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Transcription factor *MdCBF1* gene increases freezing stress tolerance in transgenic *Arabidopsis thaliana*

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Abstract

Transcription factors play vital roles in stress signal transduction and gene expression modulation. The sequence analysis shows that *MdCBF1* from *Malus domestica* Borkh. cv. Fuji contained an AP2 core domain of 56 amino acids. By comparison of deduced amino acid sequences of CBF related proteins, we deduced that *MdCBF1* is a CBF transcription factor gene which belongs to AP2/EREBP family, DREB-A1 subfamily. Further, we reported that transgenic *Arabidopsis thaliana* plants expressing the *MdCBF1* gene exhibited stronger growth than wild type plants under freezing stress. The analysis of RT-PCR for stress-responsive genes implied that *MdCBF1* over-expressing plants had a higher expression of *COR15a, RD29A*, and *RD29B* genes than wild type plants. Collectively, our results indicate that *MdCBF1* might play an important role in the response of transgenic *Arabidopsis* plants to freezing stress.

Additional key words: amino acid sequence, apple, dehydration responsive element, *Malus domestica*, RT-PCR.

Introduction

Drought, high salinity, and freezing are prevalent environmental stresses that strongly influence the survival and yields of crops. Plants respond and adapt to these stresses by a variety of biochemical and physiological mechanisms (Xiong *et al*. 2002, Jakab *et al*. 2005) which are associated with the rapid activation of signal transduction pathways. *AP2*/*ERF* genes have been implicated in abiotic stresses response (Tang *et al*. 2005).

The AP2/ERF genes constitute a large multigene family divided into four subfamilies named AP2, CBF/DREB, ERF, and RAV based on their sequence similarities and number of AP2/ERF domains (Sakuma *et al*. 2002). AP2 subfamily proteins contain two AP2/ERF domains, and genes in this subfamily participate in the regulation of developmental process (Elliott *et al*. 1996, Boutilier *et al*. 2002). The RAV subfamily proteins contain one AP2/ERF domain and one B3 domain which differ in biological functions and are involved in distinct types of transcription. Recently, the involvement of RAV subfamily members in ethylene response (Alonso *et al.* 2003), brassinosteroid response (Hu *et al*. 2004), and biotic and abiotic stress responses (Sohn *et al*. 2006) was reported. In contrast to the AP2 and RAV subfamily members, the CBF/DREB and ERF subfamily proteins contain single AP2/ERF domains. ERF subfamily transcription factors have been identified in different plant species, and several ERFs have been characterized (Onate-Sanchez and Singh 2002, Sakuma *et al*. 2002). The ERF subfamily is mainly involved in the response to biotic stresses like pathogenesis by recognizing the *cis*-acting element AGCCGCC, known as the GCC box (Hao *et al*. 1998). Many of the ERF subfamily members

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Abbreviations: ABA - abscisic acid; AP2 - apetala2; CBF - C-repeat binding factor; CBF/DREB - C-repeat binding factor/dehydration responsive element binding; DRE/CRT - dehydration responsive element/C-repeat responsive element; EDTA - ethylenediaminatetraacetic acid; ERF - ethylene responsive factor; GM - germination medium; GUS - β-glucuronidase; LEA - late embryogenesis-abundant; MDA - malondialdehyde; NCBI - National center for biotechnology information; RAV - related to ABI3/VP1; REC - relative electrical conductivity; TBARS - thiobarbituric acid reactive substances;

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also bind to DRE/CRT elements (Lee *et al.* 2004, Xu *et al.* 2007). The genes in the CBF/DREB subfamily play a crucial role in the resistance of plants to abiotic stresses by recognizing the dehydration responsive or cold-repeat element (DRE/CRT) with a core motif of A/GCCGAC (Yamaguchi-Shinozaki and Shinozaki 1994, Thomashow 1999).

In *Arabidopsis*, low temperature-inducible CBF genes have been demonstrated to regulate a large number of cold-regulated genes, whose products are thought to contribute to freezing tolerance (Wisniewski *et al.* 2011). The role of CBF in cold response and acclimation has been well documented in the following plants: birch (Welling and Palva 2008), poplar (Benedict *et al.* 2006), eucalyptus (El Kayal *et al.* 2006, Navarro *et al.* 2009), grape (Xiao *et al.* 2006), sweet cherry (Kitashiba *et al.* 2004), dwarf apple (Yang *et al.* 2011), and peach (Wisniewski *et al.* 2011). *MdCBF1*, a gene from *Malus domestica* Borkh. cv. Fuji was submitted to the National center for biotechnology information (NCBI No. DQ074478) by Hellens *et al.* (2005), however, its function remains still unclear.

By the sequence analysis, we deduced that *MdCBF1* is a transcription factor gene which belongs to AP2/ERF family, CBF/DREB subfamily. The aim was to show a direct evidence for the role of *MdCBF1* gene in freezing tolerance of *Arabidopsis thaliana*.

Materials and methods

Arabidopsis thaliana L. ecotype Columbia seedlings were grown on a germination medium (GM) solidified with agar for 2 - 3 weeks as described previously (Yamaguchi-Shinozaki and Shinozaki 1994). Then, plants were grown in pots filled with a mixture of peat moss, *Perlite*, and *Vermiculite* (1:1:1, v/v/v) in a controlled chamber at a temperature of 22 ºC, a 16-h photoperiod, and an irradiance of 100 µmol m^{-2} s^{-1}. In the first experiment, a freezing stress was conducted by placing two-week-old seedlings grown in agar under -20°C for 40, 50, 60 and 70 min, and then returned to 22 ºC for 7 d. In the second experiment, three-week-old seedlings were removed from agar, placed between damp paper towels, packed in moist sand in metal boxes, and placed in a temperature-regulated freezer at -20 °C for 60 min. The plants were subsequently removed from the sand boxes, placed in pots filled with a mixture of peat moss, *Perlite*, and *Vermiculite* (1:1:1, v/v/v) , and returned to 22 °C for 7 d.

In order to amplify full length *MdCBF1* cDNA, RT-PCR was performed on total RNA obtained by the CTAB-based method from the seedlings of *Malus domestica* Borkh. cv. Fuji. Reactions were performed using PCR primers 5'-GGATCCATGGATGGT TGCTCTAATTAC-3' and 5'-GAGCTCTTAAATGGA AAAACTCCATAAG-3'.

PCR products were cloned into the pUCm-T vector and sequenced by the Chinese National Human Genome Center in Shanghai. The *MdCBF1* cDNA was digested with *Bam* HI and *Sac* I, and the 660 bp fragment was inserted between the double CaMV 35S (D35S) promoter and nopaline synthase terminator (NOS-Ter) of binary vector pYG8406 (Fig. 1). The recombinant plasmid was introduced into *Agrobacterium tumefaciens* L. strain GV3101. The constructions were transformed by the floral dip method as described previously (Zhang *et al.* 2006) into plants of wild-type *A. thaliana* for generation of the *MdCBF1*-overexpressing transformants. Transgenic plants were selected by hygromycin resistance and confirmed by PCR (5'-GCCAGCAGTAACGCTAAG GAC-3' and 5'-AGACCACAGTGGAACATCAGC-3') and RT-PCR (5'-CTGAGGAGTCGGATGAGGTTTG-3' and 5'-CATAAGGGCACGTCA GCACAA-3') using the primers mentioned above.

For GUS staining, *Arabidopsis* leaves were immersed in a 1.5 cm^3 Eppendorf tube with 0.1 M Na_3PO_4 (pH 7.0), 10.0 mM EDTA, 0.1 % (v/v) *Triton X-100*, 1.0 mM $K_3Fe(CN)_6$, and 2.0 mM *X-Gluc*. Then the sample was wrapped in aluminum foil to keep it in the dark. After incubating at 37 °C overnight, the staining solution was removed, and the leaves were washed with 75 % (v/v) ethanol.

The extent of lipid peroxidation in leaves was estimated by measuring the amount of TBARS, as indicator of oxidation of polyunsaturated fatty acids according to Havaux *et al.* (2003). The proline content was determined using the ninhydrin assay of Bates and Teare (1973). We repeated measurements five times on independent batches of plant material.

For measurements of relative electrical conductivity (REC), leaf samples were separated into two equal groups. The first group was shaken in double-distilled water at 170 rpm and at 25 °C for 2 h, the second group was boiled for 30 min and electrical conductivity was measured as described by Xue *et al.* (2009). The conductivity in test leaves was designated as Rc and in boiled leaves as Rc′. REC [%] was calculated as Rc/Rc′ × 100.

For a reverse transcription-PCR analysis, total RNA extracted from WT plants and *35S*:*MdCBF1* plants were digested with DNase I (*Promega*, Madison, WI, USA) to remove genomic DNA. The first strand of cDNA was synthesized using 5 µg of total RNA as template with the reverse transcription system (*Promega*) in a 0.02 cm^3 reaction volume. In order to improve the reliability of RT-PCR, the *Arabidopsis actin* gene (*AtAct2*) was synthesized with two primers (AtAct2Z1: 5'-GCA CCCTGTTCTTCTTACCGAG-3' and AtAct2F1: 5'- AGT

AAGGTCACGTCCAGCAAGG-3’) and used as internal standard. The PCR was performed at 94 °C for 10 min, followed by 22 cycles at 94 °C for 20 s, at 56 °C for 20 s, and at 72 °C for 20 s, and a final extension at 72 °C for 5 min. A 660-bp fragment of the *MdCBF1* gene was amplified using two specific primers (5’-CTGAGGAGT CGGATGAGGTTTG-3’ and 5’-CATAAGGGCACG TCAGCACAA-3’) according to the sequence of the *MdCBF1* gene. *MdCBF1* was amplified using PCR conditions as follows: 94 °C for 10 min, followed by different cycles at 94 °C for 30 s, at 56 °C for 30 s, and at 72 °C for 30 s, plus a final extension at 72°C for 5 min. The PCR products were separated on 2 % (m/v) agarose gels. The ratio of the *MdCBF1* to *AtAct2* expression was analyzed with a *Shine Tech Gel* analyser (*Shine Science and Technology*, Shanghai, China). The similar results were obtained for three independent experiments. Therefore, only the result from one experiment is presented.

The specific primers of *RD29A* (5’-AAGGAAACG ACGACAAAGGAAG-3’ and 5’-CCACCACCAAAC CAGCCAGA-3’), *RD29B* (5’-GGAGTGAAGGAGACG CAACAAG-3’ and 5’-GGAATCCGAAAACCCCAT AGTC-3’), and *COR15a* (5’-AACTCTGCCGCCTTGTT TGC-3’ and 5’-ATGCGTTGATCTACGCCGCTAA-3’) were used in a semi-quantitative RT-PCR analysis (RT-sqPCR). It operated under the following conditions: 94 ºC for 10 min, followed by 30 cycles at 94 ºC for 20 s, at 56 ºC for 20 s, and at 72 ºC for 20 s, plus a final extension at 72 ºC for 10 min. The *Arabidopsis actin* gene (*ACT* GenBank: U41998) was used as positive internal control with primers 5’-GAGCTTCGTATT GCTCCTGAAGAG-3’ and 5’-AGACACACCATCACC AGAATCCAG-3’. PCR reaction was carried out with 25 cycles at 94 ºC for 15 s, 56 ºC for 15 s, and 72 ºC for 15 s, plus a final extension at 72 ºC for 5 min. The expression pattern of *RD29B* was evaluated by a *Shine Tech. Gel* analyser. The experiments were repeated three times under the same condition and one of them is presented.

For a real-time reverse transcription PCR analysis, amplifications of specific regions of targeted genes were carried out using a *iQ SYBR Green Supermix* kit (*BioRad*, Hercules, CA), and real-time detection of production was performed in a *Mini Opticon* real time PCR System (*BioRad*). The real-time PCR primer sequences were designed using the *Primer 3* software. All cDNA samples were analyzed in duplicate or triplicate, and cDNA was derived from two sets of independently grown plants. Thermal cycling conditions consisted of 38 cycles of 94 ºC for 20 s, 58 ºC for 30 s, and 72 ºC for 20 s, plus a final extension at 72 ºC for 5 min. The relative changes in gene expression were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

The SPSS software *v. 15.0J* (SPSS Inc., Chicago, USA) was used for the statistical analysis. The values with significant differences according to t-tests are indicated by *asterisks* (* - $P \leq 0.05$; ** - $P \leq 0.01$).

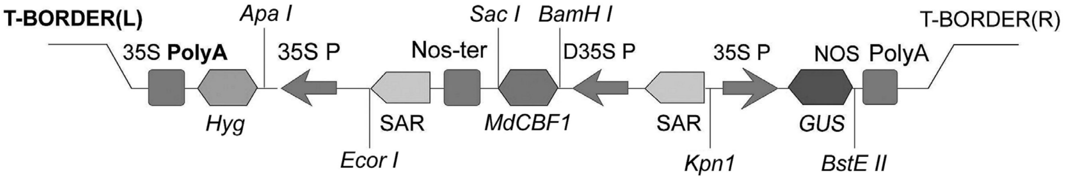

Fig. 1. Schematic diagram of the vector used in this study. The vector contains the double CaMV 35S (DCaMV35S) promoter and the tobacco mosaic virus (TMV) sequence fused to the sp1I gene. For steady transmission of the MdCBF1 gene, two Scaffold attachment regions (SAR) were fused upstream of the DCaMV35S promoter and downstream of the Nos-Terminator (Nos-T).

Results

The full-length cDNA sequence of *MdCBF1* gene isolated from apple reveals that it contained a 660 bp open reading frame encoding a polypeptide of 219 amino acids with a predicted molecular mass of 23.94 kDa and pI 5.21. The predicted MdCBF1 protein contained an AP2 domain of 56 amino acids. It contained the specific aminophenol sequence of CBF protein with PKKRAGRKKFRETRHP in the 41th position and FADSAWR in the 113th position. Two putative acidic domains in N-terminal and C-terminal regions were also detected in *MdCBF1*. A putative nuclear localization signal was located adjacent to the C-terminal acid region. It indicates that *MdCBF1* revealed the typical structure of CBF transcription factor (Fig. 2*A*). The alignment and phylogenetic tree analysis reveal that *MdCBF1* was classified into a DREB-A1 subgroup (Fig. 2*B*).

Four transgenic plants with *MdCBF1* were obtained (Fig. 3*A*), and transgenic plants of T_3 generation were confirmed by GUS staining and PCR (Fig. 3*B*,*C*). We selected two genetically stable transgenic lines (named P1 and P2) that demonstrated a relatively high expression of *MdCBF1* (Fig. 3*D*).

To demonstrate freezing tolerance, 2-week-old T_3 seedlings grown in agar were placed at -20°C for 40, 50, 60, and 70 min, and then returned to 22 ºC for 7 d. When stressed at -20°C for 70 min, 100 % wild-type plants died, but 92.2 % *35S*:M*dCBF1* plants survived and continued to grow (Fig. 4*A*). Also, 3-week-old T_3 seed-

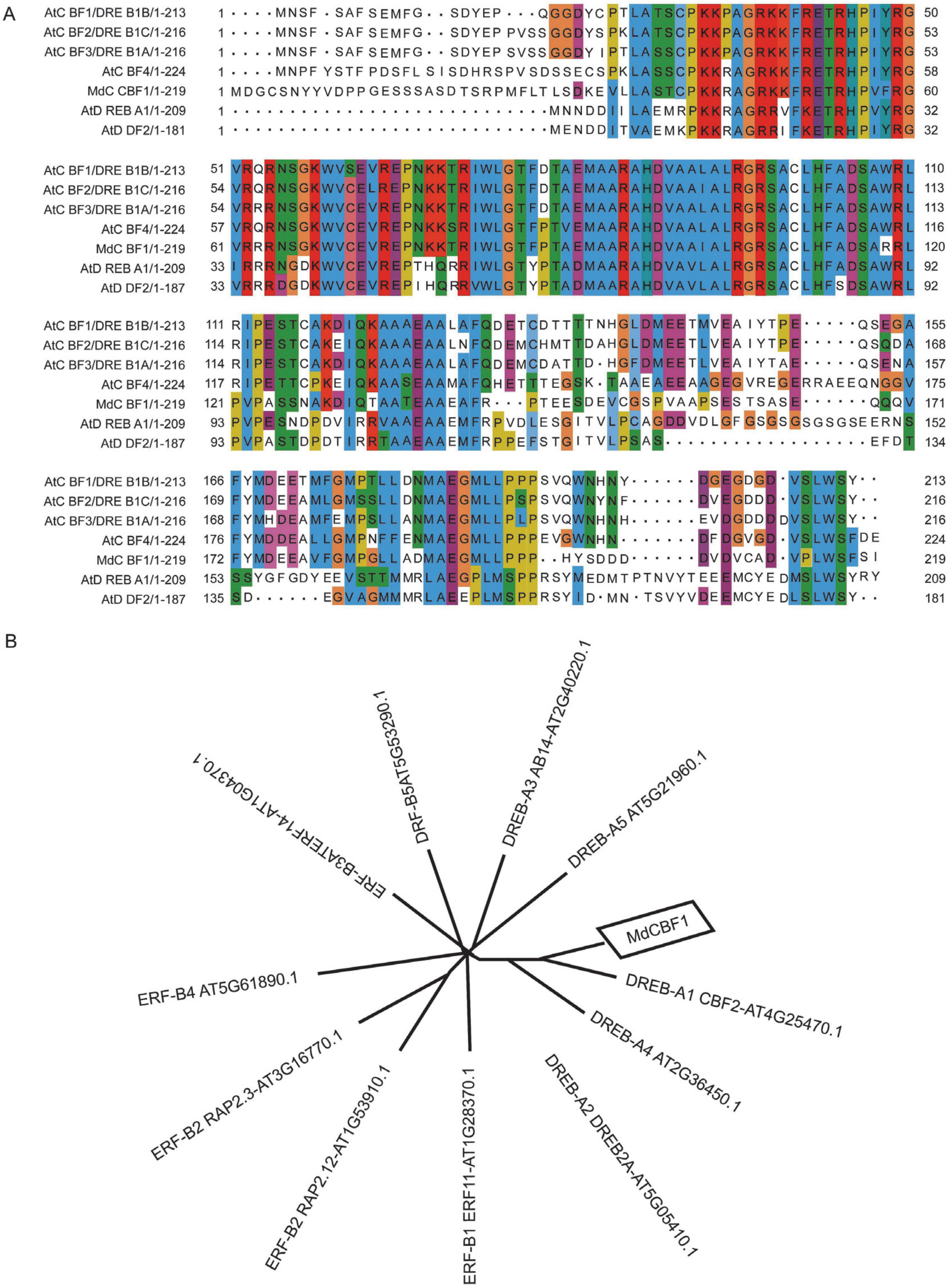

Fig. 2. Deduced amino acid sequences of AP2/EREBP proteins and phylogenic relationships of selected AP2/ERF domains from AP2/EREBP proteins. *A* - Comparison of deduced amino acid sequences of AP2/EREBP proteins that have high sequence similarity with MdCBF1. Identical amino acid residues and conservation amino acid residues are marked by *different colours*, respectively, and the conserved AP2 domain and nuclear localization signals (NLS) are marked with *overlines*. The specific amino acid sequences PKKRAGRKKFRETRHP and FADSAWR are indicated with *. *B* - Phylogenic comparison of the MdCBF1 protein and some AP2/EREBP proteins. Accession numbers for the AP2/EREBP proteins used are as follows: MdCBF1, DREB-A1 CBF2-AT4G25470.1, DREB-A4 AT2G36450.1, DREB-A2 DREB2A-AT5G05410.1, ERF-B1 ERF11-AT1G28370.1, ERF-B2 RAP2.12-AT1G53910.1, ERF-B2 RAP2.3-AT3G16770.1, ERF-B4 AT5G61890.1, ERF-B3 ATERF14-AT1G04370.1, ERF-B5 AT5G53290.1, DREB-A3 AB14-AT2G40220.1, and DREB-A5 AT5G21960.1.

lings were also treated at -20°C for 60 min. Although 59.3 % WT plants died, more than 81.5 % *35S*:*MdCBF1* plants survived and continued to grow after the freezing stress (Fig. 4*B*). The elevated tolerance of the *MdCBF1* over-expressing plants was confirmed by measuring changes in the content of TBARS and proline. The content of proline under the normal conditions was 0.265 µmol g^{-1}(f.m.) in WT and 0.276 and 0.283 $µmol^{-1}$(f.m.), respectively, in the transgenic plants P1 and P2. However, after the freezing stress, the proline content was 0.312 µmol g^{-1}(f.m.) in WT and 0.423 and 0.416 µmol g^{-1}(f.m.) in the transgenic plants. Thus, the increase of proline content in the transgenic plants was significantly higher than that in the WT plants ($P < 0.05$) under the freezing stress (Fig. 5*B*). The TBARS content in the transgenic plants was considerably lower than in WT. After the freezing stress, the TBARS content in P1 and P2 was 5.58 and 5.62 µmol g^{-1}(f.m.), respectively, and 6.19 µmol g^{-1}(f.m.) in WT ($P < 0.05$). Differences in the content of TBARS between the transgenic lines were

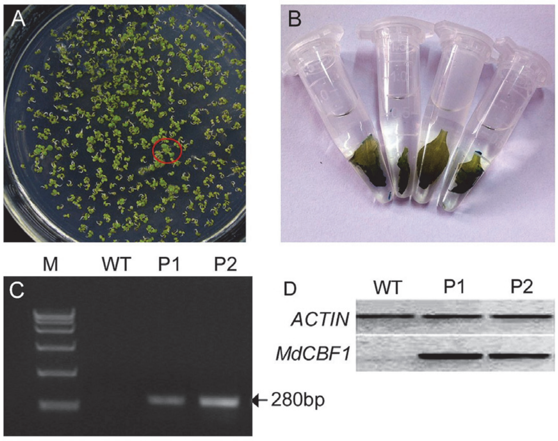

Fig. 3. The identification of transgenic plants with MdCBF1. *A* - T0 transgenic plants on a medium with hygromycin, a green seedling in the *red ellipse* means the plant carrying hygromycin resistance. *B* - GUS staining in a transgenic plant. *C* - The PCR analysis of a transgenic plant with MdCBF1; WT - wild type, P1, P2 - two transgenic lines, M - DL 2000 marker. *D* - The confirmation of MdCBF1 was expressed in transgenic lines by RT-PCR.

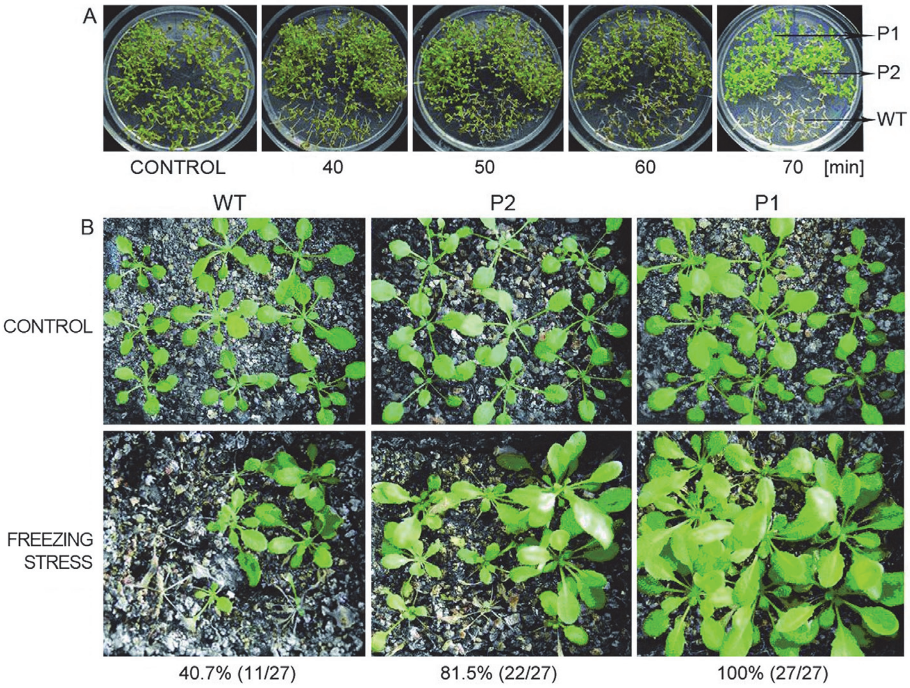

Fig. 4. The phenotypes of two-week-old wild type (WT) and transgenic (P1, P2) plants grown in normal conditions and after -20 °C for 40, 50, 60, and 70 min (*A*). The phenotypes of three-week-old WT, P1, and P2 grown in normal conditions and after -20 °C for 60 min (*B*). The percentages refer to survival rates under the freezing stress.

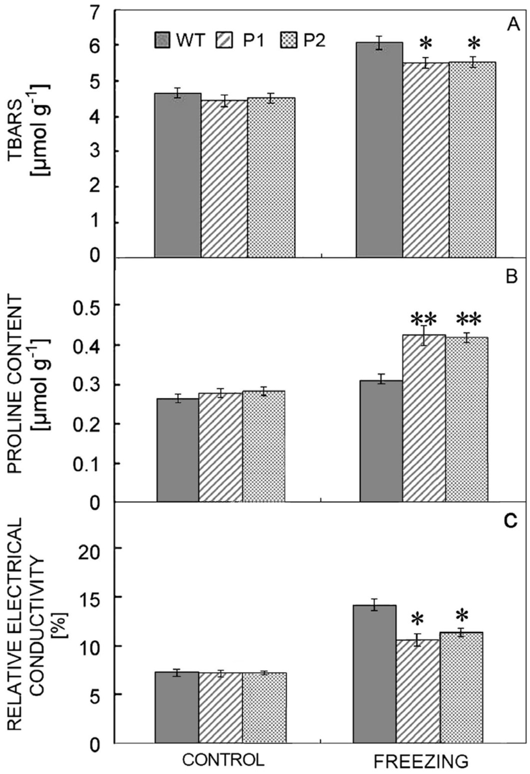

Fig. 5. Effects of freezing stress on the physiological properties in leaves of the wild type (WT) and transgenic plants (P1 and P2). TBARS (*A*), proline content (*B*), and relative electrical conductivity (*C*) after freezing at -20°C for 60 min and recovery at 22 °C for 6 h. Means ± SE, *n* = 3, ** and * indicate significant difference at $P < 0.01$ and $P < 0.05$ between the transgenic plants and WT, respectively.

consistent with the different expression of the target genes. Taken together, we conclude that *MdCBF1* is a positive regulator commonly involved in the adaptation to freezing stress (Fig. 5). The REC of transgenic plants was significantly lower than that of WT after freezing stress (Fig. 5*C*). This demonstrated a lower membrane damage in transgenic plants under freezing stress.

To further elucidate the role of the *MdCBF1* gene in response to freezing stresses, we analyzed the expression of stress-related genes in *MdCBF1* transgenic and WT plants. There was a significant difference in the expression of *COR15A, RD29A*, and *RD29B* between transgenic and WT plants also under normal conditions and, the *RD29B* mRNA showed higher accumulation in the transgenic lines compared to WT plants (Fig. 6). Under freezing stress, the expression of *COR15A* and *RD29A* in the transgenic lines increased more than in the WT plants (Fig. 6).

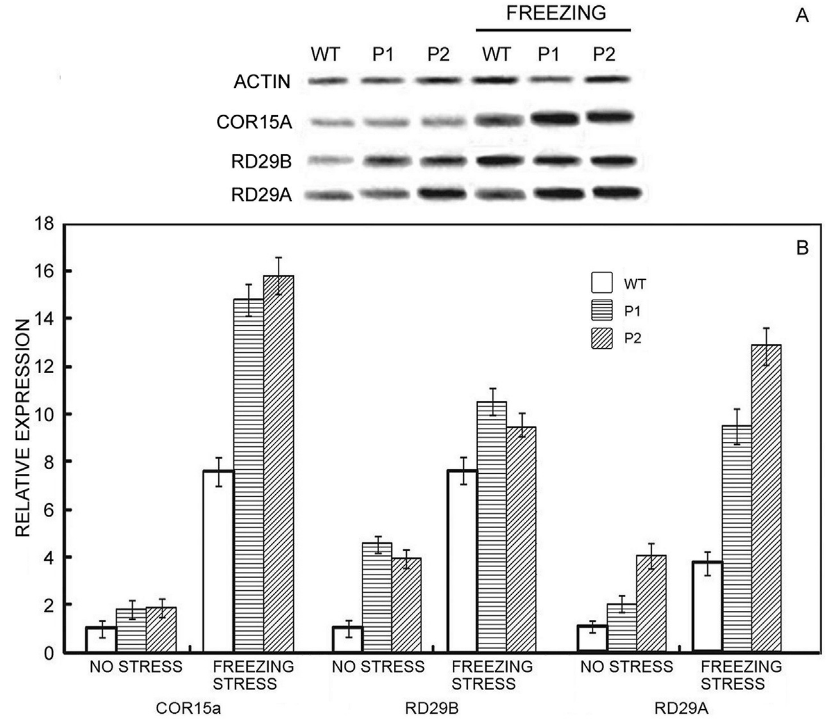

Fig. 6. The effect of freezing stress on transcription of *COR15a*, RD29B, and *RD29A* gene in WT and transgenic plants P1 and P2. *A* - semi-quantitative PCR analysis, *B* - real-time PCR analysis.

Discussion

In our study, the abiotic stress increased the TBARS content both in the wild type and transgenic line similarly to what has been found in other species (Wanner and Junttila 1999). The accumulation of TBARS is often used as indicator of lipid peroxidation. Membrane lipid peroxidation occurs due to production of reactive oxygen species and malfunction of the scavenging system. Under the freezing stress, the TBARS content was lower in the transgenic plants than in the WT plants. A similar result was found in REC. These results imply that the degree of membrane injury in the transgenic plants was lower than in the wild type plants, and that the *MdCBF1* gene could protect the cell membrane integrity of the *Arabidopsis* plants under the freezing stress. Further experiments are needed to clarify the role of *MdCBF1* gene in the maintenance of membrane ultrastructure during freezing stress.

The proline is an important factor in abiotic tolerance; it plays a major role in osmotic adjustment as well as a number of protective roles. In transgenic tobacco plants, the overproduction of proline may lead to increased tolerance against abiotic stresses (Kishor *et al*. 1995). In the present study, the proline content in the transgenic plants was higher than that in the wild type plants. The freezing tolerance and the accumulation of proline paralleled each other after the stress indicating that proline accumulation is a fundamental component of an enhanced freezing tolerance.

It was reported that GmERF3, as AP2/ERF transcription factor, may play dual roles in response to biotic and abiotic stresses in plants (Zhang *et al*. 2009). In this study, *MdCBF1*, which is also an AP2/ERF transcription factor, played an important role in the response of the transgenic *Arabidopsis* plants to the freezing stress. The analysis of RT-PCR for stress-related genes imply that *MdCBF1* over-expressing plants had a higher expression of *COR15a* and *RD29A* genes than the wild type plants, and the freezing treatments could enlarge these differences. One possible explanation for this observation is that MdCBF1 protein combing with *CRT/DRE cis*-elements up-regulated the *COR15a* and *RD29A* expressions.

The real time-PCR analysis reveals that the expression of the *RD29B* gene in the transgenic plants was higher than in the WT plants under the normal conditions. *RD29B* gene belongs to LEA genes. Although its specific mechanism is unclear, several studies have shown that *RD29B* plays a significant role in an adaptation to stresses. LEA proteins are protective biological macromolecules in plant cells, they can help to maintain the cell structure under freezing and other abiotic stresses. LEA proteins can be induced at the transcriptional level by ABA, high osmolality, *etc*. Under various abiotic stresses, plants increase the content of endogenous ABA, and ABA triggers responses to them (Tuteja 2007). The analysis of the transgenic plants suggests that the over-expression of *MdCBF1* in transgenic *Arabidopsis* made the root sensitive to ABA (unpublished data) and Zhu *et al*. (2007) have obtained similar results. These results suggest that the stress response pathway in *MdCBF1* transgenic plants may be induced by ABA. It should be emphasized that *RD29B* is also an ABA-induced gene, and its promoter region contains ABRE *cis*-acting element. It was also reported that the expression of *LcDREB3a* results in the accumulation of *AtRD29A* transcripts in transgenic *Arabidopsis* plants under normal conditions (Peng *et al*. 2011). The freezing tolerance was most likely conferred by the activation of stress-responsive genes, such as *COR15a, RD29A*, and *RD29B* in *Arabidopsis*.

References

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., Ecker, J.R.: Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. - Science **301**: 653-657, 2003.

Bates, L.S.W.R., Teare, I.D.: Rapid determination of free proline in water-stress studies. - Plant Soil **39**: 205-207, 1973.

Benedict, C., Skinner, J.S., Meng, R., Chang, Y., Bhalerao, R., Huner, N.P., Finn, C.E., Chen, T.H., Hurry, V.: The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus spp*. - Plant Cell Environ. **29**: 1259-1272, 2006.

Boutilier, K., Offringa, R., Sharma, V.K., Kieft, H., Ouellet, T., Zhang, L., Hattori, J., Liu, C.M., Van Lammeren, A.A., Miki, B.L., Custers, J.B., Van Lookeren Campagne, M.M.: Ectopic expression of *BABY BOOM* triggers a conversion from vegetative to embryonic growth. - Plant Cell **14**: 1737-1749, 2002.

El Kayal, W., Navarro, M., Marque, G., Keller, G., Marque, C., Teulieres, C.: Expression profile of CBF-like transcriptional factor genes from *Eucalyptus* in response to cold. - J. exp. Bot. **57**: 2455-2469, 2006.

Elliott, R.C., Betzner, A.S., Huttner, E., Oakes, M.P., Tucker, W.Q., Gerentes, D., Perez, P., Smith, D.R.: *AINTEGUMENTA*, an *APETALA2*-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth. - Plant Cell **8**: 155-168, 1996.

Hao, D., Ohme-Takagi, M., Sarai, A.: Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. - J. biol. Chem. **273**: 26857-26861, 1998.

Havaux, M., Lutz, C., Grimm, B.: Chloroplast membrane photostability in chlP transgenic tobacco plants deficient in tocopherols. - Plant Physiol. **132**: 300-310, 2003.

Hellens, R.P., Allan, A.C., Friel, E.N., Bolitho, K., Grafton, K., Templeton, M.D., Karunairetnam, S., Gleave, A.P., Laing, W.A.: Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. - Plant Methods **1**: 13, 2005.

Hu, Y.X., Wang, Y.X., Liu, X.F., Li. J.Y.: *Arabidopsis* RAV1 is down-regulated by brassinosteroid and may act as a negative regulator during plant development. - Cell Res. **14**: 8-15, 2004.

Jakab, G., Ton, J., Flors, V., Zimmerli, L., Metraux, J.P., Mauch-Mani, B.: Enhancing *Arabidopsis* salt and drought stress tolerance by chemical priming for its abscisic acid responses. - Plant Physiol. **139**: 267-274, 2005.

Kishor, P., Hong, Z., Miao, G.H., Hu, C., Verma, D.: Overexpression of Δ-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic Plants. - Plant Physiol. **108**: 1387-1394, 1995.

Kitashiba, H., Ishizaka, T., Isuzugawa, K., Nishimura, K., Suzuki, T.: Expression of a sweet cherry DREB1/CBF ortholog in *Arabidopsis* confers salt and freezing tolerance. - J. Plant Physiol. **161**: 1171-1176, 2004.

Lee, J.H., Hong, J.P., Oh, S.K., Lee, S., Choi, D., Kim, W.T.: The ethylene-responsive factor like protein 1 (CaERFLP1) of hot pepper (*Capsicum annuum* L.) interacts *in vitro* with both GCC and DRE/CRT sequences with different binding affinities: possible biological roles of CaERFLP1 in response to pathogen infection and high salinity conditions in transgenic tobacco plants. - Plant mol. Biol. **55**: 61-81, 2004.

Livak, K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. - Methods **25**: 402-408, 2001.

Navarro, M., Marque, G., Ayax, C., Keller, G., Borges, J.P., Marque, C., Teulieres, C.: Complementary regulation of four *Eucalyptus CBF* genes under various cold conditions. - J. exp. Bot. **60**: 2713-2724, 2009.

Onate-Sanchez, L., Singh, K.B.: Identification of *Arabidopsis* ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection. - Plant Physiol. **128**: 1313-1322, 2002.

Peng, X.J., Ma, X.Y., Fan, W.H., Su, M, Cheng, L.Q., Alam, I., Lee, B.H., Qi, D.M., Shen, S..H., Liu, G.S., Shen, S.H.: Improved drought and salt tolerance of *Arabidopsis thaliana* by transgenic expression of a novel DREB gene from *Leymus chinensis*. - Plant Cell Rep. **30**: 1493-1502, 2011.

Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K., Yamaguchi-Shinozaki, K.: 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, 2002.

Sohn, K.H., Lee, S.C., Jung, H.W., Hong, J.K., Hwang, B.K.: Expression and functional roles of the pepper pathogen-induced transcription factor RAV1 in bacterial disease resistance, and drought and salt stress tolerance. - Plant mol. Biol. **61**: 897-915, 2006.

Tang, W., Charles, T.M., Newton, R.J.: Overexpression of the pepper transcription factor CaPF1 in transgenic Virginia pine (*Pinus virginiana* Mill.) confers multiple stress tolerance and enhances organ growth. - Plant mol. Biol. **59**: 603-617, 2005.

Thomashow, M.F.: Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. - Annu. Rev. Plant Physiol. Plant mol. Biol. **50**: 571-599, 1999.

Tuteja, N.: Abscisic acid and abiotic stress signaling. - Plant Signal. Behav. **2**: 135-138, 2007

Wanner, L. A., Junttila, O.: Cold-induced freezing tolerance in *Arabidopsis*. - Plant Physiol. **120**: 391-400, 1999.

Welling, A., Palva, E.T.: Involvement of CBF transcription factors in winter hardiness in birch. - Plant Physiol. **147**: 1199-1211, 2008.

Wisniewski, M., Norelli, J., Bassett, C., Artlip, T., Macarisin, D.: Ectopic expression of a novel peach (*Prunus persica*) CBF transcription factor in apple (*Malus × domestica*) results in short-day induced dormancy and increased cold hardiness. - Planta **233**: 971-983, 2011.

Xiao, H., Siddiqua, M., Braybrook, S., Nassuth, A.: Three grape *CBF/DREB1* genes respond to low temperature, drought and abscisic acid. - Plant Cell Environ. **29**: 1410-1421, 2006.

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

Xu, Z.S., Xia, L.Q., Chen, M., Cheng, X.G., Zhang, R.Y., Li, L.C., Zhao, Y.X., Lu, Y., Ni, Z.Y., Liu, L., Qiu, Z.G., Ma, Y.Z.: Isolation and molecular characterization of the *Triticum aestivum* L. ethylene-responsive factor 1 (TaERF1) that increases multiple stress tolerance. - Plant mol. Biol. **65**: 719-732, 2007.

Xue, Y., Peng, R., Xiong, A., Li, X., Zha, D., Yao, Q.: Yeast heat-shock protein gene HSP26 enhances freezing tolerance in *Arabidopsis*. - J. Plant Physiol. **166**: 844-850, 2009.

Yamaguchi-Shinozaki, K., Shinozaki, K.: 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, 1994.

Yang, W., Liu, X.D., Chi, X.J., Wu, C.A., Li, Y.Z., Song, L.L., Liu, X.M., Wang, Y.F., Wang, F.W., Zhang, C., Liu, Y., Zong, J.M., Li, H. .: 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, 2011.

Zhang, G., Chen, M., Li, L., Xu, Z., Chen, X., Guo, J., Ma, Y.: Overexpression of the soybean *GmERF3* gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic *tobacco*. - J. exp. Bot. **60**: 3781-3796, 2009.

Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W., Chua, N.H.: *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. - Nat. Protoc. **1**: 641-646, 2006.

Zhu, S.Y., Yu, X.C., Wang, X.J., Zhao, R., Li, Y., Fan, R.C., Shang, Y., Du, S.Y., Wang, X.F., Wu, F.Q., Xu, Y.H., Zhang, X.Y., Zhang, D.P.: Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in *Arabidopsis*. - Plant Cell **19**: 3019-3036, 2007.