Identification of Rice Genes Associated With Enhanced Cold Tolerance by Comparative Transcriptome Analysis With Two Transgenic Rice Plants Overexpressing DaCBF4 or DaCBF7, Isolated From Antarctic Flowering Plant Deschampsia antarctica

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Few plant species can survive in Antarctica, the harshest environment for living organisms. Deschampsia antarctica is the only natural grass species to have adapted to and colonized the maritime Antarctic. To investigate the molecular mechanism of the Antarctic adaptation of this plant, we identified and characterized Deschampsia antarctica C-repeat binding factor 4 (DaCBF4), which belongs to monocot CBF group IV. The transcript level of DaCBF4 in D. antarctica was markedly increased by cold and dehydration stress. To assess the roles of DaCBF4 in plants, we generated a DaCBF4-overexpressing transgenic rice plant (Ubi:DaCBF4) and analyzed its abiotic stress response phenotype. Ubi:DaCBF4 displayed enhanced tolerance to cold stress without growth retardation under any condition compared to wild-type plants. Because the cold-specific phenotype of Ubi:DaCBF4 was similar to that of Ubi:DaCBF7 (Byun et al., 2015), we screened for the genes responsible for the improved cold tolerance in rice by selecting differentially regulated genes in both transgenic rice lines. By comparative transcriptome analysis using RNA-seq, we identified 9 and 15 genes under normal and cold-stress conditions, respectively, as putative downstream targets of the two D. antarctica CBFs. Overall, our results suggest that Antarctic hairgrass DaCBF4 mediates the cold-stress response of transgenic rice plants by adjusting the expression levels of a set of stress-responsive genes in transgenic rice plants. Moreover, selected downstream target genes will be useful for genetic engineering to enhance the cold tolerance of cereal plants, including rice.

Keywords: C-repeat/DRE binding factor, cold tolerance, Deschampsia antarctica, RNA-seq, transgenic plant, rice
INTRODUCTION

Plants are sessile organisms and are constantly exposed to adverse environmental conditions, to which they have adapted (Lata and Prasad, 2011; Claeyss and Inzé, 2013). The maritime Antarctic climate has low temperatures, high soil salinity, and a water deficit, which hamper the survival of angiosperms. Deschampsia antarctica Desv. (Poaceae) is one of two flowering plants that naturally inhabit Antarctica (Alberdi et al., 2002). This species has developed various adaptive mechanisms to survive in the Antarctic.

The adaptations that enable D. antarctica to survive in the harsh environment of the maritime Antarctic include changes in the leaf anatomy and the physiology of the photosynthetic apparatus (Gießwansowska et al., 2005; Sáez et al., 2017). Increased levels of nonstructural carbohydrates, apoplastic antifreeze proteins, and ice recrystallization inhibition proteins (biochemical cryoprotectants) reportedly enable plants to flourish under Antarctic conditions (Bravo and Griffith, 2005; John et al., 2009; Pastorczyk et al., 2014). In addition, transcriptome analysis of D. antarctica under abiotic stress demonstrated changes in the expression levels of stress-responsive genes (Lee et al., 2013a). However, the signaling pathways that mediate the activation of the expression of the stress-related genes responsible for the abiotic stress tolerance of this plant are unclear.

In Arabidopsis, C-repeat binding factor (CBF)/dehydration-responsive element-binding protein (DREB) is the major transcription factor responsible for induction of cold tolerance (Thomashow, 2010). Also, CBF overexpression in monocot plants resulted in enhanced tolerance to diverse abiotic stresses including cold and freezing. When overexpressed in barley, TaDREB3, TaCBF14, and TaCBF15 from wheat and HvCBF2a from barley resulted in enhanced frost tolerance by increasing the transcript levels of downstream target genes such as COR14b and DHN5 (Kovalchuk et al., 2013; Soltész et al., 2013; Jeknić et al., 2014). Moreover, TaCBF3 overexpression enhanced tolerance to frost and cold in wheat and barley (Morrán et al., 2011). Expression of cotton GhDREB in barley also resulted in enhanced cold tolerance (Gao et al., 2009). Indeed, overexpression of the CBF genes from rice (OsDREB1), barley (HvCBF4), and maize (ZmCBF3) resulted in enhanced cold tolerance in rice (Ito et al., 2006; Oh et al., 2007; Xu et al., 2011).

Followed by the discovery of AtCBF1 as the major transcriptional regulator responsible for inducing the expression of downstream COR genes in Arabidopsis (Jaglo-Ottosen et al., 1998), the CBF proteins of monocot cereal plants have been discovered genome-wide (Stockinger, 2009; Tondelli et al., 2011). Moreover, Badawi et al. (2007) divided monocot CBF proteins into groups I–IV, and group V was recently identified (Byun et al., 2015).

However, few studies have aimed to identify the downstream target genes responsible for the cold tolerance in cereal plants. Overexpression of the rice CBF homolog OsDREB1A, have shown enhanced tolerance of transgenic rice to drought, high-salt, and low-temperature stresses. A microarray analysis of 21,500 rice genes together with northern blot analysis confirmed that 12 genes, including dehydrins and a protease inhibitor, were upregulated in OsDREB1-overexpressing rice plants (Ito et al., 2006). Rice plants continuously overexpressing HvCBF4 from barley showed improved tolerance to cold, drought, and high-salt stresses. A microarray analysis revealed that the transcript levels of 15 genes, including Bowman Birk trypsin inhibitor 1 (BBTI1), were upregulated in HvCBF4 overexpressing rice plants under normal condition (Oh et al., 2007).

In previous work, we generated transgenic rice plants overexpressing DaCBF7, a CBF gene from D. antarctica, and analyzed the expression levels of downstream genes. DaCBF7 overexpression resulted in enhanced cold tolerance compared to wild-type plants (Byun et al., 2015). In this study, we found that the D. antarctica CBF4 gene (DaCBF4) encodes a homolog of cereal CBF group IV. The expression of this gene was induced in D. antarctica plants under cold and drought stress. Overexpression of DaCBF4 in rice resulted in a cold-specific phenotype similar to that of the DaCBF7-overexpressing plants. Thus, we identified the genes responsible for increasing the cold tolerance of rice by screening differentially regulated genes common to DaCBF7 and DaCBF4-overexpressing rice plants. Based on these results, we suggest that the Antarctic hairgrass DaCBF4 gene plays a crucial role in the cold tolerance in transgenic rice plants. Selected downstream target genes common to Ubi:DaCBF4 and Ubi:DaCBF7 will be useful for genetic engineering to enhance the cold-stress tolerance of cereal plants, including rice.

MATERIALS AND METHODS

Phylogenetic Analysis

The amino acid sequences of DaCBF4 and other CBF/DREB homologs from monocot crops were retrieved from the GenBank database and proofread. All downstream analyses were performed using the MEGA7 software (Kumar et al., 2016). Phylogenetic trees were constructed from the data sets by the neighbor-joining method based on the JTT matrix-based model. All positions with <95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Supports for internal branches were tested by bootstrap analyses of 1,000 replications. The accession numbers of CBF homologs from five monocot species—Antarctic hairgrass (D. antarctica), barley (Hordeum vulgare), rice (Oryza sativa), hexaploid wheat (Triticum aestivum), and einkorn wheat (Triticum monococcum)—are presented in Supplementary Table 1.

Subcellular Localization Experiment

The synthetic green fluorescent protein (sGFP)-coding region was fused in-frame to the 3’ end of the full-length DaCBF4-coding region and inserted into the pEarleyGate 100 binary
vector. The vector was transformed into Agrobacterium \textit{tumefaciens} strain GV3101 by electroporation. Tobacco \textit{(Nicotiana benthamiana)} leaves were co-infiltrated using \textit{A. tumefaciens} that contained the 35S:DaCBF4-sGFP or 35S:sGFP constructs. A 35S:nuclear-localizing signal-monomeric red fluorescent protein (35S:NLS-mRFP) construct was used as a control nuclear protein. Two days after infection, protoplasts were extracted from the tobacco leaves and visualized by fluorescence microscopy (BX51, Olympus).

**Plant Materials and Stress Conditions**

\textit{Deschampsia 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% phytagar (pH 5.7)] under a 16-h light/8-h dark photoperiod with a light intensity of 150 \(\mu\text{mol} \text{ m}^{-2} \text{ s}^{-1}\) at 15°C. For cold-stress conditions, plants grown at 15°C were transferred to a climate chamber at 4°C for the indicated periods. For drought-stress conditions, plants were transferred to filter paper and dried at 15°C. For high-salt conditions, plants were transferred to MS medium supplemented with 150 mM NaCl and incubated at 15°C. RNA was extracted from leaves and analyzed at various times after the imposition of stress. All the samplings for expression analysis was conducted at the same time to avoid variation by circadian rhythm.

**RT-PCR and DNA Gel-Blot Analysis**

Total RNA was isolated from mature leaves of wild-type and \textit{Ubi:DaCBF4} 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)]. First-strand cDNA was synthesized from 2 \(\mu\text{g}\) of total RNA using oligo(dT) primers and TOPscript Reverse Transcriptase (Enzymomics). To compare the \textit{DaCBF4} expression levels of the transgenic lines, \textit{DaCBF4} was amplified using a gene-specific primer set (Supplementary Table 2). To distinguish the independent lines of \textit{Ubi:DaCBF4} transgenic plants, genomic Southern blotting was performed. Total genomic DNA was isolated from mature rice leaves using cetyltrimethylammonium bromide (CTAB) solution, digested with \textit{BamH}I, and separated in a 0.8% agarose gel. The gel was treated sequentially with depurifying, denaturing, and neutralizing solutions, and the DNA was transferred to Hybond-N nylon membranes. The blot was hybridized with a 32P-labeled hygromycin B phosphotransferase (hph) probe under high-stringency conditions.

**Rice Plant Materials and Stress Conditions**

Dry rice seeds were sterilized with 0.4% NaClO solution for 30 min and washed several times with sterilized water. Seeds of wild-type and \textit{Ubi:DaCBF4} transgenic rice plants were germinated on half-strength MS medium containing vitamins (Duchefa Biochemie), 3% sucrose, and 0.7% phytagar. 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 as described by Byun et al. (2017). To investigate the effect of \textit{DaCBF4} on stress tolerance, 6-week-old plants grown in the growth room were subjected to various abiotic stresses, observed, and photographed. For cold-stress conditions, wild-type and \textit{DaCBF4}-overexpressing plants (independent lines #1 and #2) grown at 28°C were transferred to 4°C for 5 days, and then returned to 28°C.

**RNA-Sequencing**

RNA-sequencing (RNA-seq) was carried out using total RNA from 6-week-old wild-type and \textit{DaCBF4}-overexpressing line #1 plants under normal and cold-stress conditions (1 or 6 days after transfer to 4°C). Total RNAs were extracted from leaves of each genotype and each treatment using TRIzol reagent, treated with DNase I to remove contaminant genomic DNA, and purified using the RNasy Mini kit (Qiagen) following the manufacturer's instructions. Three different biological replicates
RESULTS

Identification and Characterization of DaCBF4

As the overexpression of barley HvCBF4 and wheat TaCBF14 (group IV) increased the cold-stress tolerance of transgenic rice and barley plants, respectively (Oh et al., 2007; Soltész et al., 2013), we cloned the D. antarctica cDNA sequence encoding the CRT-DREB-binding factor protein based on its sequence homology with HvCBF4 (Lee et al., 2013b). The gene was designated DaCBF4, and the sequence was submitted to GenBank (KM978992).

DaCBF4 encodes a protein of 216 amino acids with a conserved AP2 DNA-binding domain. To examine its phylogenetic relationships with CBF proteins from other monocot plants, we compared the amino acid sequence of DaCBF4 with those of 37 hexaploid wheat, 9 einkorn wheat, 17 barley, 9 rice, and 1 D. antarctica CBF homologs (Badawi et al., 2007; Byun et al., 2015). All members of the monocot CBF family were divided into five groups; DaCBF4 belonged, as expected, to group IV (Supplementary Figure 1). In the AP2 domain and flanking regions, DaCBF4 shares 70–75, 76–78, 67–79, 75–91, and 56–65% sequence identities with the members of groups I, II, III, IV, and V, respectively.

DaCBF4 Is a Nuclear-Localized Transcription Factor

Transcription factors must localize to the nucleus to induce the expression of downstream target genes. To examine the cellular localization of DaCBF4, an in vivo cell targeting experiment was performed. The sGFP gene was fused in-frame to the 3’ end of the DaCBF4-coding region under the control of the CaMV 35S promoter. DaCBF4-sGFP and NLS-mRFP (a nuclear marker) constructs were co-expressed in tobacco (N. benthamiana) leaves using the Agrobacterium-mediated infiltration method. The protoplasts were extracted and visualized by fluorescence microscopy. The fluorescence signal of sGFP was uniformly distributed throughout the cell (Figure 1). In contrast, the DaCBF4-sGFP fusion protein was found primarily in the nucleus, and its signal merged with that of NLS-mRFP, indicating that DaCBF4 is targeted to the nucleus of plant cells.

Induction of DaCBF4 Expression in D. antarctica by Cold and Drought Stresses

The expression of numerous monocot CBF genes is reportedly induced in response to abiotic stresses in plants (Dubouzet et al., 2003; Vágújfalvi et al., 2005; Byun et al., 2015). To investigate the effects of abiotic stresses on the DaCBF4 transcript level in Antarctic hairgrass, DaCBF4 mRNA levels were analyzed by RT-qPCR using total RNA isolated from leaves of D. antarctica plants subjected to cold (4°C), salt (150 mM NaCl), or drought (air-dried on filter paper) stress. The DaCBF4 transcript level increased in response to cold-stress treatment. DaCBF4 expression was induced from 30 min after cold stress, peaked at 4 h, and then declined but remained high until 48 h. Drought stress resulted in a similar pattern of DaCBF4 expression. In contrast, salt stress did not activate the transcription of DaCBF4.
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(Figure 2). The DalRIP, DaP5CS, and DaDHN1 genes were used as positive controls for the cold, salt, and drought stresses, respectively.

Generation and Molecular Analysis of Ubi:DaCBF4 Transgenic Rice Plants

Genetic transformation is widely used to investigate the functional roles of specific genes in plants. However, transformation of genes into D. antarctica is not yet possible. Therefore, we overexpressed DaCBF4 in rice, a monocot model plant of the same family as D. antarctica.

We generated transgenic rice plants constitutively overexpressing DaCBF4 under the control of the maize ubiquitin (Ubi) promoter (Figure 3A). Semi-quantitative RT-PCR confirmed that two different Ubi:DaCBF4 transgenic plants overexpressed DaCBF4 (Figure 3B). DNA gel-blot analysis indicated that these Ubi:DaCBF4 transgenic plants were independent lines (Figure 3C). Transgenic Arabidopsis plants overexpressing AtCBF1, AtCBF2, or AtCBF3 at high levels exhibit the dwarf phenotype (small stature and slow growth) under normal conditions (Gilmour et al., 2004). Moreover, in cereals, transgenic rice overexpressing OsDREB1 and transgenic barley overexpressing TaCBF14, TaCBF15, or HvCBF2A, showed moderate retarded development under normal condition compared to wild-type plants (Ito et al., 2006; Soltész et al., 2013; Jeknić et al., 2014). In contrast, the two independent Ubi:DaCBF4 transgenic lines (#1 and #2) exhibited...
wild-type vegetative growth under our experimental conditions (Figure 3D).

**Effect of DaCBF4 Overexpression on Abiotic Stress Tolerance in Transgenic Rice Plants**

Overexpression of heterologous CBF genes in rice results in tolerance to cold (Byun et al., 2015), drought and high salt (Oh et al., 2005; Cui et al., 2011), or cold, drought, and high salt (Oh et al., 2007; Xu et al., 2011). To investigate whether overexpression of DaCBF4 is correlated with tolerance to abiotic stresses in transgenic plants, we assessed the effect of cold stress on Ubi:DaCBF4 transgenic rice plants. Wild-type and Ubi:DaCBF4 transgenic plants were grown at 28°C for 6 weeks in the growth room and transferred to the cold room (4°C). After 5 days of cold-stress conditions, wild-type plants showed wilting and did not recover after removal from the cold-stress conditions, while most of the Ubi:DaCBF4 transgenic rice plants recovered, appeared healthy, and continued to grow (Figure 4A). The survival rate of the wild-type rice plants was 11.8% (12 of 101), and the survival rates of Ubi:DaCBF4 lines #1 and #2 were 63.6% (35 of 55) and 41.3% (19 of 46), respectively (Figure 4B).

As DaCBF4 expression was also induced by drought (Figure 2), we assessed the tolerance of wild-type and Ubi:DaCBF4 rice plants to drought stress. Six-week-old wild-type and Ubi:DaCBF4 plants were grown without a water supply for 9 days. Both wild-type and DaCBF4-overexpressing plants were severely wilted, and most plants did not recover and died even after re-watering. Next, we compared the salt tolerance of the wild-type and Ubi:DaCBF4 transgenic lines. 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 this condition, both...
wild-type and transgenic plants were almost entirely bleached and unable to recover. Therefore, constitutive expression of DaCBF4 increased the tolerance of rice to cold stress, but had no effect on the tolerance to dehydration or salt stress.

Transcriptome Analysis of Ubi:DaCBF4 Transgenic Plants Under Cold-Stress Conditions

As DaCBF4-overexpressing rice plants were more tolerant to cold stress than wild-type plants, we next performed a transcriptomic analysis of Ubi:DaCBF4 and wild-type plants to identify the downstream target genes of DaCBF4 responsible for the enhanced cold tolerance. We conducted RNA-seq analysis using the total RNA from 6-week-old leaves of wild-type and Ubi:DaCBF4 (transgenic line #1) plants grown under normal (before cold treatment) and cold-stress (1 and 6 days at 4°C) conditions. Transgenic line #1 was selected for RNA-seq analysis because it displayed a higher survival rate after cold stress than line #2 (Figure 4). The raw reads were mapped to the rice reference genome. The expression values were measured in FPKM and the differentially expressed genes (DEGs) were determined.

We reported previously that constitutive expression of DaCBF7, one of the CBF genes of D. antarctica, enhanced the cold-stress tolerance of transgenic rice plants by modulating the expression levels of downstream genes (Byun et al., 2015). Because the DaCBF4-overexpressing plants showed a cold-specific phenotype similar to that of the DaCBF7-overexpressing plants, we identified the genes responsible for the increased cold tolerance by screening for genes up- or down-regulated in both transgenic rice plants.

Genes Upregulated Under Normal Conditions

Under normal growth conditions, 33 genes were upregulated in Ubi:DaCBF4 line #1 compared to wild-type rice plants (Figure 5 and Supplementary Table 3). This indicated that constitutive expression of DaCBF4 influenced the gene expression profile of transgenic plants even before cold treatment. Among them, nine genes were also upregulated in Ubi:DaCBF7 plants compared to wild-type plants (Table 1), including those encoding a protease inhibitor family protein (BBTI12, Os01g04050) and non-specific lipid transfer proteins (LTPL114, Os03g01300; LTPL12, Os12g02320). The expression of three genes annotated as expressed proteins was highly induced in both transgenic plants. Furthermore, the induction of the expression of the genes encoding dirigent (Os11g10850), thaumatin (Os12g43380), and ribonuclease T2 family domain-containing protein (Os09g36680) differed between the two transgenic plants under normal conditions.

Genes Upregulated Under Cold-Stress Conditions

After 1 day of cold-stress conditions, 59 genes were upregulated in the Ubi:DaCBF4 transgenic rice plants compared to the wild type (Figure 5 and Supplementary Table 4). Of these, 15
were upregulated in both *Ubi:DaCBF4* and *Ubi:DaCBF7* after 1 day of cold-stress conditions (Table 1). The gene encoding AP domain-containing protein (*Os02g43970*) exhibited the greatest fold-change in expression in both transgenic lines compared to wild-type plants, except for five genes annotated as expressed proteins. This indicates that activation of the expression of downstream COR genes in transgenic plants is a multi-step process. Three transcription factor-encoding genes, dehydration-responsive element-binding protein (*Os09g35030*), ethylene-insensitive 3 (*Os07g48630*), and helix-loop-helix DNA-binding domain containing protein (*Os01g67480*), were upregulated, suggesting a role in the cold tolerance of rice. In addition, the expression of two late embryogenesis-abundant protein (LEA) genes encoding *OsLEA9* (*Os01g21250*) and *OsLEA24* (*Os03g45280*) were induced under cold-stress conditions. The expression of *OsLEA24* was induced after 1 and 6 days of cold-stress conditions, but *OsLEA9* expression was induced only after 6 days of cold-stress conditions.

### Genes Downregulated in Transgenic Rice Plants

In total, 130 genes were downregulated in *Ubi:DaCBF4* under normal and cold-stress conditions (Supplementary Table 5). To identify enriched Gene Ontology (GO) terms for these genes, over-represented GO categories were analyzed using AgriGO (Fisher's exact test *p* < 0.05) (Tian et al., 2017).

In the Biological Process category, the GO terms photosynthesis, generation of precursor metabolites and energy, and translation were significantly enriched in downregulated genes in *Ubi:DaCBF4* plants; the most highly enriched GO term was photosynthesis with the lowest corrected *p*-value of FDR, 2.21e−15 (Supplementary Figure 2). Six of the 14 genes included in the GO term photosynthesis were significantly downregulated in *Ubi:DaCBF7* plants under cold-stress compared to normal conditions. These comprised the genes encoding chlorophyll A-B binding protein (*Os01g41710* and *Os07g37240*), photosynthetic reaction center protein (*Os08g35420*), photosystem II 10 kDa polypeptide chloroplast precursor (*Os08g10020*), photosystem II 44 kDa reaction center protein (*Os04g16874*), and photosystem II D2 protein (*Os04g16872*) (Table 2).

### Validation of DEGs in *Ubi:DaCBF4* Transgenic Plants

To validate the DEG results, 12 genes that were differentially expressed in *Ubi:DaCBF4* transgenic plants under normal and cold-stress conditions were subjected to RT-qPCR (Figure 6A). Under normal conditions, the expression patterns of nine genes—including *BBTI2* (*Os01g04050*), *RNase T2* (*Os09g36680*), *LTPL114* (*Os03g01300*), *Thaumatin* (*Os12g43380*), *LTPL12* (*Os12g02320*), *Dirigent* (*Os11g10850*)—and three unknown genes—*Os01g15270*, *Os03g02470*, and *Os10g22630*—were in overall agreement with those determined by RNA-seq. The fold-changes in expression determined by RNA-seq and RT-qPCR differed slightly for *LTPL114*, *Thaumatin*, and *Os10g22630*, but their expression was increased in *Ubi:DaCBF4* line #1 and line #2 compared to wild-type plants. Under cold-stress conditions, the expression patterns of five genes—including *OsLEA24* (*Os03g45280*), *OsBI* (*Os02g03280*), *OsMDH* (*Os10g33800*)—and two unknown genes—*Os03g02470* and *Os10g22630*—were in agreement with those determined by RNA-seq, and the expression of these genes was higher in independent two lines of *Ubi:DaCBF4* compared to wild-type plants.

Promoter analysis represented that *OsLEA24* and *Os10g22630* contain putative CRT/DRE cis-acting elements in their promoter regions (Figure 6B). In addition, the upstream regions of the *OsBI* and *Os03g02470* have a putative low temperature responsive element (LTER). To investigate the function of DaCBF4 as a transcription factor, we conducted gel retardation analysis with 32P-labeled promoter regions of the target gene promoter. As shown in Figure 6B, the His-DaCBF4 protein bound to DNA and formed nucleoprotein complex. These results are in agreement with the fact that the above-mentioned genes are regulated by ectopic expression of *DaCBF4* in rice plants.

### Differential Expression of AP2 Genes in *Ubi:DaCBF4* and *Ubi:DaCBF7*

In addition to searching for genes responsible for increased cold tolerance, investigating the specificity of DaCBF4 to its downstream targets is important for understanding the molecular mechanism of regulation for downstream genes by the group IV CBF. We could make a list of 72 genes which
TABLE 1 | List of differentially induced genes in both transgenic rice plants Ubi:DaCBF4 and Ubi:DaCBF7 under normal control or cold stress conditions when compared to wild type plants.

| Locus ID               | Annotations — Description                                           | RNA-seq analysis; Baggerley’s test | Ubi:DaCBF4 vs. wild-type | Ubi:DaCBF7 vs. wild-type |
|------------------------|---------------------------------------------------------------------|-----------------------------------|--------------------------|--------------------------|
|                        |                                                                     | Fold change | FDR p-value correction | Fold change | FDR p-value correction |
| NORMAL CONDITION       |                                                                     |           |                           |             |                           |
| Os11g10850             | Dirigent                                                           | 15.42      | 3.26E−02                  | 210         | 1.09E−03                 |
| Os12g43380             | Thaumatin                                                          | 5.17       | 1.54E−04                  | 11.21       | 0                        |
| Os09g36680             | Ribonuclease T2 family domain containing protein                   | 3.06       | 0                         | 2.89        | 0                        |
| Os01g04050             | BBTI12—Bowman-Birk type trypsin inhibitor                          | 2.26       | 2.43E−04                  | 3.90        | 1.50E−03                 |
| Os12g2320              | LTPL12—Protease inhibitor family protein                           | 1.60       | 1.51E−02                  | 1.94        | 1.11E−10                 |
| Os03g01300             | LTPL114—Protease inhibitor family protein                          | 1.60       | 2.89E−11                  | 11.98       | 2.92E−08                 |
| Os10g22630             | Expressed protein                                                  | 614.27     | 1.13E−11                  | 453.35      | 0                        |
| Os03g02470             | Expressed protein                                                  | 211.86     | 1.41E−02                  | 457         | 8.73E−09                 |
| Os01g15270             | Expressed protein                                                  | 1.77       | 9.15E−08                  | 2.04        | 4.90E−06                 |
| COLD STRESS, 1 DAY    |                                                                     |            |                           |             |                           |
| Os02g43970             | AP2 domain containing protein                                      | 30.60      | 0                         | 385.13      | 2.84E−07                 |
| Os03g45280             | Dehydrin (OsLEA24)                                                 | 5.22       | 0                         | 1.36        | 1.60E−11                 |
| Os09g35030             | Dehydration-responsive element-binding protein                     | 2.91       | 1.81E−05                  | 1.77        | 9.62E−11                 |
| Os08g37670             | Plastocyanin-like domain containing protein                        | 2.82       | 0                         | 4.26        | 0                        |
| Os01g09220             | Transposon protein putative CACTA Env/Spm sub-class                | 2.16       | 6.39E−07                  | 3.44        | 0                        |
| Os07g48630             | Ethylene-insensitive 3                                              | 2.06       | 7.36E−12                  | 1.39        | 2.01E−02                 |
| Os01g67480             | Helix-loop-helix DNA-binding domain containing protein             | 2.00       | 1.97E−03                  | 1.67        | 2.92E−02                 |
| Os09g36680             | Ribonuclease T2 family domain containing protein                   | 1.69       | 3.44E−04                  | 3.56        | 0                        |
| Os01g21250             | Late embryogenesis abundant protein (OsLEA9)                       | 1.59       | 0                         | 1.53        | 0                        |
| Os04g88710             | AMP-binding domain containing protein                               | 1.22       | 2.21E−07                  | 1.97        | 0                        |
| Os01g06882             | Expressed protein                                                  | 1154.57    | 3.62E−03                  | 378         | 3.61E−07                 |
| Os04g01330             | Expressed protein                                                  | 535.30     | 1.17E−06                  | 385.13      | 0                        |
| Os10g22630             | Expressed protein                                                  | 279.51     | 9.38E−05                  | 388.62      | 0                        |
| Os03g02470             | Expressed protein                                                  | 191.20     | 6.65E−03                  | 291         | 2.63E−05                 |
| Os06g09900             | Expressed protein                                                  | 1.79       | 1.32E−10                  | 1.25        | 9.13E−03                 |
| COLD STRESS, 6 DAY    |                                                                     |            |                           |             |                           |
| Os02g43970             | AP2 domain containing protein                                      | 106.23     | 2.94E−08                  | 99.36       | 1.90E−05                 |
| Os02g03280             | Transmembrane BAX inhibitor motif-containing protein               | 3.84       | 0                         | 1.27        | 1.26E−03                 |
| Os10g33800             | Lactate/malate dehydrogenase                                        | 2.26       | 6.68E−04                  | 1.82        | 8.47E−10                 |
| Os03g45280             | Dehydrin (OsLEA24)                                                 | 1.93       | 6.41E−11                  | 1.72        | 7.26E−11                 |
| Os04g16770             | Photosynthetic reaction center protein                              | 1.81       | 0                         | 1.39        | 0                        |
| Os09g25320             | Ubiquitin family protein                                            | 1.79       | 4.38E−02                  | 1.45        | 2.82E−03                 |
| Os08g35420             | Photosynthetic reaction center protein                              | 1.50       | 0                         | 2.67        | 0                        |
| Os01g68300             | Expressed protein                                                  | 2.99       | 1.30E−02                  | 2.90        | 0                        |
| Os12g39930             | Expressed protein                                                  | 1.62       | 0                         | 5.96        | 0                        |
| Os10g21190             | Expressed protein                                                  | 1.59       | 0                         | 4.66        | 0                        |

Results for Ubi:DaCBF7 were adopted from Byun et al. (2015).

were upregulated exclusively in Ubi:DaCBF4 under cold stress condition, compared to Ubi:DaCBF7. When over-represented GO categories were analyzed using AgriGO (Fisher’s exact test \( p < 0.05 \)) (Tian et al., 2017), the GO term regulation of metabolic process was the only significantly enriched one (corrected \( p \)-value of FDR = 0.022). Notably, seven genes included in this group were encoding AP2 domain containing protein (Supplementary Table 6). For comparative analysis, we also identified 7 more AP2 genes, which were induced exclusively in Ubi:DaCBF7 under cold stress. As a result of promoter analysis, we could identify 9 DRE/CRT and 4 LTRE elements from DaCBF4 induced 7 AP2 genes, and 5 DRE/CRT and 8 LTRE elements from DaCBF7 induced 7 AP2 genes, which imply that different CBF homolog protein may prefer different type of cis-element in promoters of target genes.
Byun et al. (2015)
Ito et al., 2006; Wei et al., 2017).
Constitutive expression of
RT-qPCR analyses revealed
In this study, constitutive
We reported previously that the overexpression
Oh et al., 2007; Badawi et al., 2007
(Poaceae; Pooideae). Phylogenetic analysis revealed that DaCBF4
−Os07g37240 Chlorophyll A-B binding protein
−Os08g35420 Photosynthetic reaction center protein
−Os01g41710 Chlorophyll A-B binding protein
temperate habitats, suggesting possible roles in adaptation to low
temperature environment of the Antarctic.

Phenotype of Ubi:DaCBF4 Plants Under Normal and Cold-Stress Conditions
We generated CBF-overexpressing transgenic plants to assess the roles of these genes in tolerance to abiotic stresses. Most of the transgenic plant lines exhibited enhanced tolerance to cold, drought, and salt stresses; this was mediated by modulating the expression of downstream stress-responsive genes (Lata and Prasad, 2011). Constitutive expression of CBF transcription factors generally enhances stress tolerance, but in some cases results in retarded normal growth (dwarf phenotype) and delayed flowering (Ito et al., 2006; Wei et al., 2017). In contrast, other

CBF-overexpressing transgenic plants displayed no differences in phenotype under normal growth conditions (Oh et al., 2007; Xu et al., 2011; Byun et al., 2015). In this study, constitutive expression of DaCBF4 from D. antarctica enhanced cold-stress tolerance without affecting development. The distinct effects of CBF overexpression on vegetative growth may be due to different generation of transgenic plants analyzed (Oh et al., 2007). However, the molecular mechanisms underlying the different growth phenotypes of the various transgenic plant lines remain to be elucidated.

Discussion
Cloning and Characterization of DaCBF4 From D. antarctica
We isolated DaCBF4 from the Antarctic plant D. antarctica (Poaceae; Pooideae). Phylogenetic analysis revealed that DaCBF4 is grouped within the cereal CBF group IV (Badawi et al., 2007). Because cereal CBF group IV is found only in the Pooideae, members of this group are considered to have evolved during the recent translocation of these plants from tropical to temperate habitats, suggesting possible roles in adaptation to low temperature (Badawi et al., 2007). RT-qPCR analyses revealed that the expression levels of DaCBF4 in D. antarctica under cold and drought stresses were increased, and cold stress resulted in an increase in expression of greater magnitude than did drought stress (Figure 2). Induction of CBF IV group gene expression by cold stress also occurs in barley, wheat, and Brachypodium distachyon (Kume et al., 2005; Skinner et al., 2005; Chen et al., 2016). Together with the cellular localization of 3SS:DaCBF4-sGFP in the nucleus of tobacco protoplasts (Figure 1), DNA binding affinity of DaCBF4 (Figure 6B) represented possible roles of this protein as a transcription factor in Antarctic hairgrass. The considerable induction of DaCBF4 expression under cold stress suggests that DaCBF4 regulates development of cold tolerance in D. antarctica during adaptation to the low-temperature environment of the Antarctic.

Comparative RNA-Seq Analysis of Ubi:DaCBF4 and Ubi:DaCBF7
Overexpression of CBF reportedly enhances the cold tolerance of barley, rice, and wheat (Ito et al., 2006; Oh et al., 2007; Gao et al., 2009; Morran et al., 2011; Xu et al., 2011). However, only a few studies have performed high-throughput transcriptome analysis of downstream genes regulated by overexpressed CBF transcription factors, all of them in rice (Oh et al., 2005, 2007; Ito et al., 2006). We reported previously that the overexpression of DaCBF7, a CBF homolog from D. antarctica, resulted in enhanced tolerance to cold in rice, similar to DaCBF4 in this study. Moreover, the transcriptome of Ubi:DaCBF7 was analyzed by RNA-seq to identify downstream genes involved in promoting cold tolerance in transgenic rice plants (Byun et al., 2015). Therefore, in this study we focused on downstream target genes regulated by both DaCBF7 and DaCBF4 that enhance the cold tolerance of rice.

Genes Upregulated in Ubi:DaCBF4 and Ubi:DaCBF7 Plants Under Normal Conditions
Nine genes were upregulated in both Ubi:DaCBF4 and Ubi:DaCBF7 transgenic rice plants under normal conditions (Table 1), the majority are abiotic stress-responsive genes in various plant species. LTP112 (Os12g02320) and LTP114 (Os03g01300) encode non-specific lipid transfer proteins (LTPs) involved in lipid metabolism. In addition, LTP expression is

TABLE 2 | List of differentially down-regulated genes belonging to GO term “Photosynthesis” (GO:0015979) in both transgenic rice plants Ubi:DaCBF4 and Ubi:DaCBF7 under normal control or cold stress condition when compared to wild type plants.

| Locus ID | Annotations—description | RNA-seq analysis; Baggerley’s test |
|----------|------------------------|----------------------------------|
|          |                        | **Ubi:DaCBF4 vs. wild-type** | **Ubi:DaCBF7 vs. wild-type** |
|          |                        | Fold change | FDR p-value correction | Fold change | FDR p-value correction |
| NORMAL CONDITION | | | | |
| Os01g41710 | Chlorophyll A-B binding protein | −1.760 | 1.782E−22 | −2.044 | 0 |
| Os08g10020 | Photosystem II 10 kDa polypeptide chloroplast precursor | −1.391 | 8.340E−06 | −1.591 | 2.654E−12 |
| COLD STRESS, 1 DAY | | | | |
| Os08g35420 | Photosynthetic reaction center protein | −2.455 | 2.961E−72 | −10.319 | 3.584E−18 |
| Os07g37240 | Chlorophyll A-B binding protein | −2.367 | 2.021E−06 | −1.244 | 3.942E−10 |
| Os04g16874 | Photosystem II 44 kDa reaction center protein | −1.741 | 9.320E−06 | −5.394 | 1.856E−16 |
| Os03g16872 | Photosystem II D2 protein | −1.428 | 1.597E−02 | −4.161 | 6.194E−19 |

Results for Ubi:DaCBF7 were adopted from Byun et al. (2015).
induced in plant cells upon exposure to abiotic stresses. The bromegrass LTP gene *BG-14* was strongly induced during cold acclimation (Wu et al., 2004), and the expression of two barley *LTP4* genes, *HvLtp4.2*, and *HvLtp4.3*, was increased under cold-stress conditions (Molina et al., 1996). Overexpression of the pearl millet *DREB2A* transcription factor gene in tobacco and of the rice *DREB1A* gene in rice plants enhanced abiotic stress tolerance and induced the expression of *LTP* homologs under cold-stress conditions (Ito et al., 2006; Agarwal et al., 2010). The LTP-mediated resistance of plants to cold is associated with a decrease in thylakoid membrane lipid fluidity (Sror et al., 2003). Dirigent (Os11g10850) encodes a protein that modulates lignin biosynthesis and cell wall metabolism during exposure to abiotic stresses (Paniagua et al., 2017). Dirigent proteins are implicated in modulation of lignification levels upon exposure to abiotic stresses. The expression of several of the DIR-like genes from *Brassica rapa* and the resurrection plant *Boea hygrometrica* (*BhDIR1*) was influenced by water and cold stress (Wu et al., 2009; Arasan et al., 2013). Therefore, modulation of plant cell wall metabolism and fluidity is crucial for the cold-stress tolerance of plants. However, the molecular mechanisms underlying the effects of dirigent proteins on plant abiotic stress tolerance are unclear.

**BBTI12** (*Os01g04050*) belongs to the Bowman-Birk family, which consists of compound inhibitors comprising one to six inhibitor units that target serine proteases (Rawlings, 2010). **BBTI1** and **BBTI2**, rice BBTI homologs, are induced under normal conditions in barley *CBF4* -- or *Arabidopsis*
CBF3-overexpressing transgenic rice plants (Oh et al., 2005, 2007). Proteins are influenced by abiotic stressors and are the major players in plant responses to stress (Kidrič et al., 2014); thus, BBTI-mediated fine control of protein degradation may be required for survival of plants exposed to abiotic stressors. Expression of OsRNS4, which encodes the ribonuclease T2 family domain-containing protein (Os09g36680) was increased in response to biotic stresses, such as insects and Xanthomonas oryzae, and abiotic stresses, such as wounding and high-salt conditions (Hillwig, 2009). Overexpression of OsRNS4 enhanced tolerance to a high salt concentration, but the effect on cold tolerance was not evaluated (Zheng et al., 2014). Because OsRNS4 is an inactive RNase (Hillwig, 2009), its role in the response to abiotic stresses may be independent of its enzymatic activity. In this study, the expression of *Thaumatin*, a member of the pathogenesis-related protein 5 (PR5) family, was induced in transgenic rice plants. Moreover, the expression in winter wheat (*T. aestivum* L.) of *TaTLP*, which encodes a thraumatín-like protein (TLP), was increased during cold acclimation of wheat seedlings (Kuwabara et al., 2002). Overexpression of *ObTLP1*, a thraumatín-like protein from basil, in *Arabidopsis* enhances tolerance of plants to multiple abiotic stresses as well as the phytopathogenic fungi, *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Misra et al., 2016).

To summarize, DaCBF4 and DaCBF7 enhance cold-stress tolerance by activating the expression of target genes with roles in biotic and abiotic stress responses. Interestingly, most of the genes upregulated in both plants under normal conditions are reportedly responsive to both biotic and abiotic stresses. Therefore, the pathogen resistance of *Ubi:DaCBF4* and *Ubi:DaCBF7* plants warrants further investigation.

**Genes Upregulated in *Ubi:DaCBF4* and *Ubi:DaCBF7* Under Cold-Stress Conditions**

Fifteen and ten genes were upregulated in both transgenic plants in response to 1 day and 6 days of cold stress, respectively (Table 1). Among them, two were LEA (late embryogenesis abundant) genes, which respond to cold, drought, salinity, and ABA stresses during the vegetative stage of growth (Liu et al., 2017). LEA genes are abundant in plant genomes; the *Arabidopsis* and rice genomes harbor at least 51 and 34 LEA genes, respectively (Wang et al., 2007; Hundertmark and Hincha, 2008). LEA proteins are classified into seven groups according to their motifs (Battaglia et al., 2008). The diverse gene expression levels and protein subcellular distributions suggest that different LEAs are involved in the responses to diverse environmental stimuli (Hundertmark and Hincha, 2008; Candat et al., 2014). LEA proteins may be involved in protection of proteins or endomembrane structures under stress conditions (Koag et al., 2003; Drira et al., 2013). LEA expression enhances stress tolerance in transgenic plants (Kosová et al., 2014). Overexpression of wheat *WC116* or *Arabidopsis Cor15A* leads to increased freezing tolerance in transgenic plants (Artus et al., 1996; Sasaki et al., 2014). Heterologous expression of individual *PmLEAs* genes in tobacco conferred enhanced tolerance to cold and drought stress (Bao et al., 2017). *OsDhn1*—overexpressing plants displayed enhanced drought- and salt-stress tolerance due to increased scavenging of reactive oxygen species (Kumar et al., 2014). Transcriptome analysis of *Ubi:DaCBF4* and *Ubi:DaCBF7* plants showed specific changes in two LEA proteins, OsLEA9 (group III) and OsLEA24 (group II [dehydron], characterized by a K-segment). These genes have a DRE/CRT sequence in their upstream regions and may be directly regulated by DaCBF proteins (Byun et al., 2015). Elevated expression levels of *OsLEA9* and *OsLEA24* are positively correlated with cold-stress tolerance in transgenic rice plants. Overall, these results suggest that the *OsLEA9* and *OsLEA24* genes, which are downstream of *DaCBF*, can be used as target genes for genetic engineering of crops with enhanced stress tolerance.

In addition to cold responsive genes, the induction of a rice gene *Os02g43970*, encoding AP2 domain containing protein, was highly strong under cold condition, but was not significant in the normal condition (Table 1). Moreover, 7 more genes encoding AP2 domain containing protein were significantly upregulated exclusively in *Ubi:DaCBF4* under cold condition, but not in *Ubi:DaCBF7* under normal or cold conditions (Supplementary Table 6). Based on results that those CBF genes still can exhibit a cold responsive expression pattern, we can assume that their induction in *Ubi:DaCBF4* is caused by additive effect of *DaCBF4* overexpression and native CBF response to cold treatment. Similar phenomena were also observed in previous studies on transgenic barley overexpressing *TaCBF23*, *TaCBF14/15*, and *HvCBF2A* (Morran et al., 2011; Soltész et al., 2013; Jeknič et al., 2014).

For more investigation on difference of target specificities between two transgenes *DaCBF4* and *DaCBF7*, we examined cis-element sequences of two AP2 gene groups, *DaCBF4* induced and *DaCBF7* induced group. DaCBF4 induced AP2 genes had more DRE/CRT (9) and less LTRE elements (4) than DaCBF7 induced 7 AP2 genes with 5 DRE/CRT and 8 LTRE elements. Obviously further researches on the genome wide analysis of rice AP2 genes and their distribution profile of promoter elements are still necessary, activation of different subset of AP2 genes by DaCBF4 and DaCBF7 can be an indirect evidence of distinctive specificity and biological activity for each CBF protein in plants under abiotic stresses.

**Genes Downregulated in *Ubi:DaCBF4* and *Ubi:DaCBF7* Plants**

In total, 130 genes were downregulated in the *Ubi:DaCBF4* transgenic line under normal and cold-stress conditions. Photosynthesis was the most enriched GO term (Supplementary Figure 2). Six genes encoding photosystem proteins were significantly downregulated in both *Ubi:DaCBF4* and *Ubi:DaCBF7* (Table 2). Cold stress generally reduces photosynthetic activity and downregulates the expression of photosynthesis-related genes (Allen and Ort, 2001; Fowler and Thomashow, 2002; Tsonev et al., 2003). In addition, photosynthesis-related genes are downregulated in *CBF*-overexpressing transgenic plants compared to
wild-type plants. However, most studies did not identify the downregulated genes as upregulated genes are more important in terms of stress tolerance. An RNA-Seq study revealed that genes with diverse functions were downregulated in AtCBF2- and AtCBF3-overexpressing Arabidopsis lines, but photosynthesis-related genes were not overrepresented among the downregulated genes (Li et al., 2017). However, in this study, the expression of photosynthesis-related genes was decreased in both transgenic rice lines; therefore, the mechanism by which DaCBFs regulate the expression of genes involved in photosynthesis warrants further investigation.

CONCLUSION

Our results showed that heterologous expression of Antarctic hairgrass DaCBF4 increased the tolerance of transgenic rice plants to low-temperature stress. A comparative transcriptome analysis identified common downstream targets of DaCBF4 and DaCBF7 that resulted in the same phenotype. The genes identified in this study will facilitate genetic engineering of cereal plants with enhanced cold tolerance.

REFERENCES

Agarwal, P., Agarwal, P. K., Joshi, A. J., Sopory, S. K., and Reddy, M. K. (2010). Overexpression of PgDREB2A transcription factor enhances abiotic stress tolerance and activates downstream stress-responsive genes. Mol. Biol. Rep. 37, 1125–1135. doi: 10.1007/s11303-009-9885-8

Alberdi, M., Bravo, L. A., Gutiérrez, A., Gidekel, M., and Corcuera, I. J. (2002). Ecophysiology of Antarctic vascular plants. Physiol. Plant. 115, 479–486. doi: 10.1046/j.1399-3054.2002.1150401.x

Allen, D. J., and Ort, D. R. (2001). Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends Plant Sci. 6, 36–42. doi: 10.1016/S1360-1385(00)01808-2

Aranas, S. K. T., Park, J. I., Ahmed, N. U., Jung, H. J., Hur, Y., Kang, K. K., et al. (2013). Characterization and expression analysis of dirigent family genes related to stresses in Brassica. Plant Physiol. Biochem. 67, 144–153. doi: 10.1016/j.plaphy.2013.02.030

Artus, N. N., Uemura, M., Stepoukous, P. L., Gilmour, S. J., Lin, C., and Thomashow, M. F. (1996). Constitutive expression of the cold-regulated Arabidopsis thaliana COR15a gene affects both chloroplast and protoplast freezing tolerance. Proc. Natl. Acad. Sci. U.S.A. 93, 13404–13409. doi: 10.1073/pnas.93.23.13404

Badawi, M., Danyluk, J., Boucho, B., Houde, M., and Sarhan, F. (2007). The CBF gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal CBFs. Mol. Genet. Genomics 277, 533–554. doi: 10.1007/s00438-006-0206-9

Bao, F., Du, D., An, Y., Yang, W., Wang, J., Cheng, T., et al. (2017). Overexpression of P. mume dehydrin genes in tobacco enhances tolerance to cold and drought. Front. Plant Sci. 8:151. doi: 10.3389/fpls.2017.00151

Battaglia, M., Bravo-Carrillo, Y., GarciaRRubio, A., Campos, F., and Covarrubias, A. A. (2008). The enigmatic LEA proteins and other hydrophilins. Plant Physiol. 148, 6–24. doi: 10.1104/pp.108.120725

Bravo, L. A., and Griffith, M. (2005). Characterization of antifreeze activity in Antarctic plants. J. Exp. Bot. 56, 1189–1196. doi: 10.1093/jxb/eri112

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00601/full#supplementary-material

AUTHOR CONTRIBUTIONS

Byun, M. Y., and Kim, W. T. (2014). Suppression of OsRAD51D results in defects in reproductive development in rice (Oryza sativa L.). Plant J. 79, 256–269. doi: 10.1111/tpj.12558

Byun, M. Y., Lee, J., Cui, L. H., Kang, Y., Oh, T. K., Park, H., et al. (2015). Constitutive expression of DaCBF7, an Antarctic vascular plant Deschampsia antarctica CBF homolog, resulted in improved cold tolerance in transgenic rice plants. Plant Sci. 236, 61–74. doi: 10.1016/j.plantsci.2015.03.020

Candat, A., Paszkiewicz, G., Neveu, M., Gastier, R., Logan, D. C., Avelange-Macherel, M. H., et al. (2014). The ubiquitous distribution of late embryogenesis abundant proteins across cell compartments in Arabidopsis offers tailored protection against abiotic stress. Plant Cell 26, 3148–3166. doi: 10.1105/tpc.114.127316

Chen, L., Han, J., Deng, X., Tan, S., Li, L., Li, L., et al. (2016). Expansion and stress responses of AP2/EREBP superfamily in Brachypodium distachyon. Sci. Rep. 6:21623. doi: 10.1038/srep21623

Claeys, H., and Inzé, D. (2013). The agony of choice: how plants balance growth and development. Plant Cell 25, 1768–1779. doi: 10.1105/tpc.113.1122092

Cui, M., Zhang, W., Zhang, Q., Xu, Z., Zhu, Z., Duan, F., et al. (2011). Induced over-expression of the transcription factor OsDREB2A improves drought tolerance in rice. Plant Physiol. Biochem. 49, 1384–1391. doi: 10.1016/j.phyto.2011.09.012

Dirra, M., Saibi, W., Brini, F., Gargouri, A., Masmoudi, K., and Hanin, M. (2013). The K-segments of the wheat dehydrin DHN-5 are essential for the protection of lactate dehydrogenase and β-glucosidase activities in vitro. Mol. Biotechnol. 54, 643–650. doi: 10.1007/s12033-012-9606-8

Dubozet, J. G., Sakuma, Y., Ito, Y., Kasuga, M., Dubozet, E. G., Miura, S., et al. (2003). OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J. 33, 751–763. doi: 10.1046/j.1365-313X.2003.01661.x

Fowler, S., and Thomashow, M. F. (2002). Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14, 1675–1690. doi: 10.1105/tpc.003483

Gao, S. Q., Chen, M., Xia, L. Q., Xu, H. J., Xu, Z., Li, L. C., et al. (2009). A cotton (Gossypium hirsutum) DRE-binding transcription factor gene, GhDREB,
confers enhanced tolerance to drought, high salt, and freezing stresses in transgenic wheat. Plant Cell Rep. 28, 301–311. doi: 10.1007/s00299-008-0623-9 Gielwanowska, L., Szczuka, E., Bednara, J., and Górecki, R. (2005). Anatomical features and ultrastructure of Deschampsia antarctica (Poaceae) leaves from different growing habitats. Ann. Bot. 96, 1109–1119. doi: 10.1093/abo/mci262

Gilmour, S. J., Fowler, S. G., and Thomashow, M. F. (2004). Arabinosid transcription activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol. Biol. 54, 767–781. doi: 10.1023/B:PLAN.0000040902.06881.84

Hillwig, M. S. (2009). Regulation, Function, and Evolution of T2 RNases. Graduate theses and dissertations. Available online at: http://fibrr.iastate.edu/etd/11095 Hundermark, M., and Hincha, D. K. (2008). LEA (late embryogenesis abundant) proteins and their encoding genes in Arabidopsis thaliana. BMC Genomics 9:118. doi: 10.1186/1471-2164-9-118

Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., et al. (2006). Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant Cell Physiol. 47, 141–153. doi: 10.1093/pcp/pcti230

Jaglo-Ottosen, K. R., Gilmour, S. J., Zarka, D. G., Schabenberger, O., and Thomashow, M. F. (1998). Arabinosid CBF1 overexpression induces COR genes and enhances freezing tolerance. Science 280, 104–106. doi: 10.1126/science.280.5360.104

Jeknić, Z., Pillman, K. A., Dhillon, T., Skinner, J. S., Veisz, O., Cuesta-Marcos, A., et al. (2014). Hv-CBF2A overexpression in barley accelerates COR gene transcript accumulation and acquisition of freezing tolerance during cold acclimation. Plant Mol. Biol. 84, 67–82. doi: 10.1007/s11103-013-0119-9

John, U. P., Polotnianka, R. M., Sivakumaran, K. A., Chew, O., Mackin, L., Kuiper, J. M., et al. (2009). Ice recrystallization inhibition proteins (IRIPs) and freeze tolerance in the cryophilic Antarctic hair grass Deschampsia antarctica E. Desv. Plant Cell Environ. 32, 336–348. doi: 10.1111/j.1365-3040.2009.01925.x

Kidrič, M., Kos, J., and Sabotić, J. (2014). Proteases and their endogenous inhibitors in the plant response to abiotic stress. Bot. Serb. 38, 139–158

Koagr, M. C., Fenton, R. D., Wilkens, S., and Close, T. J. (2003). The binding of late embryogenesis abundant proteins and their encoding genes in Arabidopsis thaliana. BMC Genomics 4:118. doi: 10.1186/1471-2164-9-118

Lee, J., Noh, E. K., Park, H., and Lee, H. (2013b). Transcription factor profile analysis of the Antarctic vascular plant Deschampsia antarctica Desv. (Poaceae). Genes Genom. 35, 575–586. doi: 10.1007/s13358-013-0116-6

Li, A., Zhou, M., Wei, D., Chen, H., You, C., and Lin, J. (2017). Transcriptome profiling reveals the negative regulation of multiple plant hormone signaling pathways elicited by overexpression of C-repeat binding factors. Front. Plant Sci. 8:1647. doi: 10.3389/fpls.2017.01647

Liu, Y., Song, Q., Li, D., Yang, X., and Li, D. (2017). Multifunctional roles of plant dehydrins in response to environmental stresses. Front. Plant Sci. 8:1018. doi: 10.3389/fpls.2017.01018

Misra, R. C., Kamthan, M., Kumar, S., and Ghosh, S. (2016). A thraumat-like protein of Ocimum basilicum confers tolerance to fungal pathogen and abiotic stress in transgenic Arabidopsis. Sci. Rep. 6:25340. doi: 10.1038/srep25340

Molina, A., Diaz, I., Carbonero, P., García-Olmedo, F., and Vasil, I. K. (1996). Two cold-inducible genes encoding lipid transfer protein LTP4 from barley show differential responses to bacterial pathogens. Mol. Gen. Genet. 252, 162–168. doi: 10.1007/BF02173216

Morran, S., Eini, O., Pyvovenenko, T., Parent, B., Singh, R., Ismagul, A., et al. (2011). Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotechnol. J. 9, 230–249. doi: 10.1111/j.1467-7652.2010.00547.x

Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621–628. doi: 10.1038/nmeth.1226

Oh, S. J., Kwon, C. W., Choi, D. W., Song, S. I., and Kim, J. K. (2007). Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice. Plant J. 5, 646–656. doi: 10.1111/j.1365-7652.2007.02272.x

Oh, S. J., Song, S. I., Kim, Y. S., Jang, H. J., Kim, S. Y., Kim, M., et al. (2005). Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. Plant Physiol. 138, 341–351. doi: 10.1109/p11.04380

Pastorczyk, M., Gielwanowska, L., and Lahuta, L. B. (2014). Changes in soluble carbohydrates in polar Caryophyllaceae and Poaceae plants in response to chilling. Acta Physiol. Plant. 36, 1771–1780. doi: 10.1007/s11738-014-1551-7

Rawlings, N. D. (2010). Pepidase inhibitors in the MEROPS database. Biochimie 92, 1463–1483. doi: 10.1016/j.biochi.2010.04.013

Sánchez, P. L., Bravo, L. A., Cavieres, L. A., Vallejos, V., Sanhueza, C., Font-Carrascosa, M., et al. (2017). Photosynthetic limitations in two Antarctic vascular plants: importance of leaf anatomical traits and Rubisco kinetic parameters. J. Exp. Bot. 68, 2871–2883. doi: 10.1093/jxb/erx148

Sasaski, K., Christov, N. K., Tsuda, S., and Imai, R. (2014). Identification of a novel LEA protein involved in freezing tolerance in wheat. Plant Cell Physiol. 55, 136–147. doi: 10.1093/pcp/pcu164

Scherzer, L. V., Gusta, K. K., and M. E. Wisniewski (Cambridge: CAB International), 119–130.

Tanino, and M. E. Wisniewski (Cambridge: CAB International), 119–130.

Tanino, and M. E. Wisniewski (Cambridge: CAB International), 119–130.

Tillmann, M., and Hincha, D. K. (2008). LEA (late embryogenesis abundant) proteins and their encoding genes in Arabidopsis thaliana. BMC Genomics 9:118. doi: 10.1186/1471-2164-9-118

Tolonen, K. A., Pillman, K. A., Dhillon, T., Skinner, J. S., Veisz, O., Cuesta-Marcos, A., et al. (2014). Hv-CBF2A overexpression in barley accelerates COR gene transcript accumulation and acquisition of freezing tolerance during cold acclimation. Plant Mol. Biol. 84, 67–82. doi: 10.1007/s11103-013-0119-9

Tian, L., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., et al. (2017). agrigGO v2.0: A GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res. 45, W122–W129. doi: 10.1093/nar/gkx382
Tondelli, A., Francia, E., Barabaschi, D., Pasquariello, M., and Pecchioni, N. (2011). Inside the CBF locus in Poaceae. *Plant Sci.* 180, 39–45. doi: 10.1016/j.plantsci.2010.08.012

Tsonev, T., Velikova, V., Georgieva, K., Hyde, P. F., and Jones, H. G. (2003). Low temperature enhances photosynthetic downregulation in French bean (*Phaseolus vulgaris* L.) plants. *Ann. Bot.* 91, 343–352. doi: 10.1093/oxb/1mcg20

Vágújfalvi, A., Aprile, A., Miller, A., Dubcovsky, J., Delugu, G., Galiba, G., et al. (2005). The expression of several Cbf genes at the Fr-A2 locus is linked to frost resistance in wheat. *Mol. Genet. Genomics* 274, 506–514. doi: 10.1007/s00438-005-0047-y

Wang, X. S., Zhu, H. B., Jin, G. L., Liu, H. L., Wu, W. R., and Zhu, J. (2007). Genome-scale identification and analysis of LEA genes in rice (*Oryza sativa* L.). *Plant Sci.* 172, 414–420. doi: 10.1016/j.plantsci.2006.10.004

Wei, T., Deng, K., Zhang, Q., Gao, Y., Liu, Y., Yang, M., et al. (2017). Modulating AtDREB1C expression improves drought tolerance in *Salvia miltiorrhiza*. *Front. Plant Sci.* 8:52. doi: 10.3389/fpls.2017.00052

Wu, G., Robertson, A. J., Liu, X., Zheng, P., Wilen, R. W., Nesbitt, N. T., et al. (2004). A lipid transfer protein gene BG-14 is differentially regulated by abiotic stress, ABA, anisomycin, and sphingosine in bromegrass (*Bromus inermis*). *J. Plant Physiol.* 161, 449–458. doi: 10.1078/0176-1617-01259

Wu, R., Wang, L., Wang, Z., Shang, H., Liu, X., Zhu, Y., et al. (2009). Cloning and expression analysis of a dirigent protein gene from the resurrection plant *Boea hygrometrica*. *Prog. Nat. Sci.* 19, 347–352. doi: 10.1016/j.pnsc.2008.07.010

Xu, M., Li, L., Fan, Y., Wan, J., and Wang, L. (2011). ZmCBF3 overexpression improves tolerance to abiotic stress in transgenic rice (*Oryza sativa*) without yield penalty. *Plant Cell Rep.* 30, 1949–1957. doi: 10.1007/s00299-011-1103-1

Zheng, J., Wang, Y., He, Y., Zhou, J., Li, Y., Liu, Q., et al. (2014). Overexpression of an S-like ribonuclease gene, OsRNS4, confers enhanced tolerance to high salinity and hyposensitivity to phytochrome-mediated light signals in rice. *Plant Sci.* 214, 99–105. doi: 10.1016/j.plantsci.2013.10.003

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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