Plant Science 236 (2015) 61–74

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Constitutive expression of *DaCBF7*, an Antarctic vascular plant *Deschampsia antarctica* CBF homolog, resulted in improved cold tolerance in transgenic rice plants

Mi Young Byun[a,1], Jungeun Lee[b,1], Li Hua Cui[a], Yoonjee Kang[b], Tae Kyung Oh[a], Hyun Park[b], Hyoungseok Lee[b,**], Woo Taek Kim[a,*]

[a] Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea
[b] Division of Life Sciences, Korea Polar Research Institute, Incheon 406-840, Republic of Korea

ARTICLE INFO

Article history:
Received 6 January 2015
Received in revised form 7 March 2015
Accepted 26 March 2015
Available online 3 April 2015

Keywords:
C-repeat/DRE binding factor
Cold stress tolerance
Deschampsia antarctica
RNA-seq analysis
Transgenic rice (*Oryza sativa*) plant

ABSTRACT

Deschampsia antarctica is an Antarctic hairgrass that grows on the west coast of the Antarctic peninsula. In this report, we have identified and characterized a transcription factor, *D. antarctica* C-repeat binding factor 7 (DaCBF7), that is a member of the monocot group V CBF homologs. The protein contains a single AP2 domain, a putative nuclear localization signal, and the typical CBF signature. DaCBF7, like other monocot group V homologs, contains a distinct polypeptide stretch composed of 43 amino acids in front of the AP2 motif. DaCBF7 was predominantly localized to nuclei and interacted with the C-repeat/dehydration responsive element (CRT/DRE) core sequence (ACCGAC) in vitro. *DaCBF7* was induced by abiotic stresses, including drought, cold, and salinity. To investigate its possible cellular role in cold tolerance, a transgenic rice system was employed. *DaCBF7*-overexpressing transgenic rice plants (*Ubi:DaCBF7*) exhibited markedly increased tolerance to cold stress compared to wild-type plants without growth defects; however, overexpression of *DaCBF7* exerted little effect on tolerance to drought or salt stress. Transcriptome analysis of a *Ubi:DaCBF7* transgenic line revealed 13 genes that were up-regulated in *DaCBF7*-overexpressing plants compared to wild-type plants in the absence of cold stress and in short- or long-term cold stress. Five of these genes, *dehydrin*, *remorin*, *Os03g63870*, *Os11g34790*, and *Os10g22630*, contained putative CRT/DRE or low-temperature responsive elements in their promoter regions. These results suggest that overexpression of *DaCBF7* directly and indirectly induces diverse genes in transgenic rice plants and confers enhanced tolerance to cold stress.

1. Introduction

The Antarctic, characterized by freezing temperatures and markedly different seasonal levels of irradiation and photoperiod, is a harsh environment for terrestrial plants. *Deschampsia antarctica* Desv. (Poaceae) is one of the just two species of flowering plants that have evolved mechanisms for coping with these severe conditions. *D. antarctica* is an Antarctic hairgrass that grows along the west coast of the Antarctic peninsula. Its population size has expanded, apparently a consequence of global warming [1].

Several studies have shed light on some of the defense mechanisms that *D. antarctica* has developed against abiotic stresses, especially cold temperature. This plant displays maximal photosynthetic activity at 13 °C and retains 30% of its maximal photosynthesis at 0 °C [2]. During its growth period, it produces antifreeze proteins and accumulates non-structural carbohydrates, presumably as part of its mechanism of freezing tolerance [3,4]. Recrystallization inhibition (RI) activity, which suppresses the growth of small ice crystals into damaging large ones, enables the Antarctic grass to survive and maintain freezing tolerance [5]. These features suggest that *D. antarctica* is an important and valuable genetic resource of genes related to stress tolerance.

Abbreviations: AP2, APETALA2; CaMV, cauliflower mosaic virus; CBF, C-repeat binding factor; CDPK, calcium/calmodulin-dependent protein kinase; CRT, C repeat; DEG, differentially expressed gene; DRE, dehydration responsive element; FDR, false discovery rate; GO, gene ontology; LTRE, low-temperature responsive element; MAPK, mitogen-activated protein kinase; NLS, nuclear localization signal; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RNA-seq, RNA sequencing; RPKM, reads per kilobase of exon model per million mapped reads; sGFP, synthetic green fluorescent protein; Ubi, ubiquitin.

* Corresponding author. Tel.: +82 2 2123 2661; fax: +82 2 312 5657.
** Corresponding author. Tel.: +82 32 760 5570; fax: +82 32 760 5575.
E-mail addresses: soulaid@kopri.re.kr (H. Lee), wtkim@yonsei.ac.kr (W.T. Kim).
[1] The first two authors contributed equally to this work.

http://dx.doi.org/10.1016/j.plantsci.2015.03.020
0168-9452/

Various environmental stresses, such as drought, high salinity, and extreme temperatures, profoundly affect growth, development, and productivity of higher plants [6,7]. To endure and survive in such harsh conditions, plants have developed defense mechanisms that involve physiological and biochemical changes, which are adjusted by stress-responsive gene expressions at the molecular levels [8,9]. Stress-responsive transcription factors bind to *cis*-acting elements in the promoter regions of target genes and modulate stress signal transduction and responses [10]. C-repeat binding factor (CBF)/dehydration-responsive element binding protein (DREB) is one subfamily of the APETALA2/ethylene-responsive element binding factor (AP2/ERF) family, which is a large group of plant-specific transcription factors. CBF/DREB has significant roles in plant abiotic stress responses. The transcription factor interacts with *cis*-acting C-repeat/dehydration-responsive element (CRT/DRE) and thus up-regulates or down-regulates diverse sets of stress-related gene expression [9,11]. *Arabidopsis DREB1A*/*CBF3*, *DREB1B*/*CBF1*, and *DREB1C*/*CBF2* are induced rapidly in response to low temperature, while *Arabidopsis DREB2* gene is induced by drought and high salinity but not cold [12]. Overexpression of *Arabidopsis CBF/DREBs* induces cold-responsive genes and results in enhanced tolerance to freezing, drought, and high salinity [12,13]. Heterologous expression of *Arabidopsis CBF/DREBs* improves stress tolerance in transgenic canola, tobacco, and rice plants [14–16]. Cold-responsive *CBF* homologs have also been identified in other plant species, including tomato, wheat, barley, rice, and moss [14,17–19]. The existence of stress-inducible *CBFs* in divergent plant kingdom and the effect of these genes on stress response suggest that CBFs play conserved regulatory hub in cold tolerance of land plants [9].

In this study, we identified *D. antarctica CBF7* gene (*DaCBF7*) encoding a putative homolog of *CBF7* from wheat (*Triticum monococcum* L.) [19]. The *DaCBF7* gene was induced in response to drought, cold, and high salinity in *D. antarctica*. To investigate its possible cellular role, the *DaCBF7* gene was constitutively expressed in rice, a monocot model crop. Phenotypic analysis of transgenic rice plants (*Ubi:DaCBF7*) indicated that overexpression of *DaCBF7* enhanced tolerance to cold stress without growth defects in rice. Transcriptome analysis of a *Ubi:DaCBF7* transgenic line showed that various stress-related genes were up-regulated before and after cold treatment in transgenic rice plants. Overall, our results suggest that an Antarctic hairgrass *DaCBF7* plays a critical role in cold stress response in transgenic rice plants.

2. Materials and methods

2.1. Phylogenetic analysis

Amino acid sequences of DaCBF7 and other CBF/DREB homologs from monocot crops were retrieved from the GenBank database and proofread. All downstream analyses were performed using the program MEGA6 [20]. Phylogenetic trees were constructed from the data sets by using the maximum likelihood method based on the JTT matrix-based model. The initial tree for the heuristic search was obtained by applying the neighbor-joining method to a matrix of pair-wise distances, estimated using a JTT model. Supports for internal branches were tested by the bootstrap analyses of 1000 replications.

2.2. Subcellular localization experiment

The synthetic green fluorescent protein (*sGFP*) coding region was fused in-frame to the 3′ end of the full-length *DaCBF7* coding region and inserted into the pEarleyGate 100 (pEG100) binary vector. The vector was transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Tobacco (*Nicotiana benthamiana*) leaves were co-infiltrated using *Agrobacterium* that contained the *35S:DaCBF7-sGFP* or 35S:nuclear localizing signal-monomeric red fluorescent protein (*35S:NLS-mRFP*) construct. NLS-mRFP was used as a control for a nuclear protein. Two days after infection, protoplasts were extracted from the tobacco leaves and visualized by fluorescence microscopy (BX51, Olympus, Tokyo, Japan).

2.3. Plasmid construction for protein expression in Escherichia coli and gel retardation assay

For recombinant protein expression, a full-length coding region of *DaCBF7* was inserted into the pProEx-HTa protein expression vector (Invitrogen, Carlsbad, CA, USA). Recombinant protein expressed in *E. coli* BL21 (DE3) cells was purified by affinity chromatography using nickel-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) according to the manufacturer's protocols.

Gel retardation assays were conducted as described previously [21] with minor modifications. The DNA fragments containing the CRT/DRE core repeat or low-temperature responsive element (LTRE) sequence were labeled with ^{32}P-dCTP and incubated with recombinant DaCBF7 proteins in binding buffer [10 mM Tris–HCl, pH 8.0, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton-X 100, and 12.5% glycerol (v/v)]. After incubation for 15 min on ice, components of the reaction mixtures were separated on 6% non-denatured polyacrylamide gels in 0.5× Tris–borate EDTA buffer. Gels were dried and visualized by autoradiography. For the competition assay, non-radiolabeled competitors of the probe were pre-incubated with DaCBF7 proteins for 10 min on ice, and then the radiolabeled probes were added. The reaction mix was incubated on ice, and the components were separated on the polyacrylamide gel as described above. Plant telomeric repeats (PTRs) were used as a negative control. The gel was dried and autoradiographed.

2.4. Plant materials and stress treatments of D. antarctica

D. antarctica was collected near the King Sejong Antarctic Station (62°14′29″S; 58°44′18″W) on the Barton Peninsula of King George Island in January 2007. The plants were cultured in vitro in tissue culture medium [Murashige and Skoog (MS) medium; 2% sucrose and 0.8% phytoagar at pH 5.7] under a 16-h light/8-h dark photoperiod with a light intensity of 150 μmol m^{-2} s^{-1} at 15 °C.

For cold-stress treatment, plants were transferred to a chamber at 4 °C. For the dehydration-stress treatment, plants were transferred to a filter paper and dried at 15 °C. For high-salinity treatment, plants were transferred to MS medium supplemented with 150 mM NaCl and incubated at 15 °C. RNA was extracted and analyzed from leaves at various times after imposition of stress.

2.5. Total RNA extraction and real-time qRT-PCR analysis

Total RNA was isolated from leaves of five different *D. antarctica* plants with four tillers each (longest tiller = 5 cm) using RNeasy Plant Mini Kit in conjunction with the RNase free DNase set (Qiagen) according to the manufacturer's instructions. The quantity and quality of total RNA were determined by spectroscopic measurements at 230, 260, and 280 nm using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was checked by electrophoresis in 2% agarose gel.

Single-strand cDNA was synthesized from 2 μg of total RNA using Superscript III (Invitrogen). Real-time qRT-PCR was performed in 20 μL of reaction mixtures that included 1 μL of a 1:15 diluted cDNA template, 2 μM of each primer, and 10 μL of QuantiFast SYBR Green PCR Kit (Qiagen). Amplified signals were

monitored continuously with the Mx3000P qPCR System (Stratagene, La Jolla, CA, USA). The amplification protocol was as follows: 5 min of denaturation and enzyme activation at 95 °C followed by 40 cycles at 95 °C for 5 s, 58 °C for 20 s, and 72 °C for 15 s. The *DaEF1a* gene was used as an internal control [22]. DNA sequences of primers used for PCR amplification are listed in Table S1.

2.6. Generation of DaCBF7-overexpressing transgenic rice plants

To generate *Ubi:DaCBF7* transgenic rice plants, the *DaCBF7* coding region of 813 bp was inserted into the pGA2897 vector. The binary vector was transformed into *A. tumefaciens* strain LBA4404 by electroporation and used for rice transformation as described previously [23]. Callus tissue was induced from wild-type rice (*Oryza sativa* L. japonica variety 'Dong-Jin') seeds, co-cultivated with *Agrobacterium*, and selected on callus induction medium containing antibiotics (40 mg/L hygromycin B and 250 mg/L carbenicillin). Selected callus tissue was transferred to a regeneration medium. Transgenic T0 plants were transplanted to soil in a greenhouse and T2 plants were used for phenotypic analysis.

2.7. RT-PCR and DNA gel blot analysis

Total RNA was isolated from mature leaves of wild-type and *Ubi:DaCBF7* transgenic rice plants using TRIzol reagent [38% equilibrated phenol, pH 4.3, 1 M guanidine thiocyanate, 1 M ammonium thiocyanate, 0.3 M sodium acetate, pH 5.2, and 5% glycerol (v/v)]. Single-strand cDNA was synthesized from 2 μg of total RNA with oligo(dT) primers and TOPscript Reverse Transcriptase (Enzynomics, Daejeon, Korea). To compare the *DaCBF7* gene expression level between different transgenic lines, *DaCBF7* was amplified using a gene-specific primer set. DNA sequences of gene-specific primers are listed in Table S1.

To distinguish the independent lines of *Ubi:DaCBF7* transgenic plants, genomic Southern blotting was performed. Total genomic DNA was isolated from mature rice leaves using CTAB solution, digested with *Bam*HI restriction enzyme, and separated on 0.8% agarose gel. The gel was treated sequentially with depurifying, denaturing, and neutralizing solutions, and transferred to Hybond-N nylon membranes. The blot was hybridized with ^{32}P-labeled hygromycin B phosphotransferase (hph) probe under high-stringency conditions.

2.8. Plant materials and stress treatments of rice plants

Dry rice seeds were sterilized with 0.4% NaClO solution for 30 min and washed with sterilized water several times. Seeds of wild-type and *Ubi:DaCBF7* transgenic rice plants were germinated on half-strength MS medium including vitamins (Duchefa Biochemie, Haarlem, The Netherlands), 3% sucrose, and 0.7% phytoagar. Seedlings were grown for 2 weeks at 28 °C under a 16-h light/8-h dark photoperiod and then transplanted to soil in a greenhouse.

To investigate the effect of *DaCBF7* on stress tolerance, 6-week-old plants grown in the growth room were subjected to various abiotic stresses, observed, and photographed. For cold-stress treatment, wild-type and *DaCBF7*-overexpressing plants (independent lines #1, #2, #3, and #10) grown at 28 °C were transferred to 4 °C for 8 days, after which they were grown at 28 °C. Electrolyte leakage analysis was conducted with the leaves of 10-day-old plants after 3, 5, and 8 days of cold stress. The first tiller of each plant, approximately 7 cm long, was detached, chopped, and shaken in a test tube with 2 mL of distilled water on an orbital shaker at 200 rpm at 28 °C for 18 h. The conductivity of each sample was measured before and after autoclaving with a conductivity meter (Twin Cond B-173, Horiba, Japan). Drought stress was imposed by growing wild-type and T2 *Ubi:DaCBF7* (independent lines #1 and #10) plants without water for 9 days, after which the normal watering regime was resumed [24]. To measure water loss rate, detached leaves from 6-week-old plants were placed on filter paper and weighed periodically at room temperature [25]. The rate was calculated as the percentage of initial fresh weight. Salt stress was imposed by irrigating plants with water supplemented with 200 mM NaCl. After 10 days, watering with tap water was resumed [26].

2.9. RNA sequencing and bioinformatic analysis

Total RNA was extracted using TRIzol reagent, treated with DNase I to remove contaminant genomic DNA, and purified using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. The integrity and concentration of RNA were determined using Bioanalyzer (RIN > 6) (Agilent Technologies, Santa Clara, CA, USA) and a Qubit® RNA Broad-range Assay Kit (Life Technologies, Carlsbad, CA, USA), respectively. To construct the sequencing library, 1.5 μg of total RNA from each sample was used as input for the TruSeq RNA sample prep kit v2 (Illumina, San Diego, CA, USA). The libraries were validated, quantified by the Bioanalyzer and the library q-PCR quantification method, multiplexed with equal ratio, and loaded on the flow cell of Illumina MiSeq Reagent Kit v3 (2 × 75 run). The sequencing was performed on a MiSeq Sequencer system (Illumina) and a total of 3 Gb (40 M paired end reads) of sequencing data were generated (Q30 > 98%).

Bioinformatic analyses were performed using the CLC Genomics Workbench v7.5 software. After quality and adapter trimming, the raw reads were mapped to the rice reference genome using a gene model annotation file from the Michigan State University rice genome annotation project database v7 (http://rice.plantbiology.msu.edu/). The expression values were measured in reads per kilobase of exon model per million mapped reads (RPKM) normalized values [27] at the gene level. For statistical analysis, *t*-tests and Baggerley's tests were performed using original and normalized read counts. In addition, several relevant values for analysis, such as *p*-values, corrected *p*-values for multiple correction, and test statistics, were calculated in the "multi-group comparison" option. Through the statistical analysis, the differentially expressed genes were determined from a cut-off value (*p*-value < 0.05, corrected *p*-value of false discover rate (FDR) < 0.05, and difference $\neq 0$) from pair-wise comparisons of RPKM values among six samples. Gene Ontology (GO) mapping and annotation were performed with an annotation cutoff of $E < 1 \times 10^{-10}$. GO enrichment analysis was performed using AgriGO, a web-based GO analysis toolkit and Fisher's exact test ($p < 0.05$) [28].

3. Results

3.1. Identification and characterization of DaCBF7

Although the *Arabidopsis* genome contains three cold-inducible CBFs (CBF1, CBF2, and CBF3) [9], the cereal CBF family is larger and more complex. For instance, hexaploid wheat (*Triticum aestivum* L.) has at least 65 different *CBF* genes in 18 groups classified according to phylogenetic origin and structural characteristics [19,29]. Barley (*Hordeum vulgare*) contains 20 *CBF* genes divided into three subgroups: *HvCBF1s*, *HvCBF3s*, and *HvCBF4s* [17]. Recent studies revealed that CBFs are associated with frost tolerance in cereal crop plants [30,31]. *D. antarctica* is an Antarctic monocot hairgrass that is phylogenetically related to *Brachypodium distachyon*, oat, and wheat [32]. *D. antarctica* has distinct defense mechanisms against severe environmental conditions, especially extremely low temperature [3], and these may involve *CBF*-related gene expression. Because the expressed sequence tag (EST) database for *D. antarctica*

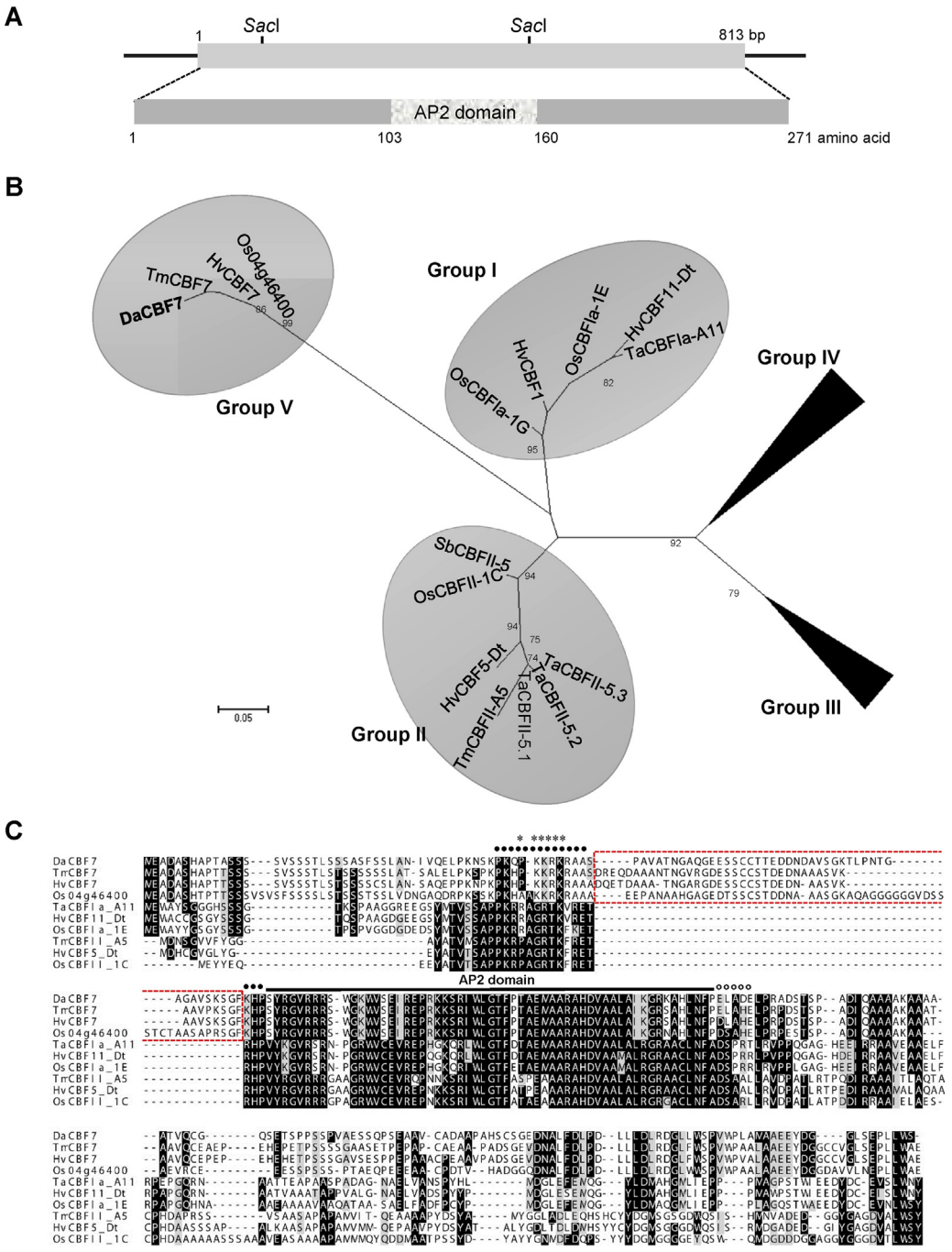


Fig. 1. Sequence analysis of DaCBF7. (A) Schematic structure of the *DaCBF7* cDNA and its deduced protein. Solid lines indicate 5′- and 3′-untranslated regions. The gray bar represents the coding region. The AP2 DNA binding domain is indicated. (B) Phylogenetic relationship of DaCBF7 homologs from monocot rice, sorghum, barley, and wheat. DaCBF7 is represented in bold letters and clades are labeled based on the work of Badawi et al. [29]. The monocot CBF members are divided into five different groups. DaCBF7, along with rice (*O. sativa*, Os04g46400), barley (*H. vulgare*, HvCBF7), and wheat (*T. monococcum*, TmCBF7) homologs, belong to group V. A more detailed phylogenetic tree is presented in Fig. S1. The bootstrap values (>70) were presented near the corresponding branch. (C) Multiple sequence alignment of DaCBF7 and other monocot CBF7 homologs. The solid lines denote the AP2 domain. A distinct array of amino acid sequences found in group V CBF homologs is highlighted in a red box. Asterisks indicate a putative nuclear localization signal. Two typical CBF signature sequences at immediate upstream and downstream of the AP2 domain are indicated by close circles and open circles, respectively. Accession numbers of CBF homologs from six monocot species, including Antarctic hairgrass (*D. antarctica*), barley (*H. vulgare*), rice (*O. sativa*), sorghum (*Sorghum bicolor*), hexaploid wheat (*T. aestivum*), and einkorn wheat (*T. monococcum*) are represented in Table S2.

is available [33,34], we searched for its homologs of *CBF*. One of the *D. antarctica CBF* homologs shared considerable sequence identity with *TmCBF7* of wheat [19] and was named *DaCBF7* (*D. antarctica* CRT Binding Factor 7). The coding region of *DaCBF7* is 813 bp encoding 271 amino acids (28.4 kDa) (Fig. 1A). DaCBF7 contained a single AP2 domain in its central region, which is known to function as a DNA-binding domain in plants, and a putative nuclear localization signal (NLS). Phylogenetic analysis revealed that monocot CBF members are divided into five different groups (Fig. 1B and Fig. S1). DaCBF7 belongs to group V, which also contains homologs of rice (Os04g46400), barley (HvCBF7), and wheat (TmCBF7) (Fig. 1B and Fig. S1). These group V CBFs are structurally similar, with approximately 68–72% amino acid sequence identity, while they are considerably dissimilar to the CBF proteins in other groups.

Multiple alignment of amino acid sequences revealed that the AP2 domain in DaCBF7 was highly conserved in various AP2/ERF transcription regulators in monocot crops, including wheat, barley, and rice (Fig. 1C). Two typical CBF signature sequences at immediate upstream and downstream of the AP2 domain have shown rather low similarity with previously known dicot CBFs [14]. Additional feature of DaCBF7 is that the upstream signature sequence is split into two by a polypeptide stretch composed of 43 amino acids. The stretch is a distinct feature of group V monocot CBFs, despite its possible role is currently unknown (Fig. 1C).

3.2. *DaCBF7 is a nuclear-localized transcription factor that interacted with the CRT/DRE sequence in vitro*

To determine the cellular localization of DaCBF7, an in vivo subcellular targeting experiment was performed. The *sGFP* gene was fused to the 3′ end of the *DaCBF7* coding region in-frame under the control of the CaMV 35S promoter. The *DaCBF7-sGFP* and *NLS-mRFP* constructs were co-expressed in tobacco (*N. benthamiana*) leaves using an *Agrobacterium*-mediated infiltration method. NLS-mRFP was used as a nuclear marker protein. Protoplasts were extracted and visualized by fluorescence microscopy. Fig. 2A reveals that the fluorescence signal of sGFP was uniformly distributed throughout the cell. In contrast, the DaCBF7-sGFP fusion protein was predominantly found in the nucleus and its signal merged with that of NLS-mRFP (Fig. 2A).

It has been shown that CBF/DREB acts as a transcriptional factor that binds to the CRT/DRE sequence of stress-related downstream genes [9,11]. Previous results showed that the *DaADF* gene encoding an actin depolymerizing factor-like protein was highly up-regulated by cold stress in *D. antarctica* [34]. The promoter region of *DaADF* contained several *cis*-acting elements related to abiotic stress, including the CRT/DRE core sequence (ACCGAC). This collection of information led us to analyze the DNA binding specificity of DaCBF7 to wild-type and base-substituted CRT/DRE sequences in the DaADF promoter by gel retardation assay. When probed with wild-type, M1, and M9 probes, all of which possessed the intact CRT/DRE sequence, DaCBF7 gave rise to a high-molecular-weight nucleoprotein complex (Fig. 2B). In contrast, DaCBF7 did not form this complex with six different mutated versions (M2–M7) of the CRT/DRE motif, while DaCBF7 showed a background level of interaction with M2 (TCCGAC) and M8 (ACCGAT) probes. These results suggested that DaCBF7 contains DNA binding activity that interacts specifically with the CRT/DRE sequence in vitro.

3.3. *DaCBF7 was induced in response to cold, high salinity, and drought treatments in* D. antarctica

Since many *CBF* genes are induced in response to abiotic stresses in plants [14,18], we wished to know if *DaCBF7* transcript level is modulated by abiotic stresses in the Antarctic hairgrass. The *DaCBF7* mRNA expression profile under abiotic stress treatment was analyzed by real-time qRT-PCR. *D. antarctica* was collected near the King Sejong Antarctic Station, cultured in vitro, and subjected to various abiotic stresses. As shown in Fig. 2C, the *DaCBF7* transcript was induced in response to cold temperature (4 °C). *DaCBF7* was already up-regulated by 30 min after cold treatment, reached a maximum at 24 h, and then declined. *DaCBF7* was also induced by dehydration and salinity stress, although less so than by cold treatment (Fig. 2C). The *DaIRIP*, *DaP5CS*, and *DaDHN1* genes were used as positive controls for cold, salt, and drought stresses, respectively [34]. As previously found, these genes were markedly induced by stress treatments (Fig. 2C).

3.4. *Generation and molecular analysis of Ubi:DaCBF7 transgenic rice plants*

We next wished to investigate the cellular roles of DaCBF7. Rice, a major food crop worldwide, was a logical choice for this purpose as it is a monocot model plant and belongs to the same family as *D. antarctica*. We generated transgenic rice plants in which the *DaCBF7* gene was constitutively overexpressed under the control of the maize ubiquitin (*Ubi*) promoter (Fig. 3A). RT-PCR analysis indicated that *DaCBF7* transcripts were highly detectable in different T2 transgenic lines under normal growth conditions (Fig. 3B). DNA gel-blot analysis indicated that these *Ubi:DaCBF7* transgenic plants were independent lines (Fig. 3C). Constitutive expression of the *CBF* genes in *Arabidopsis* often caused a dwarf phenotype under normal growth conditions [35,36]. We examined the growth phenotype of *Ubi:DaCBF7* transgenic rice plants. As shown in Fig. 3D, the *Ubi:DaCBF7* transgenic line #1, with two copies of the transgene, lines #2 and #10, with a single copy, and line #3, with three copies, had wild-type vegetative growth under our experimental conditions.

3.5. *Effect of overexpression of DaCBF7 on abiotic stress tolerance in transgenic rice plants*

As *DaCBF7* was induced by abiotic stress, we next subjected wild-type and transgenic rice plants to cold, drought, and salinity stresses and assessed the effect of constitutive expression of *DaCBF7* on stress tolerance. Both wild-type and *DaCBF7*-overexpressing plants were grown at 28 °C for 6 weeks in the growth room and transferred to the cold room at 4 °C. After 8 days of cold stress, wild-type plants were wilted and unable to recover after removal from stress, while most of the *Ubi:DaCBF7* transgenic rice plants recovered, appeared healthy, and continued to grow (Fig. 4A). The survival rate of the wild-type rice plants was $11.4 \pm 8.6\%$, and the survival rates of four independent *Ubi:DaCBF7* lines #1, #2, #3, and #10 were 57.0 ± 15.2, 27.0 ± 5.3, 53.9 ± 0.9, and $79.4 \pm 7.6\%$, respectively (Fig. 4B). Electrolyte leakage was also measured from leaves of 10-day-old cold-stressed plants. The results are consistent with those from whole plants: leaves from transgenic lines #1 and #10 had lower rates of electrolyte leakage than wild-type leaves in response to low temperature (Fig. 4C). These results suggest that the *DaCBF7*-overexpressing transgenic rice plants were more tolerant to severe cold stress than wild-type plants.

We showed earlier that the *DaCBF7* transcript was induced by drought and salinity stress, although less so than by cold stress (Fig. 2C). Therefore, it was of interest to assess the tolerance of wild-type and *Ubi:DaCBF7* rice plants to these stresses. Six-week-old wild-type and T2 *Ubi:DaCBF7* (independent lines #1 and #10) plants were further grown without a water supply for 9 days. Both wild-type and *DaCBF7*-overexpressing plants displayed severe wilting and had discolored, pale green leaves (Fig. S2A). After re-watering, most plants did not resume growth and eventually died. In addition, the rate of water loss from detached, mature leaves was indistinguishable between the two groups during 6 h incubation at room temperature under dim light (Fig. S2B). Thus, essentially no phenotypic differences in terms of drought resistance were detectable between wild-type and *Ubi:DaCBF7* plants.

Next, salt tolerance of wild-type and T2 transgenic lines was compared. Six-week-old plants were irrigated with water supplemented with 200 mM NaCl for 10 days, re-watered with tap water, and their growth patterns were observed. Under salinity stress, both wild-type and transgenic plants were almost completely bleached and unable to recover (Fig. S2C). Overall, these results suggested that constitutive expression of *DaCBF7* increased tolerance of rice to cold stress but had

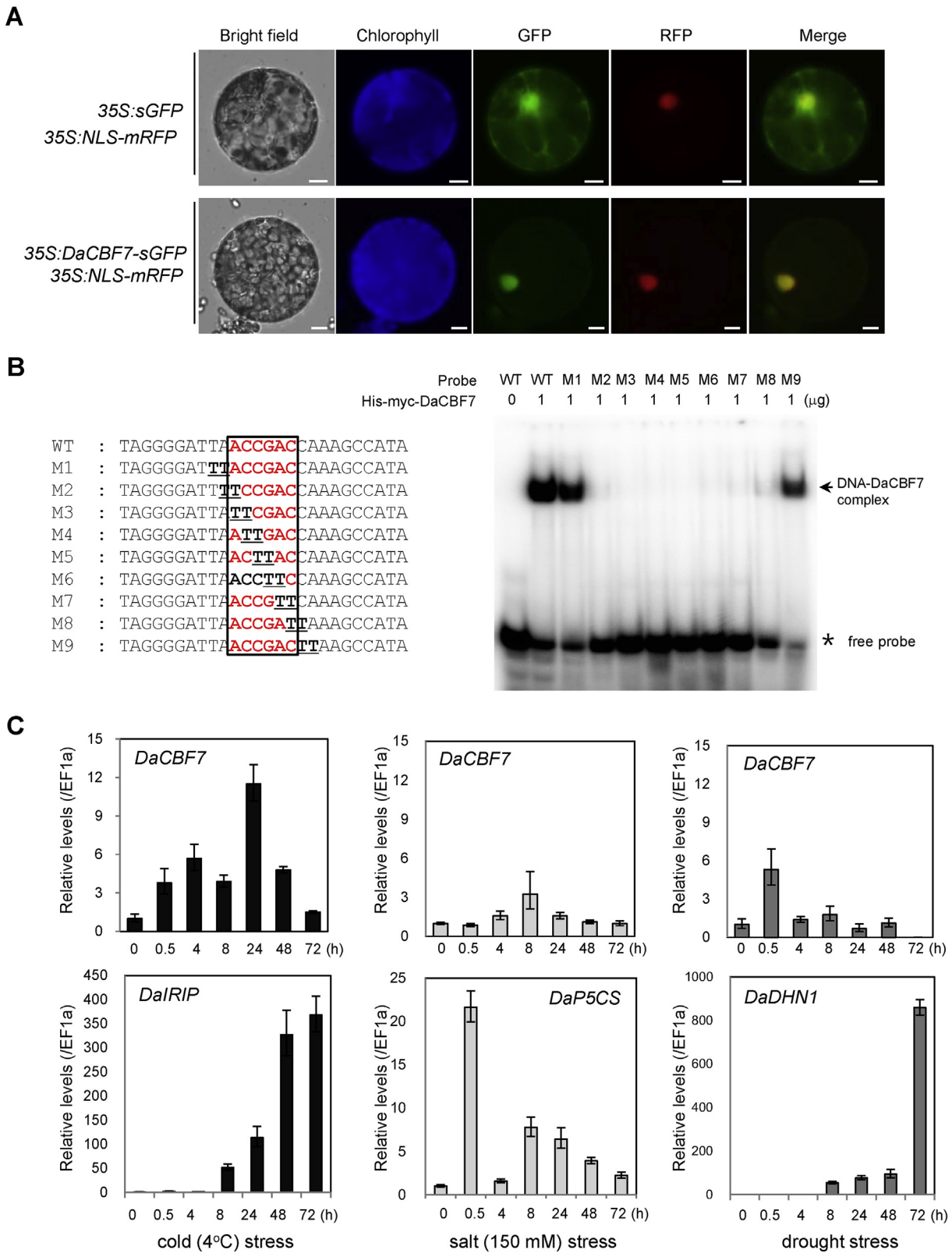


Fig. 2. Molecular characterization of DaCBF7. (A) Subcellular localization of DaCBF7. Tobacco (*N. benthamiana*) leaves were co-infiltrated using *Agrobacterium* that contained the *35S:DaCBF7-sGFP* or *35S:NLS-mRFP* construct. NLS-mRFP was used as a nuclear marker protein. Scale bar: 5 μm. (B) Gel retardation assay of DaCBF7. A series of mutants of the CRT/DRE motif were prepared and analyzed by gel retardation assays. Each probe possessed dinucleotide substitutions as indicated. Wild-type (WT) indicates intact (ACCGAC) CRT/DRE core sequence. M1–M9 indicate mutated CRT/DRE sequences. (C) Real-time qRT-PCR analysis of *DaCBF7*. Induction profiles of *DaCBF7* transcript in response to different abiotic stresses, including cold, high salinity, and drought, were monitored in *D. antarctica*. Relative transcript abundance was calculated and normalized with respect to the internal *DaEF1a* transcript level. Error bars indicate ±SD from three independent experiments. The *DaIRIP*, *DaP5CS*, and *DaDHN1* genes were positive controls for cold, high salt, and drought stresses, respectively. DNA sequences of gene-specific primers used for qRT-PCR are shown in Table S1.

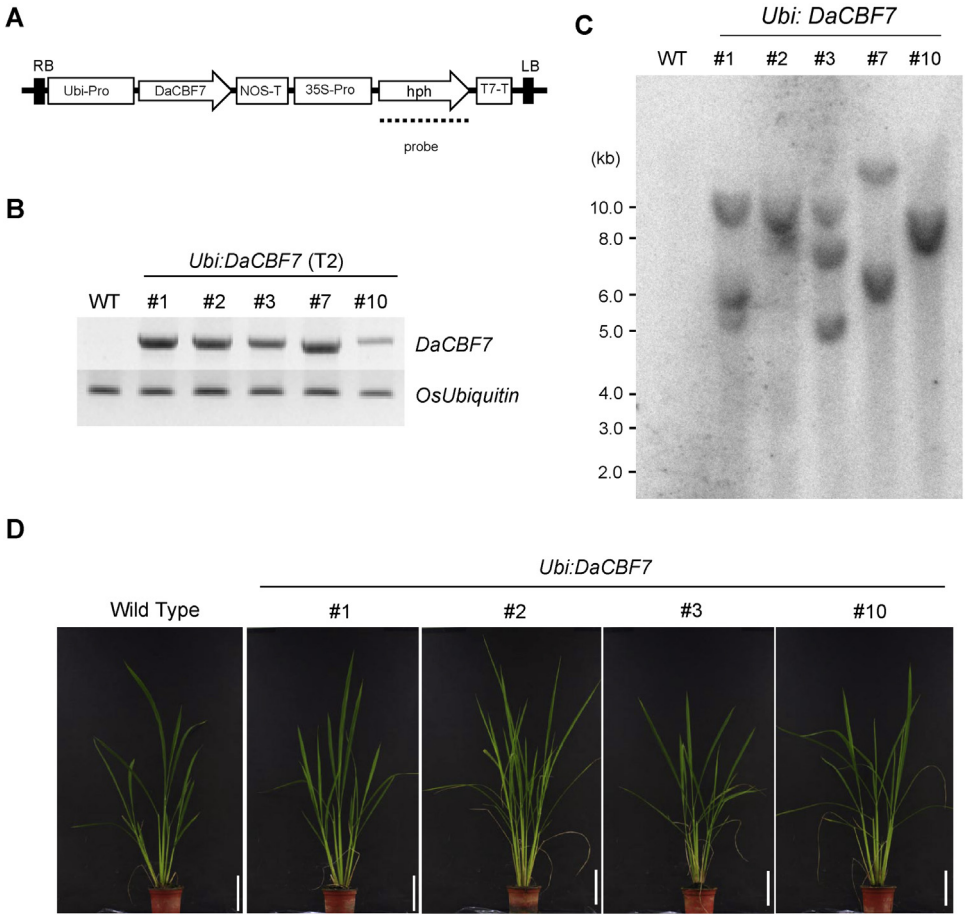


Fig. 3. Generation and molecular characterization of *DaCBF7*-overexpressing transgenic rice plants. (A) Schematic representation of the binary vector pGA2897 used for *DaCBF7* overexpression under the control of the maize ubiquitin promoter. RB, right border; Ubi-Pro, ubiquitin promoter; NOS-T, terminator sequence from nopaline synthase gene; 35S-Pro, CaMV 35S promoter; hph, hygromycin B phosphotransferase; T7-T, T7 terminator; LB, left border. (B) RT-PCR analysis of 6-week-old wild-type and five independent *Ubi:DaCBF7* T2 transgenic plants (lines #1, #2, #3, #7, and #10). (C) Genomic Southern blot analysis. Total leaf genomic DNA was isolated from wild-type and T2 *Ubi:DaCBF7* transgenic rice plants. DNA was digested by *Bam*HI and hybridized with ^{32}P-labeled hph probe under high-stringency conditions. (D) Overall morphology of 2-month-old wild-type and T2 *Ubi:DaCBF7* transgenic (independent lines #1, #2, #3, and #10) rice plants. Rice plants were grown under greenhouse conditions. Scale bar: 10 cm.

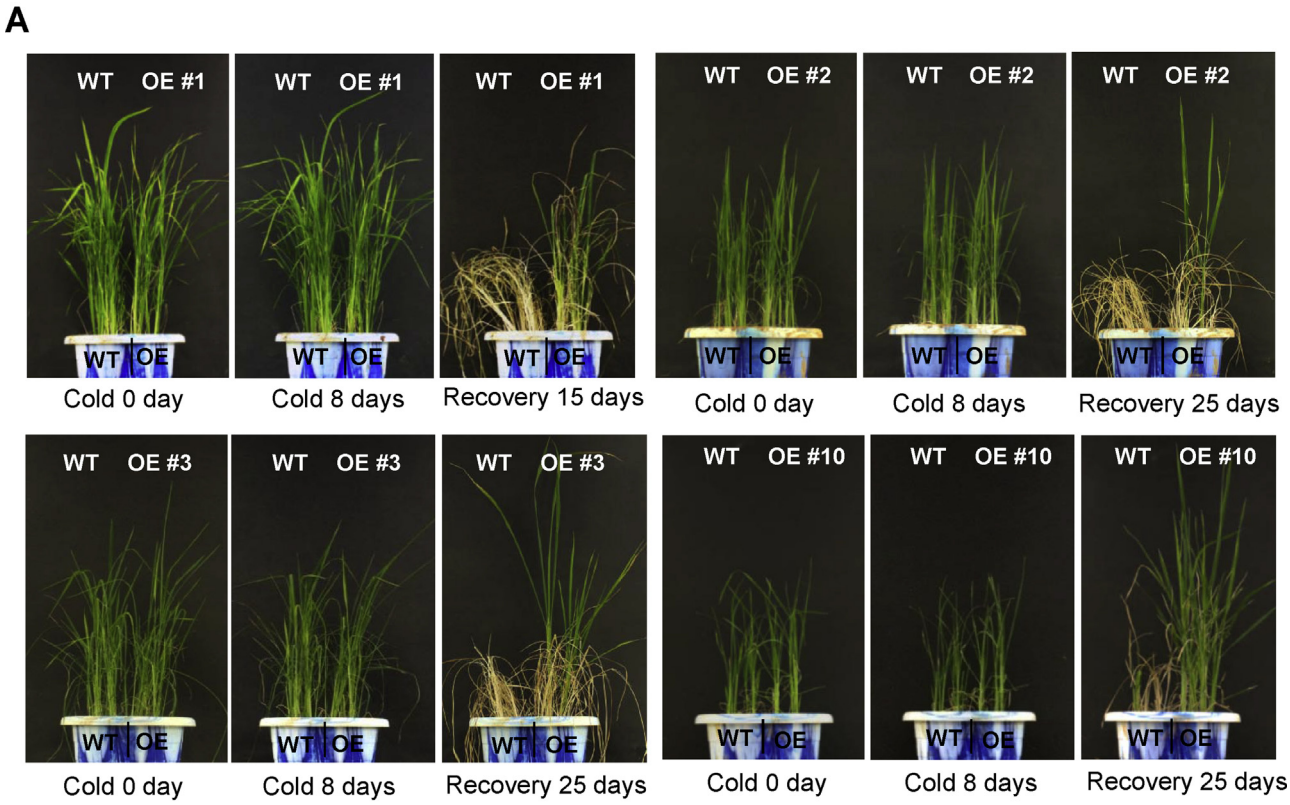
little or no effect on its tolerance to dehydration and salinity stress.

3.6. Transcriptome analysis of Ubi:DaCBF7 transgenic line under cold stress

Our observation that rice plants overexpressing *DaCBF7* were cold-tolerant (Fig. 4), together with the established role of CBFs as transcription factors that induce stress-related genes, points to the possibility that *DaCBF7* altered stress-related gene expression in transgenic rice plants. Therefore, we next compared transcription in wild-type and transgenic plants under normal and cold-stress conditions. Specifically, we conducted transcriptome analysis using an RNA sequencing (RNA-seq) technique [37]. Total RNAs were prepared from 6-week-old leaves of wild-type and *Ubi:DaCBF7* (transgenic line #10) plants grown under normal (before cold treatment) and cold-stress (1 day and 6 days treatments at 4 °C) conditions and used to construct a sequencing library. Transgenic line #10 was chosen for RNA-seq analysis because it displayed the highest survival rate after cold treatment among the lines tested (Fig. 4). The sequencing was performed and the raw reads were mapped to the rice reference genome. The expression values were measured in reads per kilobase of exon model per million mapped reads (RPKM) and the differentially expressed genes (DEGs) were determined from a cutoff value (p-value < 0.05, corrected p-value of FDR < 0.05).

Analysis of DEGs revealed that, under normal growth conditions, 245 genes were up-regulated and 124 genes were down-regulated in the transgenic line #10 compared to wild-type rice plants (Fig. 5A and Table S3). This indicated that constitutive expression of *DaCBF7* altered the gene expression profile before cold treatment in transgenic rice plants. After 1 day of cold stress, 251 genes were up-regulated and 90 genes were down-regulated in the transgenic plants compared to the wild-type (Fig. 5A). After 6 days of cold stress, 158 genes were up-regulated and 462 were down-regulated in the transgenic line compared to the wild-type plant (Fig. 5A).

Comparative GO analysis within a category “biological processes” was conducted with selected DEGs and the set of whole rice genome. As a result, GO terms of metabolic process, biosynthetic process, response to abiotic stimulus, gene expression, response to biotic stimulus, and photosynthesis were enriched in selected DEGs (Fig. 5B and Table S4). Of these terms, only the GO term of photosynthesis contained more up-regulated genes than down-regulated genes in *Ubi:DaCBF7* rice plants. While the GO term of signal transduction was not enriched in DEGs, it contained several genes encoding calmodulin and calcium/calmodulin-dependent protein kinases (CDPKs), and mitogen-activated protein kinases (MAPKs)



B

Genotype	Survival rate
Wild Type	11.4 ± 8.6% (n=7)
Ubi:DaCBF7 #1	57.0 ±15.2% (n=5)
Ubi:DaCBF7 #2	27.0 ± 5.3% (n=4)
Ubi:DaCBF7 #3	53.9 ± 0.9% (n=3)
Ubi:DaCBF7 #10	79.4 ± 7.6% (n=3)

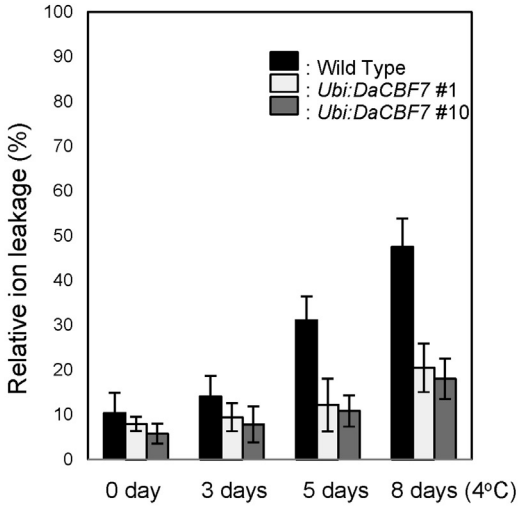
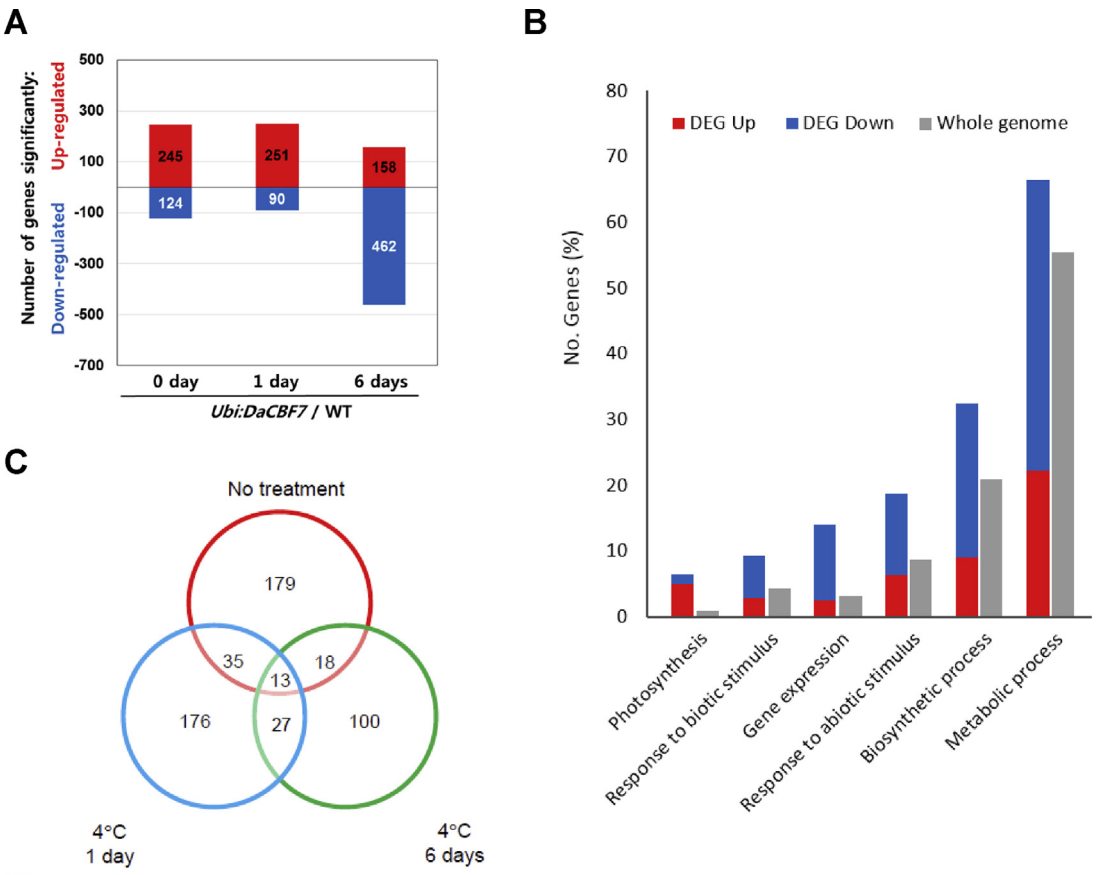


Fig. 4. Comparison of cold-stress tolerance between wild-type (WT) and *Ubi:DaCBF7* transgenic plants. (A) Six-week-old wild-type (WT) and T2 *Ubi:DaCBF7* (line #1, #2, #3 and #10) rice plants were subjected to cold treatment at 4 °C for 8 days, recovered to normal condition at 28 °C, and then compared their survival rates. (B) Survival rates of WT and four independent transgenic lines under cold-stress conditions. Results are expressed as mean ± SD from three to seven independent experiments. *n* = number of independent experiments. (C) Effect of cold-stress duration on electrolyte leakage of WT and *DaCBF7*-overexpressing transgenic (independent lines #1 and #10) plants. All data represent mean ± SD, *n* = three independent experiments.

(Table S5). Interestingly, their gene expression patterns were different in wild-type and *Ubi:DaCBF7*. Whereas their expression in wild-type showed a rapid increase between 1 day and 6 days, in a *Ubi:DaCBF7* plants, their expression showed a tendency to increase very slowly over a period from 1 day to 6 days (Fig. S3 and Table S5).

A Venn diagram revealed that among the up-regulated genes, 13 were more highly expressed in *DaCBF7*-overexpressing plants than in wild-type plants in all three treatments (normal conditions, 1 day cold treatment, and 6 days cold treatment) (Fig. 5C). These genes encode the stress-related proteins (dehydrin and remorin), the putative chaperone proteins [T-complex protein and FAD-dependent oxidoreductase (FOXRED1)], the metabolic enzyme acireductone dioxygenase (OsARD1; 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase), and eight proteins (Os02g57924, Os03g18779, Os03g63870, Os04g01330, Os07g05840, Os10g22630, Os10g24004, and Os11g34790) with unknown functions (Fig. 5D).



D

Gene ID	Annotation	Expression Level (RPKM)						Expression Pattern	
		Wild type			*Ubi:DaCBF7*			Wild type	*Ubi:DaCBF7*
		0d	1d	6d	0d	1d	6d		
LOC_Os03g18779	expressed protein	0.1	0	0.1	24.1	62.1	61.7		
LOC_Os02g57924	expressed protein	0	0	0	26.9	29.1	80		
LOC_Os03g63870	expressed protein	0	0	0.1	25.4	26.4	12.7		
LOC_Os04g01330	expressed protein	0.1	0.3	0	64.4	115.8	32.3		
LOC_Os07g05840	expressed protein	0	0	0	57.3	49.9	15.8		
LOC_Os10g22630	expressed protein	0.4	0.4	0.3	175	155.8	51.3		
LOC_Os07g38170	remorin	16.3	169.6	37	51.9	504.2	165.9		
LOC_Os10g24004	expressed protein	0	0	0	92	124.8	43.6		
LOC_Os10g28350	1,2-dihydroxy-3-keto-5-methyl-thiopentene dioxygenase protein	2.8	1	0	33.4	29	14		
LOC_Os11g34790	expressed protein	0	0	0	45.9	15	32.5		
LOC_Os06g02380	T-complex protein	256.2	273.5	129.8	441	396.1	226.1		
LOC_Os07g34570	FAD dependent oxidoreductase domain containing protein	4534	4290	306.6	4857	5845	521.3		
LOC_Os03g45280	dehydrin	1492	1049	285	1905	1432	487.4		

Fig. 5. Transcriptome analysis with RNA-seq of the cold-stress response of *Ubi:DaCBF7* transgenic plants. (A) Number of differentially expressed genes in *Ubi:DaCBF7* line #10 relative to the expression level of wild-type (WT) at each time point (0, 1, and 6 days) of cold treatment ($p < 0.05$, corrected p-value of FDR < 0.05). (B) Functional GO classification of DEGs by plant GO slim terms. Only GO terms of DEGs with significance (corrected p-value of FDR < 0.05) are presented when comparing those of rice whole genome. (C) A Venn diagram analysis of up-regulated genes in *Ubi:DaCBF7* and WT rice plants for different durations of cold treatment. A Venn diagram revealed that, among diverse up-regulated genes, 13 genes were more highly expressed in *DaCBF7*-overexpressing plants than in WT plants in all cold treatment durations (0, 1, and 6 days) tested. (D) Expression levels of the 13 selected genes from RNA-seq analysis in WT and *Ubi:DaCBF7* transgenic line #10 under normal and cold-stress conditions. The expression values were represented in reads per kilobase of exon model per million mapped reads (RPKM). The trends of expression changes over the period from 0 day to 6 days of cold treatments were indicated using spark-line chart of MS Excel program.

3.7. qRT-PCR and gel retardation analyses of DaCBF7-induced genes in Ubi:DaCBF7 transgenic rice plants

Promoter analysis indicated that, among the 13 genes in *Ubi:DaCBF7* transgenic line #10 that were consistently induced, *dehydrin* (*Os03g45280*), *Os03g63870*, *Os11g34790*, and *Os10g22630* contain putative CRT/DRE *cis*-acting elements in their promoter regions (Fig. 6A). In addition, the upstream region of the *remorin* gene (*Os07g38170*) has a putative low-temperature-responsive element (LTRE) sequence (CCGAC) [38,39]. Thus, DaCBF7 may directly activate expression of these five target genes in *Ubi:DaCBF7* plants. To investigate this possibility, qRT-PCR was conducted. Wild-type and T2 *Ubi:DaCBF7* (independent lines #1 and #10) plants were subjected to cold stress (4 °C) for 1 and 6 days. Total leaf RNA was isolated and analyzed by real-time qRT-PCR using gene-specific primers (Table S1). The results in Fig. 6B show that expressions of all five genes were higher in the two *Ubi:DaCBF7* lines than in wild-type plants, both under normal and cold-stress conditions (Fig. 6B).

Next, bacterially expressed His-myc-DaCBF7 recombinant protein was used in a gel retardation assay with ^{32}P-labeled promoter regions that contained the CRT/DRE for the *dehydrin* (*Os03g45280*), *Os03g63870*, *Os11g34790*, and *Os10g22630* genes or the LTRE for the *remorin* (*Os07g38170*) gene. As shown in Fig. 6C, with all five promoter regions, the His-myc-DaCBF7 protein produced a discrete DNA–protein complex that migrated more slowly than the free probe. The DNA binding specificity of His-myc-DaCBF7 was confirmed by a competition assay, which displayed that a 100-fold excess of unlabeled probe completely displaced the labeled probe. However, plant telomeric repeats (PTRs) did not compete with the labeled probe, implying that DaCBF7 binds specifically to the CRT/DRE or LTRE sequence of the target gene promoter in vitro. In their entirety, the results presented in Fig. 6 are consistent with the conclusion that overexpression of *DaCBF7* induced the CRT/DRE and LTRE-motif containing genes and consequently enhanced tolerance to cold stress in transgenic rice plants.

4. Discussion

The Antarctic hairgrass *D. antarctica* has successfully adapted to the freezing temperatures of its habitat, the west coast of the Antarctic peninsula. In this report, we have identified DaCBF7 in *D. antarctica*, a member of the monocot group V CBF homologs. Previously, the phylogenetic relationship between cereal CBF family members were investigated mainly with wheat and barley CBF sequences. They were divided into three major subgroups in barley [17] and four subgroups in wheat [29]. As molecular data for cereal plant species grow bigger, we constructed phylogenetic tree with more sequence data. DaCBF7, together with Os04g46400 (rice), HvCBF7 (barley), and TmCBF7 (wheat), was structurally distant to typical CBF subgroups and they were designated as a new clade, group V (Fig. 1B and Fig. S1).

Various plant CBF proteins are known to have conserved signature sequences at the immediate upstream of AP2 domain [14]. Multiple sequence alignment shows that all group V CBF proteins have an unusual insertion of amino acid stretch to divide the upstream signature sequence (Fig. 1C). This region might function in regulation of DNA-binding ability or protein–protein interaction to regulate substrate specificity, still the intensive biochemical experiments with deletion mutants should be carried out to reveal the cellular function of this region.

Because there was no report on molecular and cellular function of group V CBF homologs, we have investigated a possible cellular role of DaCBF7 in cold tolerance by using a transgenic rice system. Progeny of *DaCBF7*-overexpressing transgenic rice plants (*Ubi:DaCBF7*) were markedly more cold-tolerant than the wild-type (Fig. 4), but not more drought- or salt stress-tolerant (Fig. S2). This differential stress tolerance was somewhat unexpected, because *DaCBF7* was up-regulated by high salinity and dehydration as well as cold stress in *D. antarctica* (Fig. 2C). DaCBF7 and other group V CBF homologs contain a distinct array of 43 amino acid residues in front of the AP2 motif (Fig. 1C). Thus, it is of interest to examine that the group V CBF-specific N-terminal short amino acid sequences are involved in the response to cold stress.

The cellular function of *CBF* homologs has been investigated by transgenic approaches. Several experiments have shown that overexpression of some cereal *CBF*s improved tolerance of transgenic rice and barley plants to various abiotic stresses, offering the practical biotechnology for crop engineering. Constitutive expression of wheat *TaDREB2*, *TaDREB3*, *TaCBF14*, or *TaCBF15* increased freezing tolerance in transgenic barley [40–42]. Overexpression of barley *HvCBF4* resulted in increased tolerance to low temperature, drought, and high salinity with a cultivar-specific manner in rice [43,44]. Besides the enhanced stress tolerance, overexpression of various *CBF* genes often resulted in pleiotropic effects with retarded growth and delayed flowering in *Arabidopsis*, rice, and barley plants [40–42,45–47]. In this study, we observed that *DaCBF7*-overexpressing transgenic rice plants had strong cold tolerant phenotype without any growth defects, suggesting that extremophile-isolated genes would be potent candidates as genetic resources for crop engineering.

Regarding the issue on the reason for variation of growth retardation depending on cases, it was suggested that the phenotypic differences could be due to the differences of rice cultivars used or the difference of transgenic generations investigated [43,45]. Although these explanations appeared to be reasonable, molecular clues causing growth retardation or dwarfism in *CBF*-overexpressing plants were still missing. Vogel et al. [47] performed microarray experiment with *CBF2*-overexpressing *Arabidopsis* plants showing severe dwarfism and delayed flowering in normal condition. A total of 43 genes, including *At4g31500* (cytochrome P450) and *At4g14400* (hypothetical protein), were significantly down-regulated in transgenic lines compared to wild-type plants. Consistently, loss-of-function mutations on *At4g31500* and *At4g14400* caused severe dwarfism in *Arabidopsis* [48,49]. However, *Os12g16720* and *Os07g34830*, rice homologs to *Arabidopsis At4g31500* and *At4g14400*, respectively, are not significantly regulated in *DaCBF7*-overexpressing transgenic rice plants in this study. The differences in a range of CBF regulons in different experimental cases could be a cause of phenotypic differences on dwarfism or growth retardation.

Among DEGs comprising the GO term of signal transduction in this study, genes encoding calmodulin, CDPKs, and MAPKs showed down-regulation in *Ubi:DaCBF7* transgenic lines relative to wild-type rice plants (Fig. S3 and Table S5). In higher plants, complex interactions have been observed between CDPKs and MAPK cascades to provide fine-tuning of downstream processes involved in signaling networks to regulate diverse cellular processes, including response to biotic/abiotic stresses [50]. Besides well-known positive roles in plant defense, constitutive expression of these kinase triggered hypersensitive response-like cell death or increased ROS production in tobacco, *Arabidopsis*, and sorghum [51–54]. Based on these results, we speculated that lower expression levels of MAPKs and CDPKs in *Ubi:DaCBF7* lines relative to wild-type plants might be correlated with increased tolerance to cold stress (Fig. 4A).

RNA-seq analysis identified 13 genes that were up-regulated in *DaCBF7*-overexpressing lines compared to wild-type rice plants; this was true in both normal and cold conditions (Fig. 5C). Among these 13 selected genes, four of them (*dehydrin*, *Os03g63870*, *Os11g34790*, and *Os10g22630*) contained CRT/DRE *cis*-acting

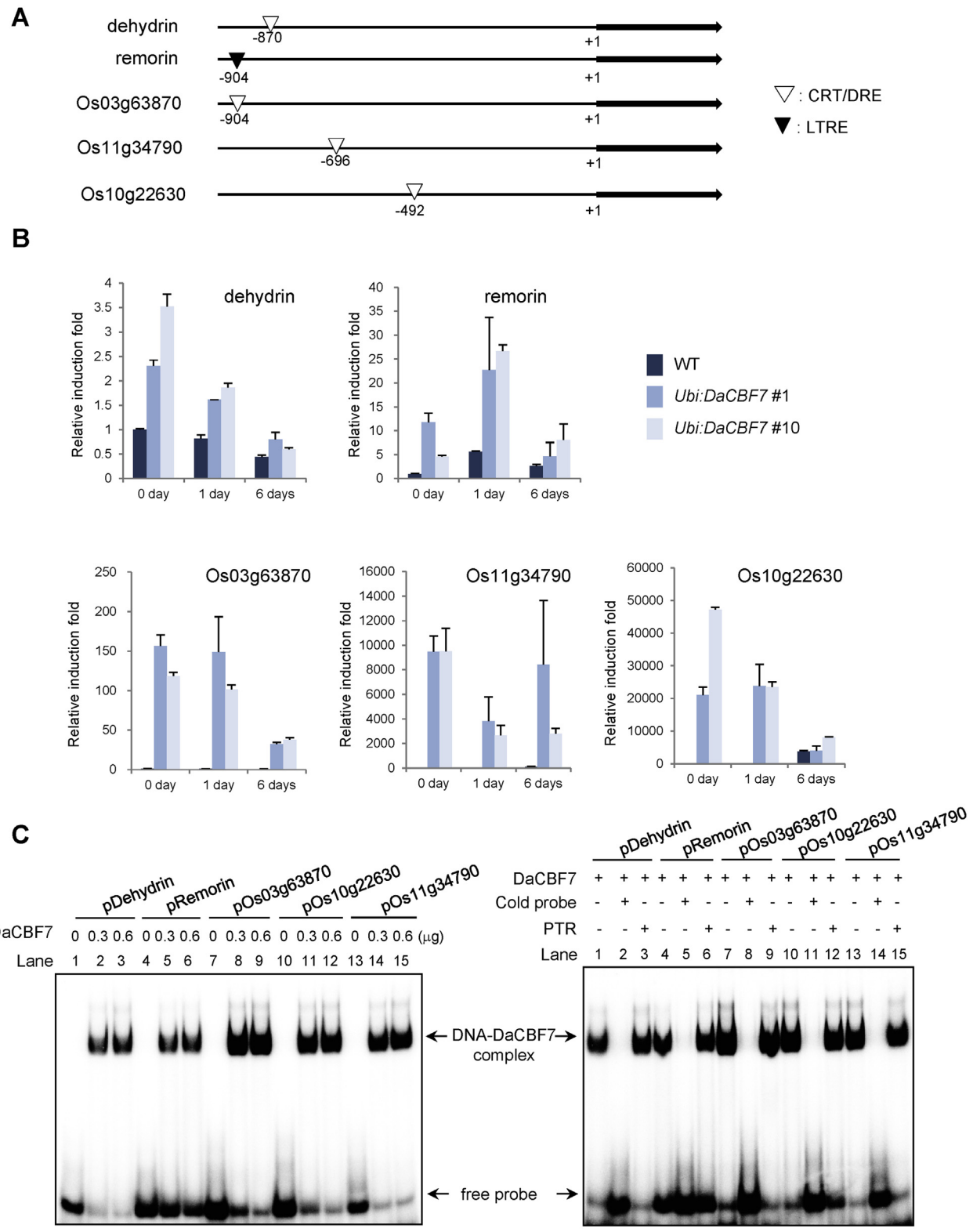


Fig. 6. qRT-PCR and gel retardation analyses of five *DaCBF7*-induced target genes in *Ubi:DaCBF7* transgenic rice plants. (A) Schematic presentation of five *DaCBF7*-induced target genes. +1 indicates a transcription initiation site. Putative CRT/DRE and LTRE motifs are indicated by ▽ and ▼, respectively. (B) Real-time qRT-PCR analysis of *DaCBF7* target genes in wild-type (WT) and T2 *Ubi:DaCBF7* (independent lines #1 and #10) plants that had been subjected to cold stress for 1 or 6 days. Total leaf RNA was analyzed by real-time qRT-PCR using gene-specific primers (Table S1). The graphs indicate a relative induction fold of the *dehydrin*, *remorin*, *Os03g63870*, *Os10g22630*, and *Os11g34790* genes as compared to the control treatment (WT with no cold-stress treatment). The mean value of three technical replicates was normalized to the levels of *OsActin* mRNA, an internal control. (C) Gel retardation assays. (Left panel) Gel retardation assays were performed with the ^{32}P-radiolabeled promoter sequence of *dehydrin* (lanes 1–3), *remorin* (lanes 4–6), *Os03g63870* (lanes 7–9), *Os10g22630* (lanes 10–12), or *Os11g34790* (lanes 13–15). Each set of lanes contained 0, 0.3, or 0.6 μg of bacterially expressed, full-length His-myc-DaCBF7 recombinant protein, respectively. (Right panel) Sequence-specific binding activity of His-myc-DaCBF7 to target gene promoters. Full-length, His-myc-DaCBF7 protein (0.6 μg) was added to each reaction mixture. Gel retardation assays were performed with the ^{32}P-radiolabeled promoter sequence of *dehydrin* (lanes 1–3), *remorin* (lanes 4–6), *Os03g63870* (lanes 7–9), *Os10g22630* (lanes 10–12), or *Os11g34790* (lanes 13–15), and titrated with cold probe (lanes 2, 5, 8, 11, and 14, respectively) or with cold PTR as a competitor (lanes 3, 6, 9, 12, and 15, respectively).

elements in their promoter regions and the *remorin* gene harbored an LTRE sequence (Fig. 6A).

Dehydrins are group 2 LEA (late embryogenesis abundant) proteins that accumulate during seed maturation [55]. In vegetative tissues, expression of *dehydrin* was induced in response to salinity, dehydration, cold, and freezing stresses [55]. Several studies report a positive role for dehydrin in tolerance to plant stress. For example, transgenic rice plants overexpressing wheat dehydrin PMA80 showed improved dehydration tolerance [56]. Transgenic *Arabidopsis* plants that constitutively expressed wheat dehydrin DHN-5 had increased tolerance to salt and osmotic stress [57]. It was recently reported that over-expression of rice dehydrin *OsDhn1* resulted in enhanced tolerance to drought and high-salinity stresses by reducing reactive oxygen species in rice plants [25]. In addition, overexpression of dehydrin elevated freezing tolerance in transgenic tobacco and strawberry plants [58,59]. In our study, qRT-PCR analysis revealed that expression levels of dehydrin in *DaCBF7*-overexpressing transgenic lines were two- to three-fold higher than those in wild-type plants (Fig. 6B), suggesting a role of dehydrin in cold tolerance in *Ubi:DaCBF7* rice plants. On the other hand, the *Os03g63870*, *Os11g34790*, and *Os10g22630* genes were greatly up-regulated in *Ubi:DaCBF7* lines relative to wild-type plants (Fig. 6B). It also appeared that bacterially expressed DaCBF7 interacted more strongly with the upstream regions of *Os03g63870*, *Os11g34790*, and *Os10g22630* than that of *dehydrin* in vitro (left panel in Fig. 6C). Based on these results, we speculated that these unknown proteins are more closely correlated with cold stress tolerance of transgenic *Ubi:DaCBF7* rice plants than of dehydrin.

Remorins are a multi-gene family found in all land plants. Transcriptome and proteome analyses suggested that the *remorin* genes were induced by a broad spectrum of biotic and abiotic stresses [60–62]. Heterologous expression of mulberry and foxtail millet remorins in *Arabidopsis* resulted in elevated tolerance to dehydration and high salinity [63,64]. In addition, *remorin* was up-regulated in *CaPIF1* (*Capsicum annuum* Pathogenesis Induced Factor 1)-overexpressing transgenic tomato plants with increased tolerance to cold stress and pathogen attack [65]. These studies indicated that remorins participate in the response of plants to diverse environmental stresses. Expression levels of *remorin* in *Ubi:DaCBF7* transgenic rice plants were 5–10-fold higher than those in wild-type plants in both normal and cold-stress conditions (Fig. 5D and 6B). Furthermore, DaCBF7 bound to the upstream region of remorin, which has a putative LTRE (Fig. 6C). Thus, it appeared that DaCBF7 was able to interact with both CRT/DRE and LTRE sequences in transgenic rice plants. Overall, these results suggest that DaCBF7 directly enhanced expression of these CRT/DRE- and LTRE-containing target genes in transgenic rice plants, which in turn resulted in enhanced tolerance to cold stress.

In addition, RNA-seq analysis identified eight other genes up-regulated in the transgenic line in normal and cold-stress conditions. These included two putative chaperones [T-complex protein and FAD dependent oxidoreductase (FOXRED1)], acireductone dioxygenase (OsARD1), and 5 unknown genes (*Os02g57924*, *Os03g18779*, *Os04g01330*, *Os07g05840*, and *Os10g24004*) (Fig. 5D). In yeast and animal systems, T-complex protein is associated with newly synthesized proteins, including actin and tubulin, and promotes their proper folding to the native state [66]. FOXRED1 is a mitochondrial complex-I-specific molecular chaperone in human [67]. Human FOXRED1 homologs were reported to participate in redox reactions [68]. Acireductone dioxygenases are unique proteins that can perform two different activities, which depend on whether they bind to iron or nickel ions. For example, OsARD1 binds to iron and catalyzes the formation of α-ketomethylthiobutyrate in the methionine cycle, which is involved in recycling of the ethylene precursor *S*-adenosylmethionine [69]. However, the possible roles of these two putative chaperone proteins and the acireductone dioxygenase enzyme in the cold-stress response are unknown. Promoter regions of these three genes, as well as the five unknown induced genes (*Os02g57924*, *Os03g18779*, *Os04g01330*, *Os07g05840*, and *Os10g24004*) did not have putative CRT/DRE motifs and thus may have been activated indirectly by overexpression of *DaCBF7* in transgenic line. Therefore, more detailed experiments may reveal the roles of these indirectly induced genes in cold tolerance.

In conclusion, our results show that heterologous expression of Antarctic hairgrass *DaCBF7* results in increased tolerance to low-temperature stress by direct and indirect induction of diverse sets of genes in transgenic rice plants. This is consistent with the notion that CBFs play regulatory roles in cold-stress responses in higher plants.

Acknowledgments

This work was supported by The Antarctic organisms: cold-adaptation mechanisms and its application grant (PE15070), funded by the Korea Polar Research Institute and a grant from the National Center for GM Crops (Project No. PJ011138) of the Next Generation BioGreen 21 Program, funded by the Rural Development Administration, Republic of Korea, to W.T.K.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.03.020.

References

[1] R.I. Lewis Smith, Vascular plants as bioindicators of regional warming in Antarctica, Oecologia 99 (1994) 322–328.
[2] F.S. Xiong, E.C. Mueller, T.A. Day, Photosynthetic and respiratory acclimation and growth response of Antarctic vascular plants to contrasting temperature regimes, Am. J. Bot. 87 (2000) 700–710.
[3] L.A. Bravo, N. Ulloa, G.E. Zuñiga, A. Casanova, L.J. Corcuera, M. Alberdi, Cold resistance in Antarctic angiosperms, Physiol. Plant. 111 (2001) 55–65.
[4] L.A. Bravo, M. Griffith, Characterization of antifreeze activity in Antarctic plants, J. Exp. Bot. 56 (2005) 1189–1196.
[5] U.P. John, R.M. Polotnianka, K.A. Sivakumaran, O. Chew, L. Mackin, M.J. Kuiper, J.P. Talbot, G.D. Nugent, J. Mautord, G.E. Schrauf, G.C. Spangenberg, Ice recrystallization inhibition proteins (IRIPs) and freeze tolerance in the cryophilic Antarctic hairgrass *Deschampsia antarctica* E. Desv, Plant Cell Environ. 32 (2009) 336–348.
[6] M.F. Thomashow, Plant cold acclimation: freezing tolerance genes and regulatory mechanisms, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 571–599.
[7] J.K. Zhu, Salt and drought stress signal transduction in plants, Annu. Rev. Plant Biol. 53 (2002) 247–273.
[8] M. Seki, T. Umezawa, K. Urano, K. Shinozaki, Regulatory metabolic networks in drought stress responses, Curr. Opin. Plant Biol. 10 (2007) 296–302.
[9] M.F. Thomashow, Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway, Plant Physiol. 154 (2010) 571–577.
[10] K. Yamaguchi-Shinozaki, K. Shinozaki, Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses, Annu. Rev. Plant Biol. 57 (2006) 781–803.
[11] J. Mizoi, K. Shinozaki, K. Yamaguchi-Shinozaki, AP2/ERF family transcription factors in plant abiotic stress responses, Biochim. Biophys. Acta 1819 (2012) 86–96.
[12] Q. Liu, M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, K. Shinozaki, 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 (1998) 1391–1406.
[13] K.R. Jaglo-Ottosen, S.J. Gilmour, D.G. Zarka, O. Schabenberger, M.F. Thomashow, Arabidopsis CBF1 overexpression induces *COR* genes and enhances freezing tolerance, Science 280 (1998) 104–106.
[14] K.R. Jaglo, S. Kleff, K.L. Amundsen, X. Zhang, V. Haake, J.Z. Zhang, T. Deits, M.F. Thomashow, Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species, Plant Physiol. 127 (2001) 910–917.

[15] M. Kasuga, S. Miura, K. Shinozaki, K. Yamaguchi-Shinozaki, A combination of the *Arabidopsis* DREB1A gene and stress-inducible *rd29A* promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer, Plant Cell Physiol. 45 (2004) 346–350.
[16] S.J. Oh, S.I. Song, Y.S. Kim, H.J. Jang, S.Y. Kim, M. Kim, Y.K. Kim, B.H. Nahm, J.K. Kim, Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth, Plant Physiol. 138 (2005) 341–351.
[17] J.S. Skinner, J. von Zitzewitz, P. Szucs, L. Marquez-Cedillo, T. Filichkin, K. Amundsen, E.J. Stockinger, M.F. Thomashow, T.H. Chen, P.M. Hayes, Structural, functional, and phylogenetic characterization of a large *CBF* gene family in barley, Plant Mol. Biol. 59 (2005) 533–551.
[18] N. Liu, N.Q. Zhong, G.L. Wang, L.J. Li, X.L. Liu, Y.K. He, G.X. Xia, Cloning and functional characterization of *PpDBF1* gene encoding a DRE-binding transcription factor from *Physcomitrella patens*, Planta 226 (2007) 827–838.
[19] S. Mohseni, H. Che, Z. Djillali, E. Dumont, J. Nankeu, J. Danyluk, Wheat *CBF* gene family: identification of polymorphisms in the CBF coding sequence, Genome 55 (2012) 865–881.
[20] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6. Molecular Evolutionary Genetics Analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[21] M.Y. Byun, J.-P. Hong, W.T. Kim, Identification and characterization of three telomere repeat-binding factors in rice, Biochem. Biophys. Res. Commun. 372 (2008) 85–90.
[22] H. Lee, J.H. Kim, M. Park, I.-C. Kim, J.H. Yim, H.K. Lee, Reference genes validation for qPCR normalization in *Deschampsia antarctica* during abiotic stresses, Antarct. Sci. 22 (2010) 477–484.
[23] M.Y. Byun, W.T. Kim, Suppression of *OsRAD51D* results in defects in reproductive development in rice (*Oryza sativa* L.), Plant J. 79 (2014) 256–269.
[24] H. Bae, S.K. Kim, S.K. Cho, B.G. Kang, W.T. Kim, Overexpression of *OsRDCP1*, a rice RING domain-containing E3 ubiquitin ligase, increased tolerance to drought stress in rice (*Oryza sativa* L.), Plant Sci. 180 (2011) 775–782.
[25] M. Kumar, S.-C. Lee, J.-Y. Kim, S.J. Kim, S.S. Aye, S.R. Kim, Over-expression of dehydrin gene, *OsDhn1*, improves drought and salt stress tolerance through scavenging of reactive oxygen species in rice (*Oryza sativa* L.), J. Plant Biol. 57 (2014) 383–393.
[26] L. Bing, C.C. Feng, J.L. Li, X.X. Li, B.C. Zhao, Y.Z. Shen, Z.J. Huang, R.C. Ge, Overexpression of the *AtSTK* gene increases salt, PEG and ABA tolerance in *Arabidopsis*, J. Plant Biol. 56 (2013) 375–382.
[27] A. Mortazavi, B.A. Williams, K. McCue, L. Schaeffer, B. Wold, Mapping and quantifying mammalian transcriptomes by RNA-Seq, Nat. Methods 5 (2008) 621–628.
[28] Z. Du, X. Zhou, Y. Ling, Z. Zhang, Z. Su, agriGO: a GO analysis toolkit for the agricultural community, Nucl. Acids Res. 38 (2010) W64–W70.
[29] M. Badawi, J. Danyluk, B. Boucho, M. Houde, F. Sarhan, The *CBF* gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal *CBF*s, Mol. Genet. Genomics 277 (2007) 533–554.
[30] A.K. Knox, C. Li, A. Vágújfalvi, G. Galiba, E.J. Stockinger, J. Dubcovsky, Identification of candidate *CBF* genes for the frost tolerance locus *Fr-Am2* in *Triticum monococcum*, Plant Mol. Biol. 67 (2008) 257–270.
[31] A. Fricano, F. Rizza, P. Faccioli, D. Pagani, P. Pavan, A. Stella, L. Rossini, P. Piffanelli, L. Cattivelli, Genetic variants of *HvCbf14* are statistically associated with frost tolerance in a European germplasm collection of *Hordeum vulgare*, Theor. Appl. Genet. 119 (2009) 1335–1348.
[32] J. Lee, Y. Kang, S.C. Shin, H. Park, H. Lee, Combined analysis of the chloroplast genome and transcriptome of the Antarctic vascular plant *Deschampsia antarctica* Desv, PLoS ONE 9 (2014) e92501.
[33] H. Lee, H.H. Cho, I.C. Kim, J.H. Yim, H.K. Lee, Y.K. Lee, Expressed sequence tag analysis of Antarctic hairgrass *Deschampsia antarctica* from King George Island, Antarctica, Mol. Cells 25 (2008) 258–264.
[34] J. Lee, E.K. Noh, H.S. Choi, S.C. Shin, H. Park, H. Lee, Transcriptome sequencing of the Antarctic vascular plant *Deschampsia antarctica* Desv. under abiotic stress, Planta 237 (2013) 823–836.
[35] S.J. Gilmour, A.M. Sebolt, M.P. Salazar, J.D. Everard, M.F. Thomashow, Overexpression of the Arabidopsis *CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation, Plant Physiol. 124 (2000) 1854–1865.
[36] J.G. Dubouzet, Y. Sakuma, Y. Ito, M. Kasuga, E.G. Dubouzet, S. Miura, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression, Plant J. 33 (2003) 751–763.
[37] Z. Wang, M. Gerstein, M. Snyder, RNA-Seq: a revolutionary tool for transcriptomics, Nat. Rev. Genet. 10 (2009) 57–63.
[38] C. Jiang, B. Iu, J. Singh, Requirement of a CCGAC *cis*-acting element for cold induction of the *BN115* gene from winter *Brassica napus*, Plant Mol. Biol. 30 (1996) 679–684.
[39] Y. Ding, Z. Chen, C. Zhu, Microarray-based analysis of cadmium-responsive microRNAs in rice (*Oryza sativa*), J. Exp. Bot. 62 (2011) 3563–3573.
[40] S. Morran, O. Eini, T. Pyvovarenko, B. Parent, R. Singh, A. Ismagul, S. Eliby, N. Shirley, P. Langridge, S. Lopato, Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors, Plant Biotechnol. J. 9 (2011) 230–249.
[41] N. Kovalchuk, W. Jia, O. Eini, S. Morran, T. Pyvovarenko, S. Fletcher, N. Bazanova, J. Harris, K. Beck-Oldach, Y. Shavrukov, P. Langridge, S. Lopato, Optimization of *TaDREB3* gene expression in transgenic barley using cold-inducible promoters, Plant Biotechnol. J. 11 (2013) 659–670.
[42] A. Soltesz, M. Smedley, I. Vashegyi, G. Galiba, W. Harwood, A. Vagujfalvi, Transgenic barley lines prove the involvement of *TaCBF14* and *TaCBF15* in the cold acclimation process and in frost tolerance, J. Exp. Bot. 64 (2013) 1849–1862.
[43] S.J. Oh, C.W. Kwon, D.W. Choi, S.I. Song, J.K. Kim, Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice, Plant Biotechnol. J. 5 (2007) 646–656.
[44] T. Lourenço, N. Saibo, R. Batista, C. Pinto Ricardo, M.M. Oliveira, Inducible and constitutive expression of *HvCBF4* in rice leads to differential gene expression and drought tolerance, Biol. Plant 55 (2011) 653–663.
[45] Y. Ito, K. Katsura, K. Maruyama, T. Taji, M. Kobayashi, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice, Plant Cell Physiol. 47 (2006) 141–153.
[46] Z. Jeknić, K.A. Pillman, T. Dhillon, J.S. Skinner, O. Veisz, A. Cuesta-Marcos, P.M. Hayes, A.K. Jacobs, T.H. Chen, E.J. Stockinger, Hv-*CBF2A* overexpression in barley accelerates *COR* gene transcript accumulation and acquisition of freezing tolerance during cold acclimation, Plant Mol. Biol. 84 (2014) 67–82.
[47] J.T. Vogel, D.G. Zarka, H.A. Van Buskirk, S.G. Fowler, M.F. Thomashow, Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis, Plant J. 41 (2005) 195–211.
[48] A.N. Stepanova, J.M. Hoyt, A.A. Hamilton, J.M. Alonso, A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in Arabidopsis, Plant Cell 17 (2005) 2230–2242.
[49] H. Lu, S. Salimian, E. Gamelin, G. Wang, J. Fedorowski, W. LaCourse, J.T. Greenberg, Genetic analysis of *acd6-1* reveals complex defense networks and leads to identification of novel defense genes in Arabidopsis, Plant J. 58 (2009) 401–412.
[50] M. Boudsocq, J. Sheen, CDPKs in immune and stress signaling, Trends Plant Sci. 18 (2013) 30–40.
[51] Y. Liu, S. Zhang, Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis, Plant Cell 16 (2004) 3386–3399.
[52] J. Shi, L. Zhang, H. An, C. Wu, X. Guo, *GhMPK16*, a novel stress-responsive group D MAPK gene from cotton, is involved in disease resistance and drought sensitivity, BMC Mol. Biol. 12 (2011) 22.
[53] G. Freymark, T. Diehl, M. Miklis, T. Romeis, R. Panstruga, Antagonistic control of powdery mildew host cell entry by barley calcium-dependent protein kinases (CDPKs), Mol. Plant Microbe Interact. 20 (2007) 1213–1221.
[54] T.K. Mall, I. Dweikat, S.J. Sato, N. Neresian, K. Xu, Z. Ge, D. Wang, T. Elthon, T. Clemente, Expression of the rice CDPK-7 in sorghum: molecular and phenotypic analyses, Plant Mol. Biol. 75 (2011) 467–479.
[55] M. Hanin, F. Brini, C. Ebel, Y. Toda, S. Takeda, K. Masmoudi, Plant dehydrins and stress tolerance: versatile proteins for complex mechanisms, Plant Signal. Behav. 6 (2011) 1503–1509.
[56] Z. Cheng, J. Targolli, X. Huang, R. Wu, Wheat LEA genes, PMA80 and PMA1959, enhance dehydration tolerance of transgenic rice (*Oryza sativa* L.), Mol. Breed. 10 (2002) 71–82.
[57] F. Brini, M. Hanin, V. Lumbreras, I. Amara, H. Khoudi, A. Hassairi, M. Pagès, K. Masmoudi, Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in *Arabidopsis thaliana*, Plant Cell Rep. 26 (2007) 2017–2026.
[58] M. Hara, S. Terashima, T. Fukaya, T. Kuboi, Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco, Planta 217 (2003) 290–298.
[59] M. Houde, S. Dallaire, D. N'Dong, F. Sarhan, Overexpression of the acidic dehydrin WCOR410 improves freezing tolerance in transgenic strawberry leaves, Plant Biotechnol. J. 2 (2004) 381–387.
[60] J.A. Kreps, Y. Wu, H.S. Chang, T. Zhu, X. Wang, J.F. Harper, Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress, Plant Physiol. 130 (2002) 2129–2141.
[61] S. Nohzadeh Malakshah, M. Habibi Rezaei, M. Heidari, G.H. Salekdeh, Proteomics reveals new salt responsive proteins associated with rice plasma membrane, Biosci. Biotechnol. Biochem. 71 (2007) 2144–2154.
[62] I.K. Jarsch, T. Ott, Perspectives on remorin proteins, membrane rafts, and their role during plant-microbe interactions, Mol. Plant Microbe Interact. 24 (2011) 7–12.
[63] V.G. Checker, P. Khurana, Molecular and functional characterization of mulberry EST encoding *remorin* (*MiREM*) involved in abiotic stress, Plant Cell Rep. 32 (2013) 1729–1741.
[64] J. Yue, C. Li, Y. Liu, J. Yu, A remorin gene *SiREM6*, the target gene of SiARDP, from foxtail millet (*Setaria italica*) promotes high salt tolerance in transgenic *Arabidopsis*, PLoS ONE 9 (2014) e100772.
[65] E.S. Seong, K. Baek, S. Oh, S.H. Jo, S.Y. Yi, J.M. Park, Y.H. Joung, S. Lee, H.S. Cho, D. Choi, Induction of enhanced tolerance to cold stress and disease by overexpression of the pepper *CaPIF1* gene in tomato, Physiol. Plant. 129 (2007) 555–566.
[66] C. Spiess, A.S. Meyer, S. Reissmann, J. Frydman, Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets, Trends Cell Biol. 14 (2004) 598–604.
[67] E. Fassone, A.J. Duncan, J.W. Taanman, A.T. Pagnamenta, M.I. Sadowski, T. Holand, W. Qasim, P. Rutland, S.E. Calvo, V.K. Mootha, M. Bitner-Glindzicz, S. Rahman, *FOXRED1*, encoding an FAD-dependent oxidoreductase complex-I-specific molecular chaperone, is mutated in infantile-onset mitochondrial encephalopathy, Hum. Mol. Genet. 19 (2010) 4837–4847.

[68] S.E. Calvo, E.J. Tucker, A.G. Compton, D.M. Kirby, G. Crawford, N.P. Burtt, M. Rivas, C. Guiducci, D.L. Bruno, O.A. Goldberger, M.C. Redman, E. Wiltshire, C.J. Wilson, D. Altshuler, S.B. Gabriel, M.J. Daly, D.R. Thorburn, V.K. Mootha, High-throughput, pooled sequencing identifies mutations in *NUBPL* and *FOXRED1* in human complex I deficiency, Nat. Genet. 42 (2010) 851–858.

[69] M. Sauter, R. Lorbiecke, B. Ouyang, T.C. Pochapsky, G. Rzewuski, The immediate-early ethylene response gene *OsARD1* encodes an acireductone dioxygenase involved in recycling of the ethylene precursor *S*-adenosylmethionine, Plant J. 44 (2005) 718–729.