PCP
PLANT & CELL PHYSIOLOGY

Isolation and Characterization of Dehydration-Responsive Element-Binding Factor 2C (MsDREB2C) from *Malus sieversii* Roem.

Kai Zhao, Xinjie Shen, Huazhao Yuan, Yun Liu, Xiong Liao, Qi Wang, Linlin Liu, Fang Li and Tianhong Li*

Department of Fruit Science, College of Agriculture and Biotechnology/Key Laboratory of Stress Physiology and Molecular Biology for Tree Fruits of Beijing, China Agricultural University, 2 Yuanmingyuan West Road, Haidian District, Beijing 100193, PR China
*Corresponding author: E-mail, lith@cau.edu.cn; Fax, +86-01062733957.
(Received March 22, 2013; Accepted June 5, 2013)

DREB2 (dehydration-responsive element-binding factor 2)-type transcription factors play a critical role in the stress-related regulation network in plants. In this study, we isolated and characterized a *DREB2* homolog from *Malus sieversii* Roem., designated *MsDREB2C* (GenBank accession No. JQ790526). *MsDREB2C* localized to the nucleus and transactivated reporter genes in yeast strain YGR-2. Quantitative real-time PCR analysis demonstrated that *MsDREB2C* was constitutively expressed and significantly induced by drought, salt, cold, heat and ABA. Transgenic Arabidopsis plants overexpressing *MsDREB2C* exhibited increased root and leaf growth and proline levels, and reduced water loss and stomatal aperture. The transcriptional level of genes that function downstream of dehydration-responsive elements was greater in the transgenic Arabidopsis plants than in wild-type plants under control and abiotic stress conditions. Furthermore, constitutive expression of *MsDREB2C* repressed the expression of pathogenesis-related (*PR*) genes and the activity of peroxidase in transgenic plants under control and pathogenic conditions. As a result, transgenic plants were more tolerant to drought, heat and cold, but more sensitive to *Pst* DC3000 (*Pseudomonas syringae* pv . tomato DC3000) infection than control plants. β-Glucuronidase expression analysis of the *MsDREB2C* promoter in transgenic tobacco plants showed that *MsDREB2C* was mainly expressed in the vascular tissues and seeds. Deletion analysis identified the regulatory regions responsible for the plant's response to drought (−831 to −680), ABA (−831 to −680 and −335 to −148), salt (−831 to −335), cold (−1,317 to −831 and −335 to −148) and heat (−335 to −148).

Keywords: Abiotic stresses • Biotic stresses • DREB • Functional analyses • *Malus sieversii* Roem. • Promoter identification.

Abbreviations: ABRE, ABA-responsive element; AP2, APETALA2; ARF, auxin response factor; CaMV, *Cauliflower mosaic virus*; CBF, C-repeat binding factor; DRE, dehydration-responsive element; DREB, dehydration-responsive element-binding factor; ELISA, enzyme-linked immunosorbent assay; ERD, early responsive to dehydration; ERF, ethylene-responsive transcription factor; GFP, green fluorescent protein; GUS, β-glucuronidase; HSE, heat shock element; HSF, heat shock factor; ICE1, inducer of CBF expression1; LEA, late embryogenesis abundant; MS, Murashige and Skoog; 4-MUG, 4-methylumbelliferyl glucuronide; NLS, nuclear localization signal; ORF open reading frame; P5CS2, delta 1-pyrroline-5-carboxylate synthetase; PR, pathogenesis-related; *Pst* DC3000, *Pseudomonas syringae* pv . tomato DC3000; RAV, related to ABI3/VP1; RT–PCR, reverse transcription–PCR; WT, wild type; ZR, zeatin riboside.

Introduction

Abiotic stresses such as drought, high salinity, cold and heat are common adverse environmental conditions that significantly limit growth and productivity of plants (Sakuma et al. 2006a). Conserved mechanisms are thought to underlie the plant's response to such environmental stresses (Liu et al. 1998). Molecular and cellular responses to abiotic stresses involve signal perception, transduction of the signal to the cytoplasm and nucleus, alteration of gene expression and, finally, metabolic changes that lead to stress tolerance (Agarwal et al. 2006). Many genes respond to drought, salt, cold and heat stress at the transcriptional level, and the products of these genes function in the stress response and tolerance acquisition (Shinozaki 2003, Sakuma et al. 2006a). Large-scale transcriptome analyses using microarray technology revealed many stress-induced genes (Bohnert et al. 2001, Fowler and Thomashow 2002, Sakuma et al. 2006b). The products of these genes can be classified into two groups, one of which is involved in stress tolerance [e.g. chaperones, late embryogenesis abundant (LEA) proteins and enzymes involved in osmolyte biosynthesis and detoxification] and the other of which influences gene expression and signal transduction (e.g. protein kinases, transcription factors and enzymes involved in phospholipid metabolism) (Yamaguchi-Shinozaki and Shinozaki 2005). Several sets of

Plant Cell Physiol. 54(9): 1415–1430 (2013) doi:10.1093/pcp/pct087, available online at www.pcp.oxfordjournals.org

trans-acting factors interact with *cis*-acting elements in the promoter regions of various stress-related genes to up-regulate the expression of many downstream genes (Xiong et al. 2002). Over 5% of the Arabidopsis genome encodes >1,500 transcription factors that are thought to be involved in regulating the expression of stress-responsive genes. Among these 1,500 transcription factors, many are unique to plants, including those belonging to the AP2/EREBP, NAC and WRKY families, and these genes are traditionally classified as belonging to the ABA-dependent and ABA-independent regulatory pathways (Reichmann et al. 2000, Xiong et al. 2002).

A total of 144 APETALA2/ethylene-responsive transcription factor (AP2/ERF)-type transcription factors were identified in the Arabidopsis genome sequence (Reichmann et al. 2000). These transcription factors are categorized into five subfamilies based on similarities in their DNA-binding (AP2) domains: AP2, RAV (related to ABI3/VP1), DREB (dehydration-responsive element-binding factor) and ERF, and a subfamily consisting of just one specific gene, AL079349. Genes included in the DREB subfamily are divided into six small subgroups (A-1 to A-6) based on similarities in the binding domain. The A-1 subgroup, which includes the DREB1/CBF (C-repeat binding factor)-like genes, are mainly induced by low temperature and activate the expression of many cold stress-responsive genes, whereas the A-2 subgroup, which is comprised of the DREB2 genes, mainly functions in osmotic stress (Sakuma et al. 2002). The dehydration-responsive element (DRE), which contains a 9 bp conserved core sequence, TACCGACAT, was first identified as a *cis*-acting promoter element in the promoter of the drought-responsive gene *rd29A* (Yamaguchi-Shinozaki and Shinozaki 1994). A similar motif was identified as the CRT (C-repeat) and LTRE (low-temperature-responsive element) in cold-inducible genes (Baker et al. 1994). Subsequently, cDNAs encoding DRE-binding proteins, CBF/DREB1s and DREB2s, were isolated using yeast one-hybrid screening (Stockinger et al. 1997, Liu et al. 1998). These proteins specifically bind to the DRE/CRT sequence and activate the transcription of genes driven by the DRE/CRT sequence in Arabidopsis. The 14th valine and 19th glutamic acid residues in the ERF/AP2 domain are highly distinctive and play a crucial role in determining DNA binding specificity (Sakuma et al. 2002).

DREB2 homologous genes have been isolated from a variety of species (reviewed by Lata and Praasd, 2011). Transgenic Arabidopsis plants overexpressing either *AtDREB2A* or *OsDREB2A* exhibited neither growth retardation nor improved stress tolerance, suggesting that the DREB2A proteins require post-translational modification (Liu et al. 1998, Dubouzet et al. 2003). It was found that overexpression of *DREB2C* induced the expression of many heat shock stress-inducible genes and conferred thermotolerance to transgenic Arabidopsis plants (Lim et al. 2007). Transgenic Arabidopsis overexpressing *OsDREB2B* exhibited enhanced tolerance to drought and heat stress (Matsukura et al. 2010). Until now, most studies on *DREB2* genes focused on their roles in abiotic stress; we still have little information on the relationship between DREB2-type genes and biotic stress. The mechanism by which CBF/DREB1 was up-regulated in response to low temperature has been well characterized (Chinnusamy et al. 2003). In addition, an ABA-responsive element (ABRE) was identified in the promoter of *DREB2A* (Kim et al. 2011), and a short promoter region of *DREB2A* was also found to repress its expression under non-stress conditions (Kim et al. 2012). A proximal promoter region of Arabidopsis *DREB2C* conferred tissue-specific expression under heat stress (Chen et al. 2012). Among the DRE-binding proteins, the DREB2 subgroup were induced by cold, drought, high salinity, heat stress and ABA (Lata and Praasd, 2011). Thus further research needs to be carried out to demonstrate the activation mechanism of DREB2-type genes in response to other kinds of stress.

Malus sieversii (Ledeb.) Roem., a wild apple species mainly distributed in the Tianshan Mountains of Central Asia, is known to be one of the most drought-tolerant apple rootstocks in China (Li 2001). DREB1 and DREB2 are involved in two separate signal transduction pathways, functioning under low temperature and dehydration, respectively (Liu et al. 1998). Most studies on the *DREB* subfamily in apple to date have centered on *DREBA-1* and *DREBA-5* genes (Champ et al. 2006, Yang et al. 2011, X.J. Zhao et al. 2012), and little is known about *DREBA-2*. We thus sought to characterize *DREBA-2* genes from *M. sieversii* in an effort to understand this plant's remarkable resistance to environmental stresses.

Results

Isolation and characterization of *DREB2C* from *M. sieversii* Roem.

The cDNA sequence of *MsDREB2C* (GenBank accession No. JQ790526) harbors a 1,197 bp open reading frame (ORF) that encodes a putative protein of 398 amino acid residues with a predicted molecular mass of 43.8 kDa and an isoelectric point of 4.93. Analysis of the deduced amino acid sequence of MsDREB2C revealed a typical AP2/ERF domain of 57 amino acids with conserved valine (V) and glutamic acid (E) residues at the 14th and 19th positions, respectively. Additionally, three β-sheets and an α-helix were found in the AP2/ERF domain (T. Zhao et al. 2012). Two short conserved sequences, KRKTRSR and KTRKVPAKGSKKG, immediately flanking the AP2/ERF domain might function as a nuclear localization signal (NLS) (Liu et al. 1998, Zhou et al. 2010). In addition, an acidic C-terminal region of MsDREB2 might act as a transcriptional activation domain (Stockinger et al. 1997, Liu et al. 1998) (**Supplementary Fig. S1**). A phylogenetic analysis was conducted to clarify the relationship between MsDREB2C and DREB-related proteins (**Fig. 1**). MsDREB2C was found to belong to the A-2 subgroup of the DREB subfamily, with the highest homology (36%) to AtDREB2C, according to the classification of AP2/ERF transcription factors in Arabidopsis (Sakuma et al. 2002).

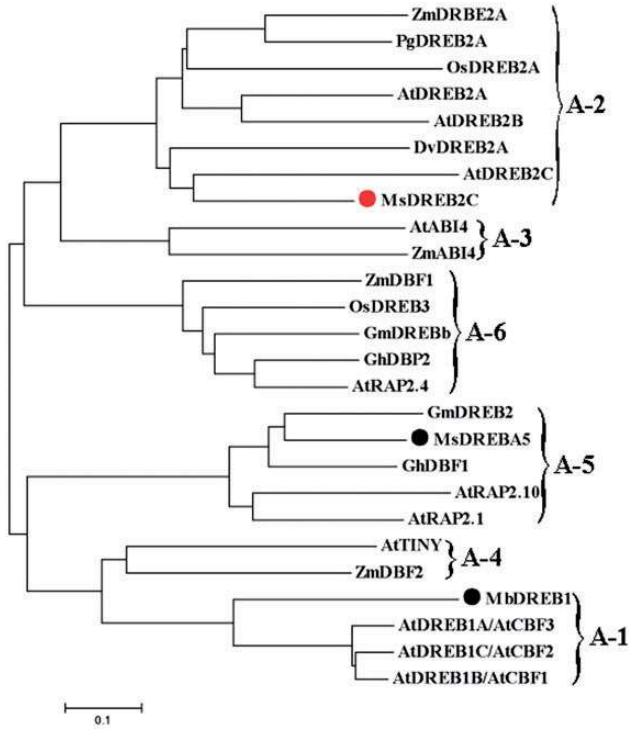

Fig. 1 Phylogenetic analysis of MsDREB2C and DREB-related proteins from different species. A phylogenetic tree of DREB proteins from various species. A-1 to A-6 indicate subgroups proposed by Sakuma et al. (2002). MsDREB2C in the A-2 subgroup is marked with a red dot. MsDREBA5 in the A-5 subgroup and MbDREB1 in the A-1 subgroup are marked with black dots. The analysis, based on minimum evolution, was performed with full-length protein sequences using the AP2 transcription factor as an outgroup. DREB-related proteins were initially aligned using ClustalW and were then used for phylogenetic analysis using MEGA version 5.05 software (http://www.megasoftware.net). The phylogenetic tree was constructed using the Neighbor–Joining method with 1,000 bootstrap replications. The accession numbers for the genes are as follows: ZmDRBE2A (AB218832), PgDREB2A (DQ227697), OsDREB2A (AF300971), AtDREB2A (AB007790), AtDREB2B (NM111939), DvDREB2A (EF633987), AtDREB2C (NM129594), MsDREB2C (JQ790526), AtABI4 (AF040959), ZmABI4 (AY125490), ZmDBF1 (AF493800), OsDREB3 (AK105877), GmDREBb (AY296651), GhDBP2 (AY619718), AtRAP2.4 (AT1G78080), GmDREB2 (DQ208968), MsDREBA5 (JQ655768), GhDBF1 (AY174160), AtRAP2.10 (AT4G36900), AtRAP2.1 (AT1G46768), AtTINY (At5g25810), ZmDBF2 (AF493799), MbDREB1 (EF582842), AtDREB1A (AB007787), AtDREB1C (AT4G25470) and AtDREB1B (AT4G25490).

Subcellular localization and transactivation of MsDREB2C

To analyze the subcellular localization of the MsDREB2C transcription factor, the full-length coding region was introduced into the pE3025-GFP (geen fluorescent protein) vector, upstream of the *GFP* reporter gene. The recombinant constructs of the *MsDREB2C-GFP* fusion gene and *GFP* alone were transformed into onion (*Allium cepa*) epidermal cells using a gene gun. The MsDREB2C–GFP fusion proteins were observed in the nucleus, whereas GFP on its own was present in both the cytoplasm and nucleus (**Supplementary Fig. S2**). These results showed that MsDREB2C is a nuclear protein, consistent with its predicted function as a transcription factor.

To test the transactivation ability of MsDREB2C, we used a yeast system. The yeast strain harbored dual reporter genes, *His3* and *LacZ*, both controlled by the GAL4 upstream activation sequence. All transformants containing pGAL4 (positive control), pBD (negative control) and pBD-MsDREB2C grew well on SD/−Trp medium. In contrast, only yeast cells harboring the fusion plasmid pBD-MsDREB2C and pGAL4 grew well on SD/−Trp/−His medium, and stained blue in X-Gal solution (**Supplementary Fig. S3**). These results indicate that MsDREB2C has transactivation capability.

Expression patterns of *MsDREB2C* in different organs and in response to various stresses

The expression pattern of *MsDREB2C* in different tissues and organs of *M. sieversii* was examined under normal conditions. *MsDREB2C* was constitutively expressed in almost all tissues and organs examined, including young leaves and roots, mature leaves and roots, xylem, phloem, flowers and fruit. The expression level of *MsDREB2C* was highest in mature leaves and roots, followed by young leaves, young roots, flowers and fruit (**Fig. 2**; **Supplementary Fig. S4**). We also tested the expression patterns of *MsDREB2C* under various stresses. Quantitative reverse transcription–PCR (RT–PCR) revealed that *MsDREB2C* expression was induced by drought, heat, cold, salt and ABA (**Fig. 3**; **Supplementary Fig. S5**). *MsDREB2C* expression was rapidly and transiently induced by heat shock treatment and peaked at 1 h. The expression declined slightly at 3 h after the start of treatment and then drastically declined to a relatively low level, which was maintained until 12 h after treatment. In contrast, *MsDREB2C* expression was gradually induced by drought and cold stress during the 12 h following treatment and peaked at 12 h. We also analyzed the effect of salt stress, and found that *MsDREB2C* expression increased slightly within 1 h of treatment, decreased from 1 h to 3 h and increased to a maximum at 6 h, before dropping down at 12 h to a level that was higher than that at 1 h. Finally, we tested the effects of exogenous ABA on *MsDREB2C* expression. The transcript level gradually increased and peaked at 3 h after treatment, and then decreased over the next 3 h to the level of the non-treated control.

Phenotype analysis of transgenic Arabidopsis lines expressing *MsDREB2C*

To establish the functional significance of *MsDREB2C*, three random homozygous T_3 lines (L-1, L-2 and L-3) containing a single T-DNA insertion were chosen for subsequent analyses. Expression of *MsDREB2C* in transgenic *Arabidopsis* was detected by RT–PCR (**Supplementary Fig. S6A**). These lines overexpressing *MsDREB2C* were morphologically different from the

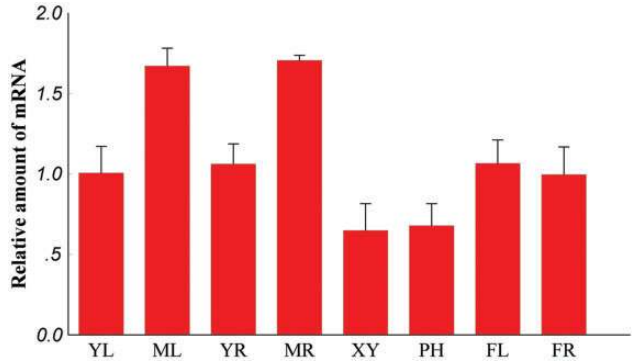

Fig. 2 The organ-specific expression of *MsDREB2C*. YL (young leaf), ML (mature leaf), YR (young root), MR (mature root), XY (xylem), PH (phloem), FL (flower), FR (fruit). 18S rRNAs were used as endogenous controls. Each column represents the mean of three replicates. Error bars on each column represent the SE of three replicates. Primers used are listed in **Supplementary Table S1**.

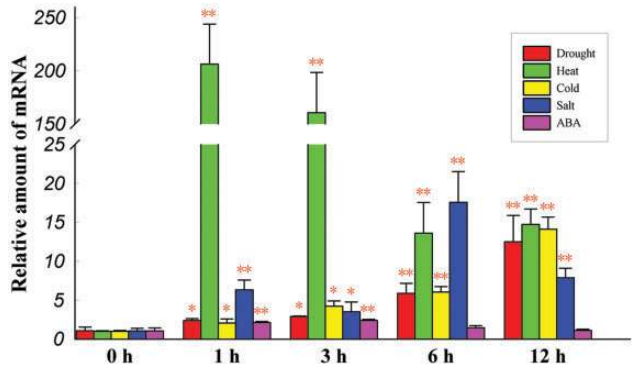

Fig. 3 Expression patterns of *MsDREB2C* in *M. sieversii* leaves subjected to various abiotic treatments. For drought, hydroponic seedlings were removed from water and placed on filter paper; for heat and cold, hydroponic seedlings were exposed to 45 and 4°C, respectively, in a growth chamber; for salt, hydroponic seedlings were transferred into Hoagland nutrient solution containing 200 mM NaCl; for ABA, leaves of hydroponic seedlings were sprayed with 200 μM ABA solution. For all treatments, functional leaves were harvested at the designated time intervals. The relative expression was calculated using 18S rRNA as an internal reference. The unstressed expression level was assigned a value of 1. Each column represents the mean of three replicates. Error bars on each column represent the SE of three replicates (Student's *t*-test, $^{**}P < 0.01$, $^{*}P < 0.05$). Primers used are listed in **Supplementary Table S1**.

wild-type (WT) plants. Transgenic seedlings growing on 1/2 Murashige and Skoog (MS) medium developed significantly longer primary roots and higher numbers of lateral roots than the WT plants. Furthermore, the above-ground parts of the transgenic seedlings were taller than those of the WT, and the fresh weight was greater (**Supplementary Fig. S6B–G**). In addition, we also analyzed the difference between the transgenic and WT seedlings grown in soil. Similar results were obtained for materials grown on 1/2 MS agar medium. In comparison with the WT plants, the transgenic lines had larger leaves, a greater number of inflorescences and heavier seeds; however, the inflorescences of transgenic seedlings were shorter than those of the WT seedlings (**Fig. 4A–C**; **Supplementary Fig. S7**). Observations of leaf cross-sections showed that transgenic plants had larger mesophyll cells, and thicker palisade tissue and spongy tissue than WT plants (**Fig. 4D**).

To investigate the underlying cause of the phenotypic alterations in the transgenic lines, we measured the content of endogenous auxin (free IAA) and cytokinin [zeatin riboside (ZR)] in WT and *MsDREB2C*-expressing transgenic Arabidopsis plants. The levels of free IAA and ZR were significantly higher in transgenic plants than in the WT (**Table 1**). Among the 22 Arabidopsis *ARF* (auxin response factor) genes, *ARF7* and *ARF19* were found to be involved in promoting leaf expansion and lateral root formation (Wilmoth et al. 2005). Thus, we tested the expression levels of both of these genes in transgenic plants expressing *MsDREB2C*, and found them to be more strongly expressed than in WT seedlings (**Fig. 5**).

Abiotic stress treatment of transgenic plants

Overexpression of *ZmDREB2A* (Qin et al. 2007), *OsDREB2B* (Matsukura et al. 2010) or *AtDREB2C* (Lim et al. 2007) has been demonstrated to enhance tolerance to drought and heat shock stress. To examine further the function of *MsDREB2C*, three soil-grown transgenic lines (L-1, L-2 and L-3) were exposed to heat, drought, salt and freezing stress. The survival rate and representative results are shown in **Supplementary Fig. S8**. The constitutive expression of *MsDREB2C* resulted in increased tolerance to heat and drought. Under heat conditions, only 4.7% of the WT plants survived, whereas survival rates of 90.9, 86.9 and 92.3% were obtained for the three transgenic lines, respectively. Similar results were obtained after drought treatment. Only 47.1% of WT plants survived drought treatment, compared with 91.2, 96.5 and 100% of the three transgenic lines, respectively. WT and transgenic seedlings subjected to salt stress had similar survival rates; 13.0% of WT seedlings survived the treatment, whereas 10% of L-1, 13.0% of L-2 and 16.7% of L-3 survived. In addition, we tested the freezing tolerance of the plants. WT plants exhibited a 38.2% survival rate upon exposure to freezing stress. While the survival rate of L-2 and L-3 plants was elevated (77.8% and 80.0%, respectively), that of L-1 plants (38.5%) was similar to that of WT plants.

Proline content accumulates in plants subjected to high-salt and cold stress conditions to confer stress tolerance (Igarashi et al. 1997, Ito et al. 2006). In addition, transgenic Arabidopsis plants overexpressing *DREB1/CBF* accumulate proline under unstressed control conditions (Gilmour et al. 2000, Ito et al. 2006). We investigated whether proline also accumulated in transgenic Arabidopsis plants overexpressing *MsDREB2C*. The proline levels of both WT and transgenic plants were significantly higher under drought stress conditions. Furthermore, the

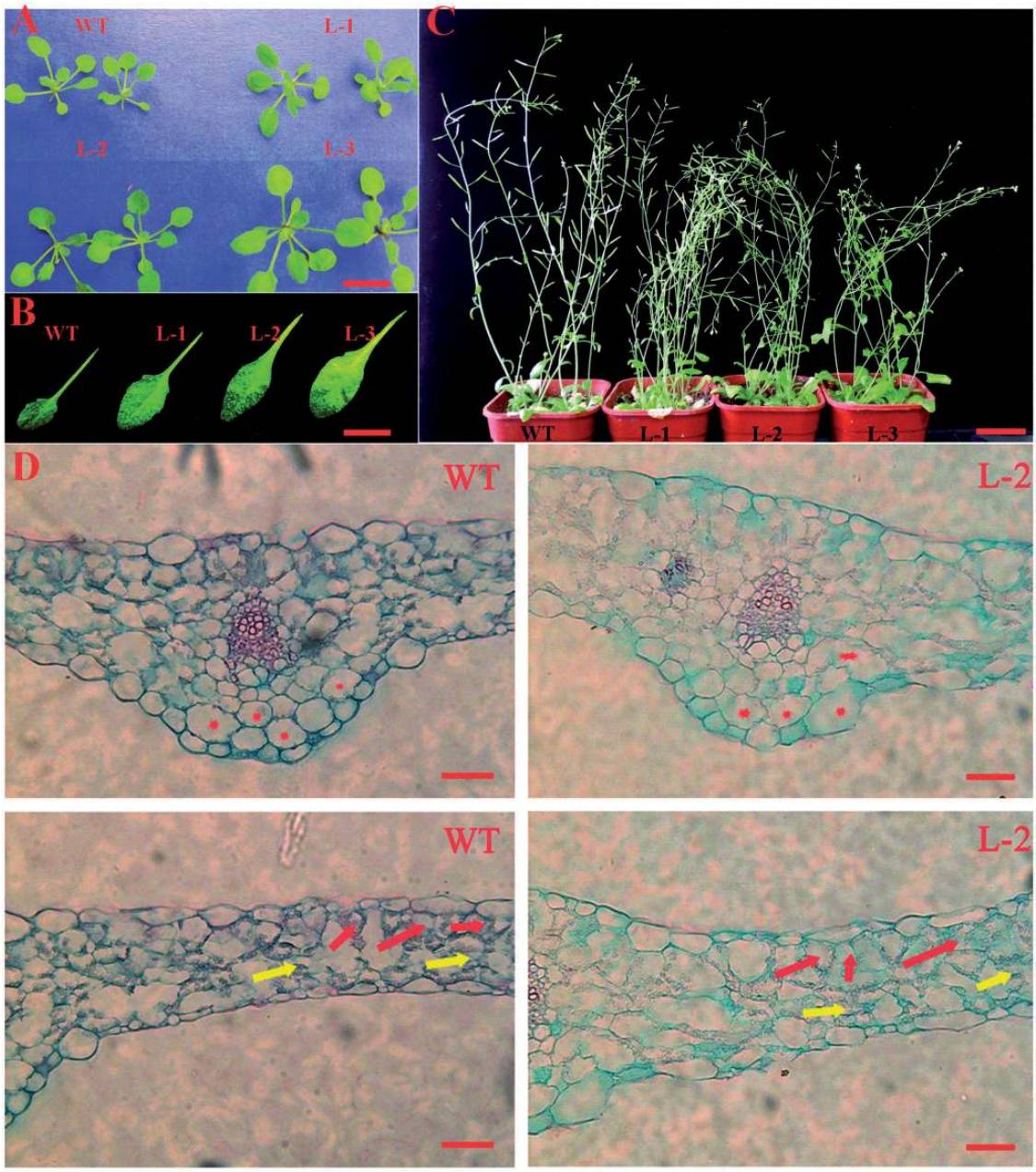

Fig. 4 Analyses of *35S:MsDREB2C* transgenic Arabidopsis lines grown in soil. WT and transgenic (L-1, L-2 and L-3) Arabidopsis plants were grown on 1/2 MS agar medium for 10 d and then transferred to soil and grown for 11 d (A), 24 d (B) and 60 d (C). The eighth 34-day-old rosette leaves from the top were chosen for transverse sections analysis (D). Mesophyll cells, palisade tissue and spongy tissue are indicated by red asterisks, red arrows and yellow arrows, respectively. Scale bars = 1 cm (A, B); 2 cm (C); 50 μm (D).

Table 1 IAA and ZR levels in WT and *MsDREB2C* transgenic Arabidopsis plants

	WT	L-1	L-2	L-3
IAA (ng g^{-1} FW)	60.78 ± 3.37	84.43 ± 4.71**	83.44 ± 5.90**	89.22 ± 4.69**
ZR (ng g^{-1} FW)	7.60 ± 0.13	8.58 ± 0.41**	8.43 ± 0.55**	8.82 ± 0.5**

IAA and ZR content were measured in plants grown in soil for 21 d under control conditions. The experiments were repeated three times.
Statistical significance compared with values of WT plants was determined by Student's *t*-test (**$P < 0.01$).

proline content of all the transgenic lines was slightly higher than that of WT plants (**Supplementary Fig. S9**). Plants over-expressing AREB/ABF (ABA-responsive element-binding factors), NAC (NAM, ATAF1-2 and CUC2) and DREB showed enhanced drought resistance as a result of smaller stomatal apertures (Kang et al. 2002, Hu et al. 2006, Bhatnagar-Mathur et al. 2007). Therefore, we tested whether the constitutive over-expression of *MsDREB2C* in transgenic Arabidopsis plants reduced stomatal closure. Our results indicated that the

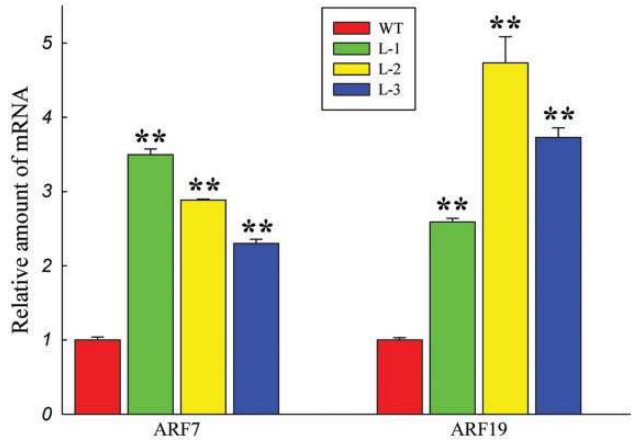

Fig. 5 Expression patterns of *ARF7* and *ARF19* in WT and *35S:MsDREB2C* plants. The expression of *ARF7* and *ARF19* was evaluated in WT and transgenic plants by quantitative RT–PCR. Total RNA was extracted from 21-day-old seedlings grown under normal conditions. *AtACTIN2* was amplified as an internal reference. Each column represents the mean of three replicates. Error bars represent the SE of three replicates (Student's *t*-test, $^{**}P < 0.01$). Primers used are listed in **Supplementary Table S1**.

stomatal aperture index of the transgenic plants was significantly lower than that of WT plants, whereas no significant difference was observed among the three transgenic lines (**Supplementary Fig. S10**). Consistent with these results, the detached leaves of WT plants lost water more quickly than those of the transgenic plants (**Supplementary Fig. S11**). Given that the *MsDREB2C* transgenic plants showed enhanced tolerance to heat, drought and freezing stress, we decided to quantify the molecular responses of seven stress-responsive genes containing DRE elements in their promoter regions that had previously been identified as the downstream targets of AtDREBs in Arabidopsis by microarray analyses (Maruyama et al. 2004, Sakuma et al. 2006a, Sakuma et al. 2006b). Among these seven genes, *RD29A*, *RD29B*, *LEA*, *KIN1* and *KIN2* were previously demonstrated to be the direct downstream genes of *AtDREB2A* (Sakuma et al. 2006a), and *RD29A*, *KIN1*, *KIN2* and *GolS3* were found to be the target genes of *AtDREB1A* (Maruyama et al. 2004). *HSFA3* was significantly induced by heat treatment (Sakuma et al. 2006b). In all the three transgenic lines, the expression level of *RD29A*, *RD29B*, *LEA7* and *HSFA3* was greater in the transgenic plants than in the WT plants under non-stressed and drought, salt, cold and heat conditions. In contrast, *KIN1*, *KIN2* and *GolS3* were expressed at similar levels in the transgenic and WT plants (**Supplementary Fig. S12**).

Biotic stress treatment for transgenic plants

To confirm the role of *MsDREB2C* in biotic stress, three F_3 homozygous transgenic Arabidopsis lines with a single insertion of the *MsDREB2C* gene were chosen for biotic resistance analysis. Both WT and transgenic plants were inoculated with *Pst* DC3000 (*Pseudomonas syringae* pv . tomato DC3000) and disease symptoms were monitored 7 d after inoculation (**Fig. 6A**). WT plants did not exhibit any apparent phenotypic abnormality compared with transgenic plants. Trypan blue staining (**Fig. 6B**) revealed that the leaves of three transgenic lines developed more severe disease symptoms than those of WT plants, exhibiting more wilting, necrosis, collapse and chlorotic lesions. Under normal growth conditions, the leaves of WT and transgenic plants showed no significant color difference after trypan blue staining. In contrast, the transgenic plants exhibited significantly more dark blue zones than WT plants after *Pst* DC3000 inoculation.

To determine why the *35S:MsDREB2C* Arabidopsis plants showed reduced tolerance to *Pst* DC3000, we selected the *PR-1* (pathogenesis-related-1) and *PR-2* genes, which had previously been identified as marker genes associated with increased resistance to pathogens (Maleck et al. 2001). Under normal conditions, both *PR-1* and *PR-2* mRNAs were more highly expressed in WT plants than in transgenic plants. When inoculated with *Pst* DC3000, the expression of *PR-1* and *PR-2* was up-regulated in both WT and transgenic plants. Interestingly, the expression level of *PR* genes remained higher in the WT than in transgenic plants (**Fig. 7A, B**). Previous studies demonstrated that enhanced resistance to pathogen attack was accompanied by peroxidase accumulation (Bindschedler et al. 2006). To investigate the roles of peroxidase activity in defense responses to *Pst* DC3000, we measured the peroxidase activity in the WT and transgenic plants (**Fig. 7C**). Our results showed that peroxidase activity in the WT was greater than that in the three transgenic lines under control conditions. Seven days after inoculation with *Pst* DC3000, all of the *35S:MsDREB2C* mutant and WT plants exhibited an increase in peroxidase activity compared with the plants grown under control conditions, and the enzyme activity of the WT remained above that of transgenic plants. Taken together, our findings herein suggested that *MsDREB2C* might have negative roles in basal resistance to the bacterial pathogen.

Isolation and activity analysis of the *MsDREB2C* promoter

To analyze the regulatory mechanism that controls *MsDREB2C* expression, p1317 transgenic tobacco plants were grown under normal conditions and subjected to histochemical staining for β-glucuronidase (GUS) activity. *MsDREB2C* promoter-driven GUS expression occurred in the roots, leaves, fruits and seeds of transgenic tobacco plants, and was especially strong in the leaves and seeds (**Fig. 8A, B, E, F, I, K**). No expression was detected in the stems and flowers (**Fig. 8C, D, G, H**).

Using the PLACE Signal Scan Search Program (Higo et al. 1999), several *cis*-acting elements were predicted to exist in the promoter of *MsDREB2C* (**Supplementary Fig. S13**), including one typical TATA-box, a CAAT-box, MYC recognition sites, ABRE/ERD and GT-1 motifs. Based on these predictions, we

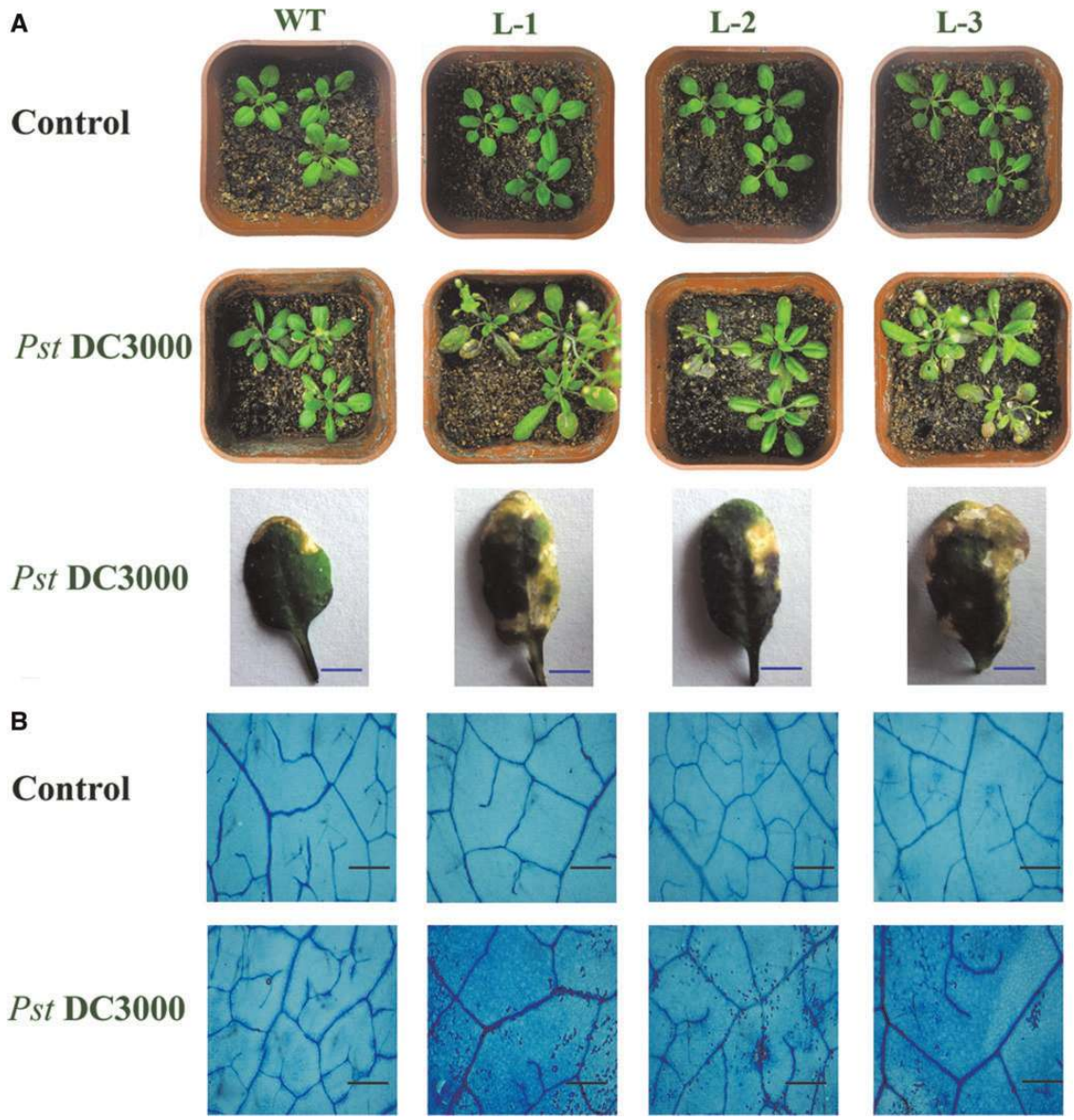

Fig. 6 *35S:MsDREB2C* Arabidopsis plants show increased susceptibility to *Pst* DC3000. Three-week-old seedlings grown under control conditions were inoculated with the pathogen. (A) Disease symptoms developed on the leaves of WT and transgenic Arabidopsis plants. Plants and infected leaves were photographed 7 d after inoculation with *Pst* DC3000 (10^7 c.f.u. ml^{-1}). Bars = 0.5 cm. (B) Trypan blue staining of leaf tissues of WT and transgenic seedlings was carried out under normal growth conditions and with *Pst* DC3000. Bars = 0.2 mm.

constructed six fusions of GUS with various truncations of the *MsDREB2C* promoter, including 5′ deletions to −1,317, −1,062, −831, −680, −335 and −148 bp relative to the transcription initiation site. Under normal conditions, the fluorometric GUS assay demonstrated that deletions from −1,062 to −831, −680 to −335 and −335 to −148 caused a moderate reduction in GUS activity. Intriguingly, the −1,317 to −831 and −1,062 to −680 deletions did not result in significant changes in GUS activity (**Fig. 9B**). These data indicate that the *MsDREB2C* promoter has three positive regulatory regions (−1,062 to −831, −680 to −335 and −335 to −148 bp) that are responsible for its basal activity. Given that *MsDREB2C* responded to abiotic treatment (drought, heat, cold, salt and ABA), we further delineated the specific promoter regions required for *MsDREB2C* expression in response to these treatments by expressing GUS fused to the series of truncated *MsDREB2C* promoter regions in transgenic tobacco plants. Induction of GUS reporter activity was observed in the leaves of transgenic plants bearing the −1,317 deletion upon exposure to drought, cold, heat, ABA and salt. The transgenic plants had similar patterns of GUS induction under drought and ABA treatment (**Fig. 9C, D**). Deletion from −1,317 to −831 bp did not impair drought and ABA responsiveness, whereas deletions from −831 to −680 and −335 to −148 bp reduced GUS activity. Thus, the

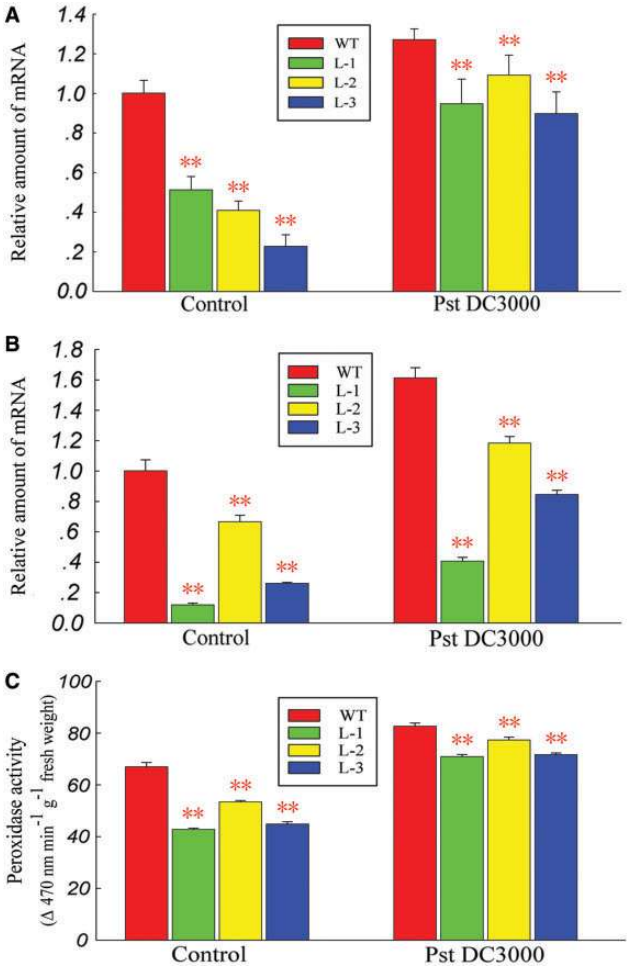

Fig. 7 Expression analysis of *PR* genes and measurement of peroxidase activity in WT and transgenic Arabidopsis plants. (A, B) Quantitative RT–PCR analysis of *PR-1* and *PR-2* expression under normal growth conditions and 7 d after inoculation with *Pst* DC3000. *AtACTIN2* was amplified as an internal reference. (C) Peroxidase activity was determined as the change in absorbance at 470 nm ($min^{-1}g^{-1}$ FW) (Mydlarz and Harvell 2007). Each column represents the mean of three replicates. Error bars represent the SE of three replicates (Student's *t*-test, $^{**}P < 0.01$). Primers used are listed in **Supplementary Table S1**.

151 bp promoter region between −831 and −680 bp, and the 187 bp promoter region between −335 and −148 bp included *cis*-acting elements involved in the dehydration- and ABA-induced expression of *MsDREB2C*. Under cold stress, GUS activity gradually declined from −1,317 to −831 bp, and deletion from −335 to −148 bp significantly impaired cold responsiveness. However, GUS activity was induced 0.78- and 1.29-fold in the −831 and −680 transformants, respectively, which showed that the fragment between −831 and −680 bp contained cold-responsive transcriptional repressor elements (**Fig. 9E**). Under heat stress, deletion from −1,317 to −335 bp did not lead to significant changes in the level of induction. However, transformants bearing the −148 deletion exhibited a lower level of expression than those bearing the −335 deletion (2.99- to 1.93-fold decrease), which suggested that the heat shock-responsive *cis*-element was located in this sequence (**Fig. 9F**). Interestingly, P-335 plants, which lacked the *MsDREB2C* promoter region between the transcription initiation site and −335 bp, had a higher level of GUS activity than P-680 plants under drought, ABA, cold and heat treatments. This indicated that the regions from −680 to −335 bp might contain common transcriptional repressor elements (**Fig. 9C–F**). Under salt treatment, the results differed from the above treatment. GUS activity was slightly higher in P-831 than in P-1317 plants (1.02- and 4.31-fold, compared with the control, respectively) and was strikingly higher in P-831 than in P-148 plants (4.31- and 0.69-fold, respectively). These observations indicate that the sequences from 1,317 to −831 bp contain salt-responsive transcriptional repressor elements and that those from −831 to −148 bp include salt-responsive *cis*-acting elements (**Fig. 9G**).

Discussion

A novel DREB member from *M. sieversii* Roem.

DREB2-type transcription factors exhibit a more complex stress-responsive pattern than DREB1-type transcription factors and may thus provide a more suitable strategy for improving tolerance to drought, heat, cold and salt stress (Lata and Prasad 2011). Therefore, we isolated and identified a DREB2 transcription factor from *M. sieversii* Roem., named MsDREB2C. No research had hitherto been conducted on *DREBA-2* genes isolated from fruit trees. The homology comparisons of DREBs showed that MsDREB2C shared relatively low similarity with two reported DREBs from apple, MbDREB1 (A-1 subgroup) from *M. baccata* and MsDREBA5 (A-5 subgroup) from *M. sieversii* (Yang et al. 2011, X.J. Zhao et al. 2012). However, MsDREB2C (A-2 subgroup) exhibited the highest level of sequence identity to AtDREB2C, with the identity being mostly limited to the AP2 domain region, suggesting that MsDREB2C possesses distinct functional characteristics. *AtDREB2C* was significantly induced by salt, whereas ABA, mannitol and cold treatment had little effect on its expression (Lee et al. 2010). *MsDREB2C* was up-regulated by drought, salt, cold, heat and ABA stress. In addition, the *MsDREB2C* transcript was induced by the biotic stress-related hormones jasmonic acid and methyl jasmonate (data not shown). These results indicate that *MsDREB2C* might play a significant role in the biotic and abiotic stress response, which is consistent with the observation that *M. sieversii* Roem. is a superior apple rootstock (Li 2001).

MsDREB2C possessed a PKK-like NLS sequence, RKVPAKGSKKG, adjacent to the AP2 domain, and an acidic C-terminal region rich in aspartic acid and glutamic acid known to function in *trans*-activation activity (Liu et al. 1998, Zhou et al. 2010). These observations are consistent with the finding that MsDREB2C is a nuclear-localized protein that exhibits *trans*-activation activity.

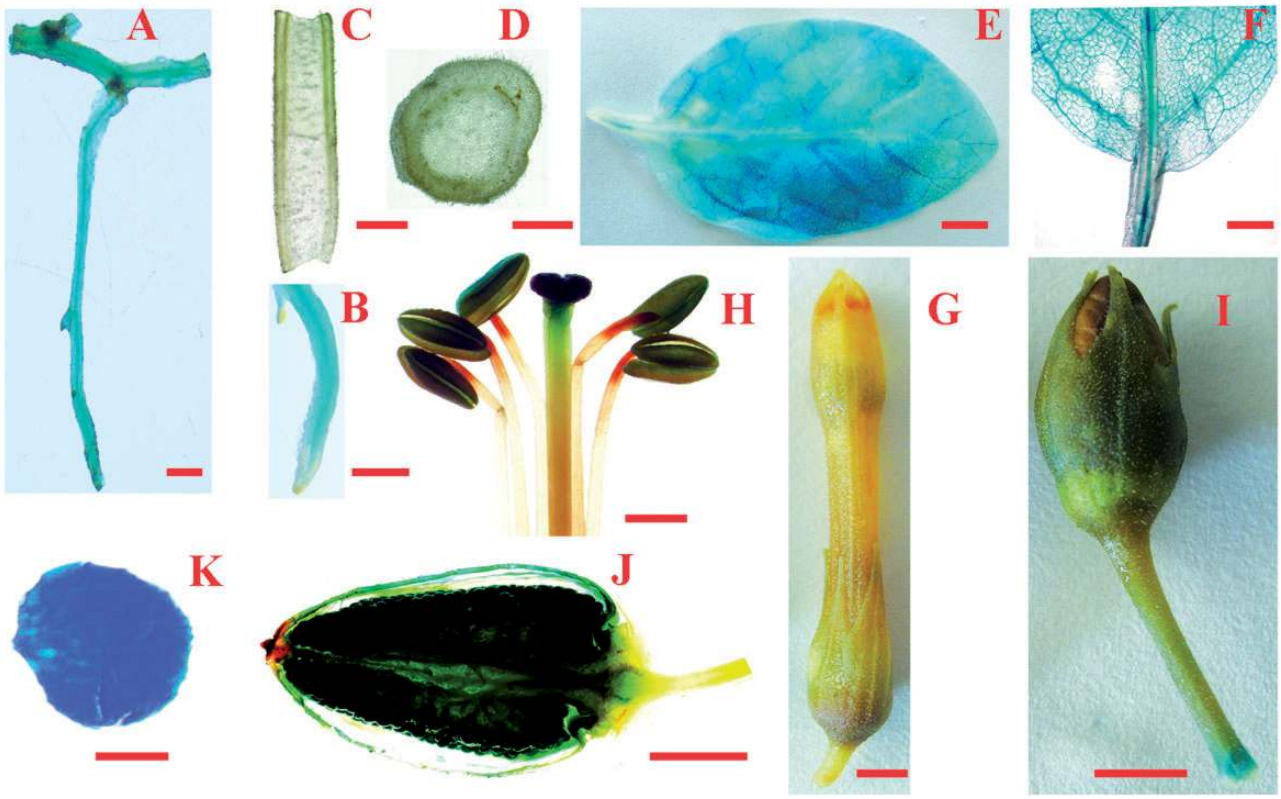

Fig. 8 Histochemical staining of GUS activity in tobacco plants transformed with *ProMsDREB2C:GUS*. Roots (A, B), stems (C, D), leaves (E, F), flower (G), stamens and pistil (H), fruit (I, J) and seed (K). Scale bars = 0.1 cm (A, B); 0.5 cm (C–J); 150 μm (K).

Auxins and cytokinins might co-regulate leaf and root development in transgenic Arabidopsis plants expressing *MsDREB2C*

In the present study, three transgenic lines were selected at random for phenotypic comparison with WT plants. All the lines exhibited a similar phenotype that differed from that of WT plants. Therefore, these phenotypic changes were not influenced by the gene insertion site, but were due to the constitutive expression of *MsDREB2C*.

The growth of *DREB2A-CA*, *DREB2C*, *ZmDREB2A* and *OsDREB2B* transgenic Arabidopsis plants was retarded relative to that of WT plants (Sakuma et al. 2006a, Qin et al. 2007, Lee et al. 2010, Matsukura et al. 2010). However, the constitutive expression of an APETALA2-family transcription factor CAP2 from *Cicerarietinum* (chickpea) enhanced the growth of transgenic tobacco (Shukla et al. 2006), resulting in a phenotype similar to that of transgenic Arabidopsis plants constitutively expressing *MsDREB2C*. These phenotype differences may be due to the fact that different target genes are directly or indirectly activated by the foreign genes. We further compared the homology in AP2 DNA-binding sequences between MsDREB2C and other DREB2 proteins. MsDREB2C had a higher level of sequence similarity with the CAP2 transcription factor (86.21%) than with DREB2C (79.31%), which was demonstrated to have the highest level of sequence similarity to MsDREB2C in this study. Therefore, MsDREB2C might activate downstream genes similar to those activated by CAP2 and result in a similar phenotype.

Cytokinins and auxins are classes of plant hormones that play roles in various aspects of plant growth and development, such as the promotion of cell division and expansion, apical dominance and the formation of the shoot and lateral root (Dubrovsky et al. 2008, Benkova et al. 2009). Auxin polar transport also promoted Arabidopsis lateral root initiation (Casimiro et al. 2001). Therefore, we analyzed the ZR and free IAA levels in both WT and transgenic Arabidopsis plants to investigate why *MsDREB2C* transgenic lines had larger shoots and more lateral roots than WT plants. Our results indicated that both the free IAA and ZR content in *MsDREB2C* transgenic plants was higher than in WT plants, which was consistent with the phenotypic change between transgenic lines and WT plants. Taken together, these results suggested that auxin and cytokinin might cooperate to regulate the morphogenesis of transgenic Arabidopsis plants, including root and shoot formation. In addition, ARFs bound to auxin-responsive promoter elements and mediated transcriptional responses to auxin. Among these ARFs, ARF7 and ARF19 were activated by auxin to induce lateral root formation and leaf expansion (Wilmoth et al. 2005). In all of the *MsDREB2C* transgenic lines, the expression levels of both *ARF7* and *ARF19* were significantly higher than in WT plants, providing further molecular evidence for the role of auxin.

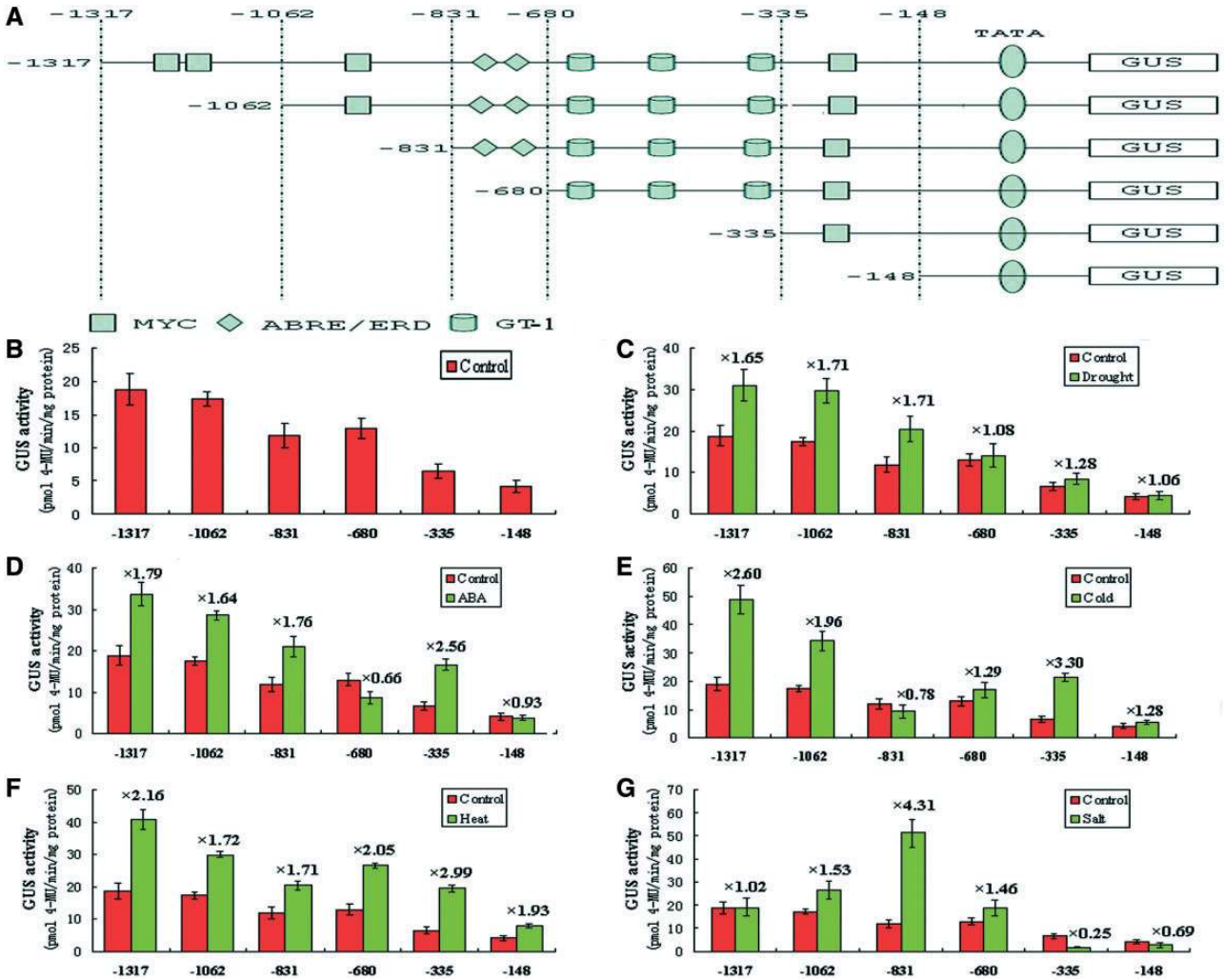

Fig. 9 Deletion analysis of the *MsDREB2C* promoter. (A) Schematic diagram of the 5′-deletion constructs used. Numbers on the left indicate the 5′ ends of the six constructs, relative to the transcriptional start site. The location of the TATA-box and *cis*-acting elements, such as MYC, ABRE/ERD and GT-1, are also shown. Relative GUS activities in transgenic tobacco seedlings (approximately 20 cm high) carrying each construct and grown under control (B) or the indicated conditions. For drought treatment, seedlings were deprived of water for 7 d (C); for ABA treatment, seedlings were sprayed with 200 μM ABA solution and then incubated for 6 h (D); for cold and thermotolerance treatment, plants were placed in a chamber at 0 and 45°C, respectively, for 6 h (E, F); for salt treatment, the seedlings were watered with 200 mM NaCl solution for 24 h (G). The relative GUS activity was calculated as the ratio of GUS activity of the deleted *MsDREB2C* promoter series under abiotic treatment to that of the same promoter series under normal conditions. For each treatment, three independent lines for each construct were repeated three times. The 10th leaves from the top were chosen for promoter deletion analysis. The error bars on each column represent the SE of three replicates.

Overexpression of *MsDREB2C* enhanced plant abiotic stress tolerance

The overexpression of *DREB2*-type genes resulted in increased tolerance to drought, salt, heat and freezing stresses (Sakuma et al. 2006a, Agarwal et al. 2007, Lim et al. 2007, Qin et al. 2007, Chen et al. 2009, Matsukura et al. 2010). The constitutive expression of *MsDREB2C* conferred enhanced tolerance to drought, heat and freezing in transgenic plants, possibly because of the overexpression of four stress-inducible DREB2-reponsive genes. LEA proteins were quite hydrophilic and were believed to protect plant cells from these stresses. *DR29A* and *RD29B* also encoded proteins similar to LEA (Dure et al. 1989). Furthermore, the activity of *LEA* genes was associated with cold stress in plants (Tunnacliffe and Wise 2007). *AtHSFA3* was a member of the *HSF* (heat shock factor) gene family and could be induced by drought and heat treatment. *AtHSFA3* expression was highest among the *HSF* genes in the constitutively active *35S:DREB2A-CA* plants (Sakuma et al. 2006a, Sakuma et al. 2006b). Overexpression of *AtHSFA3* was consistent with the improved thermotolerance of *MsDREB2C* transgenic plants. Physiological parameters, including proline content, stomatal aperture and water loss, were also measured in these plants, and the results agree with the finding that the transgenic lines were more tolerant than WT plants. *P5CS2*,

which encodes a key enzyme in proline biosynthesis (delta 1-pyrroline-5-carboxylate synthetase), seemed to contribute to the increase in proline levels. It was found that a DRE element (CCGAC) existed in the promoter of *P5CS2*, which might be under the control of *MsDREB2C* (Ito et al. 2006). Transgenic Arabidopsis plants overexpressing a drought-induced transcription factor, NFYA5, displayed reduced leaf water loss and stomatal apertures and were more resistant to drought stress than the WT (Li et al. 2008). Similar results were obtained in our experiment, and these results are consistent with the enhanced tolerance to drought of transgenic Arabidopsis plants expressing *MsDREB2C*.

MsDREB2C negatively regulates the resistance to biotic stress

Previous experiments had shown that DREBs were involved in the biotic signal pathways. DEAR1, a member of the DREB/CBF family, suppressed the induction of DREB1/CBF by cold treatment, leading to a reduction in freezing tolerance. Arabidopsis plants overexpressing *DEAR1* showed less *P. syringae* pathogen growth than the WT plants (Tsutsui et al. 2009). *OsDREB1B* from rice conferred improved abiotic and biotic stress tolerance on transgenic Arabidopsis plants through up-regulation of *PR* genes (Gutha and Reddy 2008). However, Arabidopsis plants overexpressing *MsDREB2C* showed improved abiotic stress tolerance, but reduced biotic stress tolerance by suppressing *PR* genes. It has been demonstrated that *DREB2A* was repressed in transgenic lines overexpressing *adr1*, which exhibited broad-spectrum disease resistance and significant drought tolerance (Chini et al. 2004). These data indicated that antagonism might exist between *DREB2* genes (*MsDREB2C* and *DREB2A*) and disease resistance genes; in contrast, *DREB1* genes and *PR* genes might collaborate with each other.

The promoter sequence of *MsDREB2C* contains multiple *cis*-acting elements

In this study, we detected prominent GUS activity in the vascular tissues of leaves, roots and seeds of transgenic tobacco plants. It had been demonstrated that the CT/GA-rich motifs, CTT/GAA-rich motifs and the CATGCA/CACGTG sequence were involved in vascular- and embryo-specific expression, respectively (Chandrasekharan et al. 2003, Ruiz-Medrano et al. 2011). Many CT/GA-rich motifs, CTT/GAA-rich motifs and C ATGCA/CACGTG sequences were found in the promoter of *MsDREB2C*. These observations implied that *MsDREB2C* might play a regulatory role in the vascular tissue of leaves and root and in embryo development. Although xylem and phloem belong to vascular tissues, GUS activity was hardly detected in the stem of transgenic tobacco plants, in agreement with the fact that *MsDREB2C* had the lowest transcript level in the xylem and phloem.

We demonstrated that *MsDREB2C* expression was inducible in response to multiple abiotic treatments (drought, salt, cold, heat and ABA). To analyze the expression pattern of *MsDREB2C* further, we fused various truncations of the *MsDREB2C* promoter to the GUS reporter gene. All the data were analyzed by the uniform standard of whether the induction ratios were statistically significant. A deletion assay from the 5′ end of the promoter showed that *MsDREB2C* had a similar regulation pattern under drought and ABA treatment. Deletion from −1,317 to −831 bp caused no significant change in GUS activity, whereas deletion from −831 to −680 bp resulted in a significant decrease in drought and ABA responsiveness (**Fig. 9C, D**). Deletion from −335 to −148 bp caused a remarkable decline in ABA responsiveness (2.56- to 0.93-fold) and no significant decline in drought responsiveness (1.28- to 1.06-fold). Three promoter fragments, from −1,317 to −1062, −1,062 to −831 and −335 to −148 bp, were shown to be responsive to cold stress (**Fig. 9E**). Four copies of MYC recognition sites (CANNTG), which were previously shown to bind to inducer of CBF expression1 (ICE1; Abe et al. 2003, Chinnusamy et al. 2003, Lee et al. 2005), were present in these sequences. Under heat stress, the GUS activity of transformants bearing the −148 deletion was significantly lower than that of those bearing the −335 deletion (**Fig. 9F**), suggesting that this 188 bp promoter region contains heat shock-inducible *cis*-elements. However, sequence analysis revealed several putative *cis*-elements, such as Dof sequences (Yanagisawa and Schmidt 1999) and a W-box (Yu et al. 2001), but not a heat shock element (HSE). It had been reported that a heat-responsive gene, Arabidopsis *DREB2C*, did not require an HSE (Chen et al. 2012). Thus, the induction of *DREB* genes by heat shock might not always require an HSE. Interestingly, the deletion from −680 to −335 bp led to an increase in the GUS activity ratio under drought, cold, heat and ABA treatment, implying that negatively regulated elements exist in this region. Deletion analysis showed that the response of the *MsDREB2C* promoter to salt stress differed from that to all of the above treatments. Both negative and positive elements might exist within the region from −1,317 to −831 and −831 to −148, respectively (**Fig. 9G**). In addition, three copies of a consecutive GT-1 motif (GAAAAA) were found in the −680 to −335 bp promoter region, which were previously reported to be responsive to salt treatment (Park et al. 2004), whereas we did not find any GT-1 motifs between the −831 and −680 bp regions, suggesting that other salt-regulated elements might exist in this region. Although multiple positive and negative regulatory regions were identified within the *MsDREB2C* promoter sequence, and copies of *cis*-acting elements were also predicted to exist within this sequence, further deletion and base substitution experiments are needed to reveal the core elements.

Materials and Methods

Plant materials and treatment

Rooted, micropropagated *M. sieversii* Roem. plants were precultured in pots with 1/2 Hoagland nutrient solution for 14 d and transferred into full-strength Hoagland nutrient solution

under constant conditions of 22°C and 60% relative humidity and a photoperiod of 16/8 h (day/night). Plants with heights of approximately 30 cm and with uniform vegetative growth were selected for further analyses. For dehydration stress, uniformly developed seedlings were removed from the pots and placed on filter paper at 22°C under dim light. For heat and cold stress, uniform plants were exposed to 45 and 4°C in a growth chamber. For salt stress, seedlings were transferred to Hoagland nutrient solution containing 200 mM NaCl. For ABA treatment, seedlings were sprayed with 200 μM ABA solution. For all five treatments, functional leaves were harvested from the same position of the seedlings at the given time periods (0, 1, 3, 6 and 12 h) and three biological replicates were performed. Subsequently, leaves were rapidly frozen in liquid nitrogen and stored at −80°C for further study.

Isolation and characterization of *MsDREB2C* from *M. sieversii* Roem.

A blastp analysis of the amino acid sequence of Arabidopsis DREB2A (GenBank ID: AT5G05410) was performed to search for homologs in a database of apple amino acid sequences (http://genomics.research.lasma.it/). MDP0000446783 shared the highest homology with Arabidopsis DREB2A. Therefore, MDP0000446783 was chosen as the preliminary candidate gene. Specific primers were designed to clone this gene, using the cDNA of *M. sieversii* as template (forward, 5′-TTGGTGTAT TTGGGTCTTGT-3′; reverse, 5′-AATCTGTTTCTTTGCT T TCG-3′). The cDNA sequence, named *MsDREB2C* (GenBank accession No. JQ790526), was obtained. Then, the MsDREB2C protein sequence was analyzed using Pfam (http://pfam.sanger.ac.uk/) to identify the presence of an AP2 domain, and CDD v3.08-4334 PSSMs (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Maruchler-Bauer et al.2011) to compare the sequence with that of other DREB-related proteins. The bioinformatics tools at the website (http://www.expasy.org) were used to analyze the molecular weights, isoelectric point and characteristic AP2/ERF domain of MsDREB2C. The NLS was predicted using the website http://www.moseslab.csb.utoronto.ca/. Sequence alignment of MsDREB2C and other DREB-related proteins was performed using Clustal W version 2.01. The phylogenetic tree was generated using MEGA version 5.05 (Kumar et al. 2008).

Subcellular localization and transactivation of MsDREB2C

The full-length ORF of *MsDREB2C* without the termination codon was amplified by PCR with the following primers: forward, 5′-GAATTCATGGGAGCTTATGATCAAGGCG-3′ (*Eco*RI site underlined) and reverse, 5′-GTCGACTCATTTCCATCGAATAGT TGTAACCTCC-3′ (*Sal*I site underlined). The PCR product was subcloned into the pE3025-GFP vector to generate pE3025-MsDREB2C-GFP, containing an MsDREB2C–GFP fusion construct under the control of the *Cauliflower mosaic virus* (CaMV) dual 35S promoter, as well as a *Tobacco etch virus* (TEV) enhancer. The fusion construct and control (pE3025-GFP) were transformed into onion (*A. cepa*) epidermal cells using a gene gun (Bio-Rad). GFP fluorescence was detected by confocal microscopy (Nikon) after incubation for 16 h at 22°C.

The entire coding sequence of *MsDREB2C* was cloned using two specific primers: forward, 5′-GAATTCATGGGAGCTTATGA TCAAG-3′ (*Eco*RI site underlined) and reverse, 5′-GTCGACTCAC ATTTCCATCGAATA-3′ (*Sal*I site underlined). The PCR product was subcloned into the DNA-binding domain vector pBD, a yeast expression vector with the promoter and terminator of *ADH1*, to construct the fusion plasmid pBD-MsDREB2C. The recombinant plasmid was then transferred into yeast strain YGR-2, which possesses the reporter genes *His3* and *LacZ*. The transformed yeast was streaked on plates lacking tryptophan (SD/−Trp) or both tryptophan and histidine (SD/−Trp/−His). The plates were incubated at 30°C for 3 d and subjected to a β-galactosidase assay to examine the transactivation ability of MsDREB2C.

Relative quantitative RT–PCR

Total RNA was extracted as described previously (Chang et al. 1993) from the leaves of *M. sieversii* at different treatment time points, and then treated with RNase-free DNase I (TAKARA BIO INC.) to remove genomic DNA. Total RNA (2 μg) was reverse transcribed into first-strand cDNA using the M-MLV Reverse Transcriptase Kit (Promega), according to the manufacturer's protocol, and oligo(dT) primer and random primers were used in the reverse transcription reactions. The cDNA samples were diluted 5-fold and used as template, and the primer sequences are shown in **Supplementary Table S1**.

Gene expression profiles were determined using the Applied Biosystems 7500 Real-Time PCR System. For quantitative RT–PCR, triplicate quantitative assays were performed for each cDNA dilution using UltraSYBR Mixture (CWBIO), and the means and corresponding standard errors were calculated. Forty PCR cycles were performed according to the following temperature scheme: 94°C for 10 s and 60°C for 31 s. The relative quantification value for *MsDREB2C* was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using the apple 18S rRNA and *Histone H3* gene as an internal control. Each analysis was performed in biological and technical triplicate.

Total RNA of *Arabidopsis thaliana* (ecotype Columbia) and *MsDREB2C* transgenic T_3 plants was extracted as described (Chang et al. 1993). To detect the transcript levels of putative target genes of *MsDREB2C*, *AtACTIN2* was used as a reference gene. All relevant primers used in this work are listed in **Supplementary Table S1**.

Generation of transgenic Arabidopsis

Arabidopsis thaliana (ecotype Columbia) was used in this study. To generate transgenic plants, full-length *MsDREB2C* cDNA was amplified using a specific primer pair: forward, 5′-GGATCCTTG GTGTATTTGGGTCTTGT-3′ (*Bam*HI site underlined) and reverse, 5′-TCTAGAAATCTGTTTCTTTGCTTTCG-3′ (*Xba*I site underlined). The PCR product was fused into the binary plant

transformation vector pCB302-3 under the control of the CaMV 35S promoter. The pCB302-3-MsDREB2C plasmid was introduced into *Agrobacterium tumefaciens* strain EHA105. Arabidopsis plants were transformed via the floral dip method (Clough and Bent 1998). The transgenic plants were selected by spraying with 0.1% (v/v) BASTA (Bayer CropScience). Three random homozygous T_3 lines (L-1, L-2 and L-3) containing a single T-DNA insertion were chosen for subsequent analyses.

Tissue preparation

Paraffin sectioning was performed according to Kerk et al. (2003).

Determination of IAA and ZR content

An enzyme-linked immunosorbent assay (ELISA) kit was used to determine IAA and ZR content. The ELISA procedures were conducted according to the instructions provided by the manufacturer (China Agricultural University).

Stress treatment for soil-grown plants

Three-week-old plants were exposed to the following stress treatments. For thermotolerance treatment, seedlings were placed in a chamber at 56°C for 3 h. For drought stress, water was withheld for 14 d. For freezing stress, plants were exposed to a temperature of −6°C for 2 d. For salt stress, seedlings were watered with 200 mM NaCl solution every other day for a 16 d period. After treatment, all seedlings were returned to normal conditions for 7 d and the survival rate was scored.

Measurement of proline content

Fresh leaf material (0.3 g) was extracted with 5 ml of deionized water at 100°C for 10 min, and shaken with 0.03 g of permutit for 5 min. The extract was separated by centrifugation at 3,000 r.p.m. for 10 min, and then the proline content of the aqueous extract was determined using the acid ninhydrin method (Troll and Lindsley 1955). Briefly, 2 ml of aqueous extract was mixed with 2 ml of deionized water, 1 ml of glacial acetic acid and 3 ml of acid ninhydrin reagent (12.5 mg of ninhydrin, 0.3 ml of glacial acetic acid, 0.2 ml of 6 M orthophosphoric acid) and heated at 100°C for 40 min. The solution was cooled to room temperature and extracted by vigorous shaking in 4 ml of benzene for 5 min. The organic phase was determined at 515 nm. The resulting values were compared with a standard curve constructed using known amounts of proline (Sigma).

Stomatal aperture analysis

Stomatal guard cells were observed as described by Kang et al. (2002), and the width and length of stomatal pores were measured according to Yamazaki et al. (2003).

Water loss measurement

Water loss measurement was performed as described by Li et al. (2008).

Pathogen inoculation

Pathogen inoculation was performed as described by Kim et al. (2008).

Peroxidase activity detection

Peroxidase activity was measured based on a method described by Mydlarz and Harvell (2007). Frozen samples were weighed (0.1 g) and homogenized in 10 mM sodium phosphate buffer (1 ml), pH 6.0. After centrifugation (10,000×*g* for 10 min at 4°C), 100 µl of supernatant was added to 3 ml of the assay mixture [0.25% (v/v) guaiacol (Sigma-Aldrich), 0.1 M H_2O_2 in 10 mM sodium phosphate buffer, pH 6.0]. Changes in absorbance at 470 nm were recorded, and the activity of peroxidase was represented as the change in absorbance at 470 nm g^{-1} FW min^{-1}.

Isolation and activity analysis of the *MsDREB2C* promoter

Genomic DNA was extracted from the leaves of *M. sieversii* using a DNA secure Plant Kit (TIANGEN) and used as template for PCR amplification. Promoter sequence analysis was performed using the PLACE Signal Scan Search Program (Higo et al. 1999). According to the predicted position of the *cis*-elements in the *MsDREB2C* promoter, six truncations of the promoter, −1,317, −1,062, −831, −680, −335 and −148 bp relative to the transcription initiation site, were generated and separately introduced into the pCAMBIA1301 binary vector to replace the CaMV 35S promoter upstream of the *GUS* reporter gene. These vectors were transformed into tobacco by the leaf disc transformation method (Horsch et al. 1985). The putative transgenic plantlets were confirmed by PCR and GUS staining. The verified rooted transgenic plantlets were then transferred into soil. For histochemical staining of GUS, fresh tissue samples were dissected from tobacco plants and immediately incubated in X-Gluc solution at 37°C overnight (Jefferson et al. 1987). After bleaching with 50–100% ethanol and fixation in 0.3% formaldehyde (Nakashima et al. 1998), stained samples were observed with an OLYMPUS SZX16-DP72 stereo fluorescence microscope.

For drought stress, the transgenic tobacco plants were deprived of water for 7 d. For salt stress, seedlings were watered with 200 mM NaCl solution for 24 h. For heat and cold stress, plants were placed in a chamber at 45 and 0°C for 6 h, respectively. For ABA treatment, seedlings were sprayed with 200 µM ABA solution and then incubated for 6 h. For fluorometric GUS assay, the leaves of treated plants were ground in liquid nitrogen and homogenized in freshly prepared GUS extraction buffer. After centrifugation for 10 min at 12,000 r.p.m. at 4°C, the GUS activity of the supernatant was determined using 4-methylumbelliferyl glucuronide (4-MUG) as substrate. The fluorescence of the GUS-catalyzed hydrolysis reaction product, 4-MU, was measured with the TECAN GENios system. Protein concentration in the supernatant was assessed by the Bradford (1976) method, using bovine serum albumin (BSA) as a

standard. GUS activity was normalized to the protein concentration of each supernatant extract and calculated as pmol of 4-MU mg^{-1} of soluble protein min^{-1}. The relative GUS activity was calculated as the ratio of GUS activity of the deleted *MsDREB2C* promoter series under abiotic treatment to that of the same promoter series under normal conditions. For each treatment, three independent lines were chosen for each construct and repeated three times. The 10th leaves from the top were selected for promoter deletion analysis.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the National Natural Science Foundation Project [grant No. 31171938]; the Special Fund for Agro-scientific Research in the Public Interest [grant No. 201003021].

Acknowledgments

The authors thank Dr. Tao Wang and Dr. Jiangli Dong (College of Biological Science, China Agricultural University, Beijing, China) for their technical assistance. We would like to thank Dr. Shouyi Chen (Institute of Genetics and Developmental Biology, Beijing, China) for providing us with both the yeast expression vector pBD-GAL4 and the yeast strain YRG-2. We thank Dr. Jun Fan (China Agricultural University) for providing us with the *Pst* DC3000 strain. We thank Dr. Kathleen Farquharson (University of Northern Colorado) for her critical review of the manuscript.

Disclosures

The authors have no conflicts of interest to declare.

References

Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* 15: 63–78.

Agarwal, P., Agarwal, P.K., Nair, S., Sopory, S.K. and Reddy, M.K. (2007) Stress inducible DREB2A transcription factor from Pennisetum glaucum is a phosphoprotein and its phosphorylation negatively regulates its DNA binding activity. *Mol. Gen. Genet.* 277: 189–198.

Agarwal, P.K., Agarwal, P., Reddy, M.K. and Sopory, S.K. (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Rep.* 25: 1263–1274.

Baker, S.S., Wilhelm, K.S. and Thomashow, M.F. (1994) The 5′-region of *Arabidopsis thaliana* cor15a has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol. Biol.* 24: 701–713.

Benkova, E., Ivanchenko, M.G., Friml, J., Shishkova, S. and Dubrovsky, J.G. (2009) A morphogenetic trigger: is there an emerging concept in plant developmental biology? *Trends Plant Sci.* 14 189–193.

Bhatnagar-Mathur, P., Devi, M.J., Reddy, D.S., Lavanya, M., Vadez, V., Serraj, R. et al. (2007) Stress-inducible expression of AtDREB1A in transgenic peanut (Arachis hypogaea L.) increases transpiration efficiency under water-limiting conditions. *Plant Cell Rep.* 26: 2071–2082.

Bindschedler, L.V., Dewdney, J., Blee, K.A., Stone, J.M., Asai, T., Plotnikov, J. et al. (2006) Peroxidase dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *Plant J.* 47: 851–863.

Bohnert, H.J., Ayoubi, P., Borchert, C., Bressan, R.A., Burnap, R.L., Cushman, J.C. et al. (2001) A genomic approach towards salt stress tolerance. *Plant Physiol. Biochem.* 39: 295–311.

Bradford, M.M. (1976) A rapid sensitive method for the quantitation of micrgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* 72: 248–254.

Casimiro, I., Marchant, A., Bhalerao, R.P., Beeckman, T., Dhooge, S., Swarup, R. et al. (2001) Auxin transport promotes *Arabidopsis* lateral root initiation. *Plant Cell* 13: 843–852.

Champ, K.I., Febres, V.J. and Moore, G.A. (2006) The role of CBF transcriptional activators in two Citrus species (*Poncirus and Citrus*) with contrasting levels of freezing tolerance. *Physiol. Plant.* 129: 529–541.

Chandrasekharan, M.B., Bishop, K.J. and Hall, T.C. (2003) Module-specific regulation of the beta-phaseolin promoter during embryogenesis. *Plant J.* 33: 853–866.

Chang, S., Puryear, J. and Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* 11: 113–116.

Chen, H., Je, J.Y., Song, C., Hwang, J.E. and Lim, C.O. (2012) A proximal promoter region of *Arabidopsis* DREB2C confers tissue-specific expression under heat stress. *J. Integr. Plant Biol.* 54: 640–651.

Chen, J.H., Xia, X.L. and Yin, W.L. (2009) Expression profiling and functional characterization of a DREB2-type gene from Populus euphratica. *Biochem. Biophys. Res. Commun.* 378: 483–487.

Chini, A., Grant, J.J., Seki, M., Shinozaki, K. and Loake, G.J. (2004) Drought tolerance established by enhanced expression of the CC-NBSLRR gene, ADR1, requires salicylic acid, EDS1 and ABI1. *Plant J.* 38: 810–822.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.H., Hong, X.H., Agarwal, M. et al. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.* 17: 1043–1054.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16: 735–743.

Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S. et al. (2003) OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought-, high-salt-, and cold-responsive gene expression. *Plant J.* 33: 751–763.

Dubrovsky, J.G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M.G., Friml, J., Shishkova, S. et al. (2008) Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. *Proc. Natl Acad. Sci. USA* 105: 8790–8794.

Dure, L., Crouch, M., Harada, J., David, T.H., Mundy, J., Quatrano, R. et al. (1989) Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Mol. Biol.* 12: 475–486.

Fowler, S. and Thomashow, M.F. (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* 14: 1675–1690.

Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D., Thomashow, M.F. et al. (2000) Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.* 124: 1854–1865.

Gutha, L.R. and Reddy, A.R. (2008) Rice DREB1B promoter shows distinct stress-specific responses, and the over expression of cDNA in tobacco confers improved abiotic and biotic stress tolerance. *Plant Mol. Biol.* 68: 533–555.

Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database. *Nucleic Acids Res.* 27: 297–300.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229–1231.

Hu, H., Dai, M., Yao, J., Xiao, B., Li, X.H., Zhang, Q.F. et al. (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl Acad. Sci. USA* 103: 12987–12992.

Igarashi, Y., Yoshiba, Y., Sanada, Y., Yamaguchi-Shinozaki, K., Wada, K. and Shinozaki, K. (1997) Characterization of the gene for Δ^1-pyrroline-5-carboxylate synthetase and correlation between the expression of the gene and salt tolerance in Oryza sativa L. *Plant Mol. Biol.* 33: 857–865.

Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M. et al. (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol.* 47: 141–153.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901–3907.

Kang, J.Y., Choi, H.I., Im, M.Y. and Kim, S.Y. (2002) *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell* 14: 343–357.

Kerk, N.M., Ceserani, T., Tausta, S.L., Sussex, I.M. and Nelson, T.M. (2003) Laser capture microdissection of cells from plant tissues. *Plant Physiol.* 132: 27–35.

Kim, J.K., Mizoi, J., Yoshida, T., Fujita, Y., Nakajima, J. and Ohori, T. (2011) An ABRE promoter sequence is involved in osmotic stress-responsive expression of the DREB2A gene, which encodes a transcription factor regulating drought-inducible genes in *Arabidopsis*. *Plant Cell Physiol.* 52: 2136–2146.

Kim, J.K., Mizoi, J., Kidokoro, S., Maruyama, K., Nakajima, J. and Nakashima, K. (2012) *Arabidopsis* GROWTH-REGULATING FACTOR7 functions as a transcriptional repressor of abscisic acid- and osmotic stress-responsive genes, including DREB2A. *Plant Cell* 24: 3393–3405.

Kim, K.C., Lai, Z.B., Fan, B.F. and Chen, Z.X. (2008) Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. *Plant Cell* 20: 2357–2371.

Kumar, S., Nei, M., Dudley, J. and Tamura, K. (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief. Bioinformatics* 9: 299–306.

Lata, C. and Prasad, M. (2011) Role of DREBs in regulation of abiotic stress responses in plants. *J. Exp. Bot.* 60: 121–135.

Lee, B.H., Byeong-ha, Lee, Henderson, D.A. and Zhu, J.K. (2005) The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. *Plant Cell* 17: 3155–3175.

Lee, S.J., Kang, J.Y., Park, H.J., Kim, M.D., Bae, M.S., Choi, H.I. et al. (2010) DREB2C interacts with ABF2, a bZIP protein regulating abscisic acid-responsive gene expression, and its overexpression affects abscisic acid sensitivity. *Plant Physiol.* 153: 716–727.

Lescot, M., Dehais, P., Thijs, G., Marchal, K., Moreau, Y. and Van de Peer, Y. (2002) PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* 30: 325–327.

Li, W.X., Oono, Y., Zhu, J.H., He, X.J., Wu, J.M., Lida, K. et al. (2008) The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. *Plant Cell* 20: 2238–2251.

Li, Y.N. (2001) Researches of Germplasm Resources of Malus Mill pp. 20–23. China Agriculture Press, Beijing; (in Chinese with an English abstract).

Lim, C.J., Hwang, J.E., Chen, H., Hong, J.K., Yang, K.A., Choi, M.S. et al. (2007) Over-expression of the *Arabidopsis* DRE/CRT-binding transcription factor DREB2C enhances thermotolerance. *Biochem. Biophys. Res. Commun.* 362: 431–436.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. et al. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low- temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* 10: 1391–1406.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25: 402–408.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Kay, A. et al. (2001) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nature* 26: 403–410.

Maruchler-Bauer, A., Lu, S., Anderson, J.B., Chitsaz, F., Derbyshire, M.K. and DeWeese-Scott, C. (2011) CDD: a conserved domain database for the functional annotation of proteins. *Nucleic Acids Res.* 39: D225–D229.

Maruyama, K., Sakuma, Y., Kasuga, M., Ito, Y., Seki, M., Goda, H. et al. (2004) Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems. *Plant J.* 38: 982–993.

Matsukura, S., Mizoi, J., Yoshida, T., Todaka, D., Ito, Y., Maruyama, K. et al. (2010) Comprehensive analysis of rice DREB2-type genes that encode transcription factors involved in the expression of abiotic stress-responsive genes. *Mol. Gen. Genet.* 283: 185–196.

Mydlarz, L.D. and Harvell, C.D. (2007) Peroxidase activity and inducibility in the sea fan coral exposed to a fungal pathogen. *Comp. Biochem. Physiol. A* 146: 54–62.

Nakashima, K., Satoh, R., Kiyosue, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) A gene encoding proline dehydrogenase is not only induced by proline and hypo-osmolarity but is also developmentally regulated in reproductive organs in *Arabidopsis thaliana*. *Plant Physiol.* 118: 1233–1241.

Park, H.C., Kim, M.L., Kang, Y.H., Jeon, J.M., Yoo, J.H., Kim, M.C. et al. (2004) Pathogen- and NaCl induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. *Plant Physiol.* 135: 2150–2161.

Qin, F., Kakimoto, M., Sakuma, Y., Maruyama, K., Osakabe, Y., Tran, L.S. et al. (2007) Regulation and functional analysis of ZmDREB2A in response to drought and heat stresses in Zea mays L. *Plant J.* 50: 54–69.

Reichmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C.Z., Keddie, J. et al. (2000) *Arabidopsis* transcription factor: genome wide comparative analysis among eukaryotes. *Science* 290: 2105–2110.

Ruiz-Medrano, R., Xoconostle-Cázares, B., Ham, B.K., Li, G. and Lucas, W.J. (2011) Vascular expression in Arabidopsis in predicted by the frequency of CT/GA-rich repeats in gene promoter. *Plant J.* 67: 130–144.

Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2002) 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: 998–1009.

Sakuma, Y., Maruyama, K., Osakabe, Y., Qin, F., Seki, M., Shinozaki, K. et al. (2006a) Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression. *Plant Cell* 18: 1292–1309.

Sakuma, Y., Maruyama, K., Qin, F., Osakabe, Y., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2006b) Dual function of an *Arabidopsis* transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression. *Proc. Natl Acad. Sci. USA* 103: 18822–18827.

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

Shukla, R.K., Raha, S., Tripathi, V. and Chattopadhyay, D. (2006) Expression of CAP2, an APETALA2-family transcription factor from Chickpea, enhances growth and tolerance to dehydration and salt stress in transgenic tobacco. *Plant Physiol.* 142: 113–123.

Stockinger, E.J., Gilmour, S.J. and Thomashow, M.F. (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl Acad. Sci. USA* 94: 1035–1040.

Troll, W. and Lindsley, J. (1955) A photometric method for the determination of proline. *J. Biol. Chem.* 215: 655–660.

Tsutsui, T., Kato, W., Asada, Y., Sako, K., Sato, T. and Sonoda, Y. (2009) DEAR1, a transcriptional repressor of DREB protein that mediates plant defense and freezing stress responses in Arabidopsis. *J. Plant Res.* 122: 633–643.

Tunnacliffe, A. and Wise, M.J. (2007) The continuing conundrum of the LEA proteins. *Naturwissenschaften* 94: 791–812.

Wilmoth, J.C., Wang, S., Tiwari, S.B., Joshi, A.D., Hagen, G., Guilfoyle, T.J. et al. (2005) NPH4/ARF7 and ARF19 promote leaf expansion and auxin induced lateral root formation. *Plant J.* 43: 118–130.

Xiong, L., Schumaker, K.S. and Zhu, J.K. (2002) Cell signaling during cold, drought, and salt stress. *Plant Cell* 14: S165–S183.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell* 6: 251–264.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (2005) Organization of *cis*-acting regulatory elements in osmotic- and cold-stress responsive promoters. *Trends Plant Sci.* 10: 88–94.

Yamazaki, D., Yoshida, S., Asami, T. and Kuchitsu, K. (2003) Visualization of abscisic acid-perception sites on the plasma membrane of stomatal guard cells. *Plant J.* 35: 129–139.

Yanagisawa, S. and Schmidt, R.J. (1999) Diversity and similarity among recognition sequences of Dof transcription factors. *Plant J.* 17: 209–214.

Yang, W., Liu, X.D., Chi, X.J., Wu, C.A., Li, Y.Z., Song, L.L. et al. (2011) Dwarf apple MbDREB1 enhances plant tolerance to low temperature, drought, and salt stress via both ABA-dependent and ABA-independent pathways. *Planta* 233: 219–229.

Yu, D., Chen, C. and Chen, Z.X. (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell* 13: 1527–1539.

Zhao, T., Liang, D., Wang, P., Liu, J.Y. and Ma, F.W. (2012) Genome-wide analysis and expression profiling of the DREB transcription factor gene family in Malus under abiotic stress. *Mol. Genet. Genomics* 287: 423–436.

Zhao, X.J., Lei, H.J., Zhao, K., Yuan, H.Z. and Li, T.H. (2012) Isolation and characterization of a dehydration responsive element binding factor MsDREBA5 in *Malus sieversii* Roem. *Sci. Hortic.* 142: 212–220.

Zhou, M., Ma, J., Pang, J., Zhang, Z., Tang, Y. and Wu, Y. (2010) Regulation of plant stress response by dehydration responsive element binding (DREB) transcription factors. *Afr. J. Biotechnol.* 9: 9255–9279.