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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

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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.

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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