family have binding specificity of either type I [CNGTT(A/G)] or type II [G(G/T)T(A/T)GTT(A/G)] and type IIG [G(G/T)T(A/T)GGT(A/G)], and members of different subgroups within the MYB family prefer different MYB recognition sequences (Romero *et al.*, 1998). In region −1058 to −664 of the *GmDREB3* promoter, three MYB sites (MB2, TAGTTA; MB3, TAGTAA; and MB4, TTGTTG) are more like type II MYB sites, which are more likely to be recognized by members belonging to subgroup B of the MYB family (Romero *et al.*, 1998).

HOS1, a novel RING finger protein (Lee *et al.*, 2001), and FRY2, a transcriptional repressor (Xiong *et al.*, 2002), appear to be negative regulators of *CBF* expression. In addition, Agarwal *et al.* (2006) reported that a MYB transcription factor, *MYB15*, could bind to MYB recognition sequences in the promoters of *CBF* genes. Overexpression of *MYB15* resulted in reduced expression of *CBF* genes, and its loss of function led to increased expression of *CBF* genes upon cold treatment. MYB15 preferentially binds to type II MYB or type IIG sites, and, to a much lesser extent, to the type I MYB recognition sequence (Romero *et al.*, 1998). To date, due to lack of detection of negative regulatory elements related to these negative regulators of *CBF*, its cold-regulated mechanism in plants remains to be explained. In this study, in the region

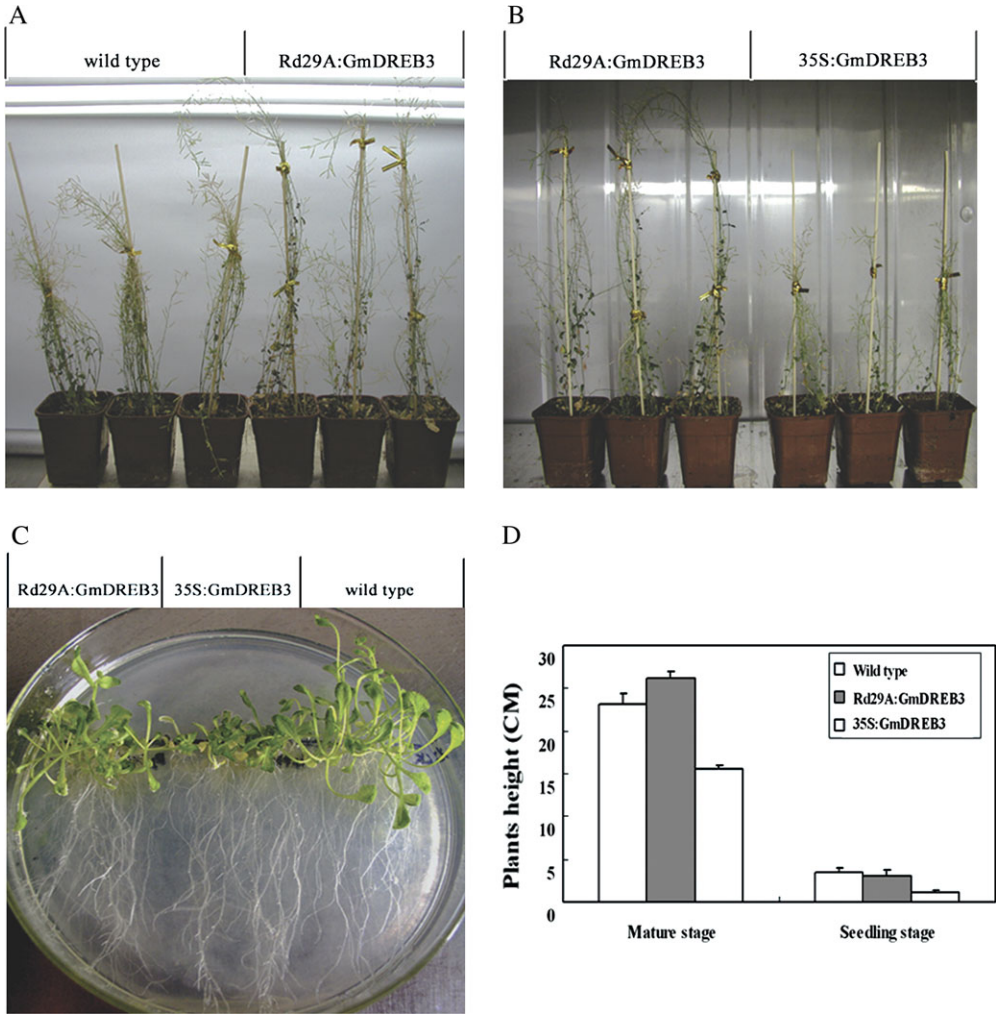

Fig. 6. Phenotypic evaluation of *GmDREB3* transgenic *Arabidopsis*. (A and B) Growth retardation was observed in *35S:GmDREB3* transgenic *Arabidopsis* grown in pots after 70 d under normal conditions, whereas *Rd29A:GmDREB3* transgenics were similar to the wild type. (C) After 22 d, the phenotype of *Rd29A:GmDREB3* transgenics grown on MS medium was similar to that of the wild type, whereas the growth of *35S:GmDREB3* transgenics was obviously retarded. (D) Average seedling and mature plant heights of transgenics are compared with those of wild-type plants on MS medium and in pots.

−1403 to −1058, three copies of consecutive MYB recognition site (TTGATA) were detected. This is similar to type II recognition sites, suggesting some MYB transcription factors might bind to these three MYB sites to regulate the expression of *GmDREB3* negatively (Fig. 2D, E). Further identification of actively regulated elements in region −1058 to −664 and negatively regulated elements in region −1403 to −1058 will be important for elucidating the regulatory mechanism of *GmDREB3* under cold stress.

Expression of GmDREB3 *controlled by the stress-inducible* Rd29A *promoter increased tolerance to drought and high salt stresses, and minimized negative effects on plant growth under normal growth conditions*

Overexpression of *PpDBF1* in transgenic tobacco plants showed enhanced tolerance to salt, osmotic, and cold stresses, and, when grown on MS medium plus 200 mM NaCl and 250 mM sorbitol, root growth rates of transgenic tobacco plants were higher than those of the wild-type (Liu *et al.*, 2007). Similarly, overexpression of *GmDREB2* in transgenic plants also showed enhanced tolerance to salt and drought stresses (Chen *et al.*, 2007). In the present work, *GmDREB3* transgenic *Arabidopsis* plants were tolerant to drought, cold, and high salt stresses, similar to *GmDREB2* transgenic plants. In addition, transcriptional activation analysis of GmDREB3 showed that yeast cells with the recombinant plasmid harbouring *GmDREB3* grew on media lacking histidine in the presence of 10, 20, 30, and 40 mM 3-AT (Fig. 3C), whereas yeast cells with *GmDREB2* grew only on media in the presence of 10 mM 3-AT (Chen *et al.*, 2007), suggesting that under the same conditions (uniform plasmids were present in the yeast cells; data not shown), transcriptional activation of GmDREB3 was higher than that of GmDREB2. The C-terminal part of GmDREB3 shared low homology with GmDREB2 (Supplementary Fig. S1A at *JXB* online). Moreover, LWST domains, characteristic domains of CBF proteins (Jaglo

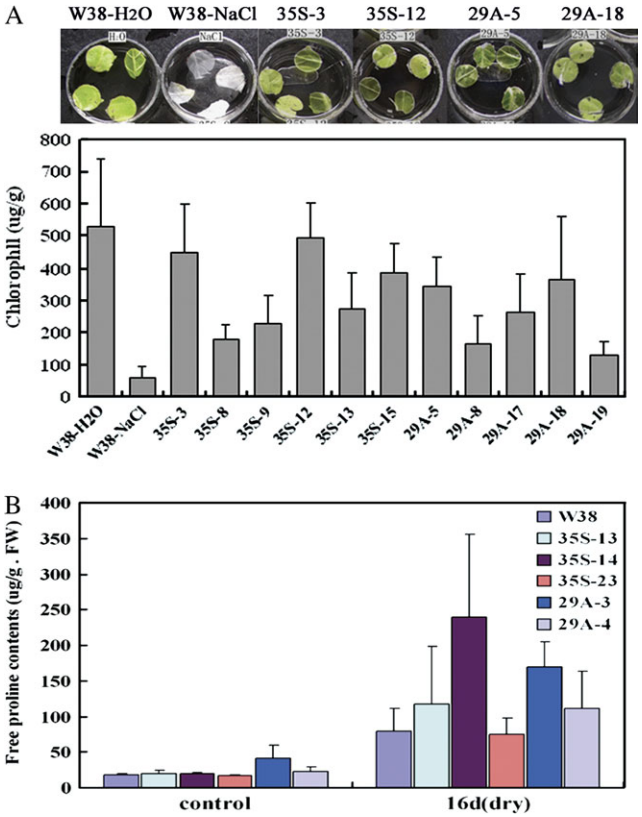

Fig. 7. Chlorophyll and free proline content analyses of wild-type and transgenic tobacco plants under high salt and drought-stressed conditions. (A) Visual differences and chlorophyll contents (μg g^{-1} fresh weight) measured after 96 h of high salt treatment. The experiments were repeated three times, each replicate with four leaf discs. W38H_2O and W38NaCl represent the wild type floated on H_2O and high salt solution, respectively. 35S-3 to 35S-15 (*35S:GmDREB3*) and 29A-5 to 29A-15 (*Rd29A:GmDREB3*) are transgenics. (B) Free proline contents of wild-type and transgenic tobacco plants under normal or drought stress conditions. The wild-type (W38) and transgenic tobacco plants were grown in pots under normal conditions for 8 weeks when leaves were harvested as control samples. Both were then deprived of water for 16 d (dry); leaves were harvested at the 16th day for proline analyses.

et al., 2001), were detected only in GmDREB3 and PpDBF1. The structural differences might cause the different transcriptional activation characteristics of GmDREB3 and GmDREB2. Thus members of the A-5 subgroup might play key roles in regulating expression of stress-related genes, and, among them, *GmDREB3*, as an important member, might be useful for improving stress tolerance in crop plants.

In this study, overexpression of *GmDREB3* in *35S:GmDREB3* transgenic plants led to reduced height of seedlings and mature plants (Fig. 6). Similar phenomena were observed with *DREB1A*, *DREB1B*, and *DREB1C* transgenic *Arabidopsis* (Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2004). Recent studies showed that expression of some genes downstream of *DREB1*, such as the GA (gibberellin) biosynthesis gene and transcription repressor STZ, might be involved in growth retardation of DREB1 transgenic plants (Nakashima *et al.*, 2006). To minimize the negative effects of *DREB1* on plant growth, the stress-inducible *Rd29A* promoter, employed to control the expression of *DREB1A*, improved the drought, salt, and freezing stress tolerance of transgenic *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1993), tobacco (Kasuga *et al.*, 2004), rice (Dubouzet *et al.*, 2003), wheat (Pellegrineschi *et al.*, 2004), and potato (Behnam *et al.*, 2006). Both the stress-inducible *Rd29A* and constitutive *CaMV 35S* promoters were used to control the expression of *GmDREB3*. Growth retardation of *Rd29A:GmDREB3* transgenic plants was reduced (Fig. 6), and the survival rate of *Rd29A:GmDREB3* transgenic *Arabidopsis* was high (Fig. 4A), providing hope that a combination of the *Rd29A* promoter and *GmDREB3* might be useful for improving tolerance to environmental stresses in crop plants.

GmDREB3 *transgenic plants accumulated higher levels of solute or free proline, enhancing the tolerance of transgenic plants to cold and drought stresses*

The fresh weights of both wild-type and *35S:GmDREB3* transgenic plants were similar before cold stress, and both decreased after cold stress; however, the transgenics retained higher levels of water than wild-type plants (P <0.005) (Fig. 5B). Moreover, after cold stress, all wild-type plants withered and died, whereas most of the transgenics remained green (Fig. 5A). Thus *35S:GmDREB3* transgenics decreased water loss from cells, leading to enhanced tolerance to cold stress.

Many plants increase their tolerance to freezing when exposed to low, but non-freezing, temperatures in an adaptive process known as cold acclimation. During cold acclimation, the accumulation of compatible solutes, such as soluble sugars, protein, and betaine, occurs in many plant species (Guy, 1999). Yoshida *et al.* (1998) reported a positive correlation between freezing tolerance and soluble sugar content in 18 wheat cultivars. Uemura *et al.* (2003) reported that the freezing sensitivity of a cold-acclimated *sfr4* mutant of *Arabidopsis* was due to its continued susceptibility to LOR (loss of osmotic responsiveness) and was associated with low sugar content in its cells. The level of freezing tolerance in canola was strongly dependent on the osmotic potential of the leaves, and the osmotic potential of acclimated leaves was lower than that of non-acclimated leaves under cold stress (−3 °C) (Gusta *et al.*, 2004). The osmotic potential (ψ_S), as a function of the molal concentration of solute, was calculated using the van't Hoff relationship: $\psi_S = -CiRT$ [where ψ_S =osmotic potential; C=osmolality of solute; i=ionization constant; R=gas constant (0.00831 kg MPa mol^{-1} K^{-1}); T=absolute temperature (K)=+273 °C] (Bhatia *et al.*, 2005). In this study, the osmolalies of wild-type and transgenic plants were similar before cold stress, whereas transgenics had significantly higher osmolalities than wild-type plants after cold stress (Fig. 5C), indicating that the osmotic potentials of transgenics were lower than those of wild-type plants after cold stress. Thus *35S:GmDREB3* transgenics are likely to accumulate higher levels of solutes, such as sugars, such

that the osmotic potentials decline faster than in wild-type plants.

Many plants accumulate free proline under drought, high salt, and cold stress conditions, and this probably functions as an osmo-protectant in stressed plants, leading to tolerance (Igarashi *et al.*, 1997). For example, transgenic *Arabidopsis* plants overexpressing *DREB1A*/*CBF3* accumulate free proline under unstressed conditions (Gilmour *et al.*, 2000). Free proline contents of *GmDREB3* transgenic tobacco plants were compared with those of wild-type plants under drought stress conditions. Among the transgenic tobacco lines, 35S-14 and 29A-3 accumulated higher levels of free proline than wild-type plants after drought stress treatment for 16 d (Fig. 7B). These results suggested that overexpression of *GmDREB3* in transgenic plants enhanced tolerance to drought stress by inducing expression of downstream genes involved in the synthesis and accumulation of higher levels of free proline.

The nucleotide sequence of the *GmDREB3* gene reported in this article has been submitted to the GenBank database under the accession number *DQ208969*.

Supplementary material

The supplementary material is available at *JXB* online.

Figure S1. shows the amino acid sequence alignment of GmDREB3 and other members in the A-5 subgroup.

Figure S2. shows the expression pattern of the *GmDREB3* gene in soybean under different abiotic stresses.

Figure S3. shows the promoter sequence alignment of *GmDREB3* and *GmDREB2* in the soybean genome.

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