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Cloning and Functional Analysis of three Cold Regulated *CBF* Genes in the Overwintering Crucifer *Boechera stricta*

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Abstract

In this research, we isolated three *CBF* (C-repeat-Binding Factors) genes from two genotypes of *Boechera stricta* with contrasting freezing tolerance and characterized their structure and expression patterns in response to cold treatment. An amino acid sequence comparison revealed that the *CBF* genes in *B. stricta* showed high conservation in the AP2 domain and PKKP/RAGR motif like other cold adaptable *Brassicaceae*. The pairwise sequence alignment of the *CBF* genes isolated from two genotypes of *B. stricta* showed non-synonymous mutations in *CBF* 2 and 3. Gene expression analysis demonstrated that *CBF* genes in *B. stricta* have expression patterns similar to *CBF*s in *A. thaliana* in response to cold treatment, while differential expression at the molecular level in *CBF* and *COR* genes was presented between two genotypes of *B. stricta*. Our results suggest that signal transduction of three *CBF* genes can be one of the central pathways in the development of freezing tolerance in *B. stricta*. © 2018 Friends Science Publishers

Keywords: *Boechera strict*; *CBF*; Gene expression; Phylogenetic tree

Introduction

Frost is one of the most important environmental factors affecting the geographical distribution of overwintering plant species. Most temperate plants enhance their freezing tolerance through an adaptive process known as cold acclimation, a response to low but non-freezing temperatures that occurs before freezing (Xin and Browse, 2000). This adaptive process involves various biochemical and physiological changes, including increased levels of solutes, the modification of membrane lipid composition and the accumulation of secondary metabolites (Guy, 1990). The precise regulation of cold acclimation is still unknown, but it has been assumed that some genes responding to low temperature can be associated with this process (Chinnusamy *et al.,* 2003; Zhu *et al.,* 2007; Winfield *et al.,* 2010; Wang *et al.,* 2013; Le *et al.,* 2014). Hence, identifying genes regulated by low temperature can improve the current understanding of mechanisms of freezing tolerance.

In *Arabidopsis thaliana* (*A. thaliana*) and *Medicago truncatula*, major QTLs responsible for a large proportion of the variation in freezing tolerance have been identified and linked to variation in C-repeat-binding factors (*CBF*s) (Alonso-Blanco *et al.,* 2005; Tayeh *et al.,* 2013). In *A. thaliana,* three *CBF*s occur in a tandem array (*CBF*1, *CBF*2 and *CBF*3) in the following order: *CBF*1 ->*CBF*3->*CBF*2. These three *CBF* genes belong to the AP2/EREBP family of DNA-binding proteins and can bind to the C-repeat (CRT)/dehydration responsive element (DRE), a cis-acting element contained in numerous downstream genes that influence the transmission of cold signals and regulates the expression of related proteins (Maruyama *et al.,* 2004; Xu *et al.,* 2011). Transgenic over-expression of *CBF*1and *CBF*3 enhanced cold tolerance by regulating approximately 100 cold-responsive (*COR*) genes and leading to the accumulation of sugar and proline (Jaglo-Ottosen *et al.,* 1998; Liu *et al.,* 1998; Kasuga *et al.,* 1999; Gilmour *et al.,* 2000). A*CBF* pathway responding to low temperature was also found in poplar, wheat, rye and *Brassica napus*, all of which are freezing tolerant (Skinner *et al.,* 2005), and even in tomato and rice which are freezing sensitive (Zhang *et al.,* 2004). The expression patterns of the *CBF* and *COR* genes in other freezing tolerant species were similar to those of *Arabidopsis* and the core regions within the *CBF* genes were highly conserved (Welling and Palva, 2008). In contrast, freezing sensitive species such as tomato exhibited a reduced *CBF* regulon and induced fewer cold-responsive genes, which likely contribute to their freezing sensitivity (Dubouzet *et al.,* 2003; Zhang *et al.,* 2004). Hence, *CBF* genes are thought to play a pivotal role in integrating the activation of multiple components for the development of freezing tolerance in plants.

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Boechera stricta (*B. stricta*) belongs to the *Brassicaceae* family and is a genetically tractable, short-lived perennial species found in mostly undisturbed habitats of the Rocky Mountains. It occurs along a wide elevational gradient and is found in locations with varying abiotic and biotic conditions (Anderson *et al.,* 2012). Recently, the LTM line, one of the *B. stricta* genotypes used in this study, has been fully sequenced with the Roche 454 platform by the Department of Energy Joint Genome Institute and with Sanger BAC end-sequences by Hudson Alpha Institute for Biotechnology (Lee and Mitchell-Olds, 2013). Previously, extensive comparative analyses with *Arabidopsis* were done for *B. stricta*, providing access to information and techniques from *Arabidopsis* and facilitating molecular genetic studies to understand ecologically important traits (Schranz *et al.,* 2007; Rushworth *et al.,* 2011). Previous studies mainly focused on understanding genetic variation of flowering time and glucosinolates (Schranz *et al.*, 2009; Prasad *et al*., 2012; Lee *et al*., 2014), but no attention has been made on freezing tolerance in *B. stricta*. In an attempt to elucidate the genetic determinants of freezing tolerance in two genotypes of *B. stricta*, we identified the QTL locus responsible for freezing tolerance in *Boechera stricta* (Heo *et al*., 2014). The major locus we have found is syntenic with a genomic region in *A. thaliana* that contains *CBF* genes. Thus, in this study, we isolated *CBF*-type genes, *BsCBF*1, 2 and 3 and characterized their expression patterns under cold treatment by polymerase chain reaction (PCR) and real-time polymerase chain reaction (RT-PCR) in order to understand development of freezing tolerance in B. *stricta* at molecular level. Based on the cDNA sequences, we inferred amino acid sequences and analyzed the structure and phylogenetic positions of these three genes. To date, this is the first study of *CBF*-type transcriptional factors in *B. stricta*. Our results could help to enhance the understanding of the evolution of cold stress-related genes in *Brassicaceae*.

Materials and Methods

Study Species and Experimental Condition

Two genotypes of *B. stricta*, LTM and SAD12, and one *A. thaliana* ecotype, Columbia (Col), were used in this study. Details about plant locations and growth environments for two genotypes of *B. stricta* were previously described by Schranz *et al.* (2007). All plants were grown on agar plates for the experiment. Seeds were surface sterilized by using 10% (v/v) bleach solution for 8 min and washed three times with deionized water. The seeds were put on 0.8% agar (Hispanagar, Burgos, Spain) and 0.5X MS media (Duchefa, Haarlem, The Netherlands) containing 30 mg L^{-1} kanamycin. Plated seeds were kept at 4°C for 7 days before they were transferred to a growth chamber and grown at 20°C under short-day photoperiods (8 h of cool-white fluorescent light, photon flux of 100 µmol m^{-2} s^{-1}). A low temperature treatment was imposed by transferring 18-day-old seedlings to a cold chamber at 4°C under the same light and photoperiodic conditions. Leaves were harvested after 0, 3 h, 8 h, 12 h, 24 h and 48 h of cold treatment. Harvested leaves were quickly frozen in liquid nitrogen and stored at −80°C until further use.

Isolation, Sequence Alignment and Phylogenetic Analysis of CBF Genes in *Boechera stricta*

A draft genome of LTM, sequenced by Department of Energy Joint Genome Institute and HudsonAlpha Institute for Biotechnology, was utilized to isolate *CBF*1, 2 and 3 genes from two genotypes of *B. stricta*. The scaffold containing *CBF*1, 2 and 3 genes in *B. stricta* was provided from Mitchell-Olds laboratory at Duke University. Primers for isolating genomic DNAs of *CBF*1, 2 and 3 from SAD12 were designed using Primer3 software based on the LTM scaffold sequence. Genomic DNAs of LTM and SAD12 genotypes were isolated from DNeasy Plant Mini Kit (Qiagen) and the products were purified using GeneJET PCR Purification Kit according to the manufacturer's instruction (Thermo Scientific). DNA sequencing of the products was done by GATC Biotech, Germany. After comparing the alignment of genomic DNAs between LTM and SAD12, full-length cDNAs of three *CBF* genes in two genotypes of *B. stricta* were synthesized from RNA of leaves of LTM and SAD12 exposed to cold, from which amino acid sequences were inferred.

The amino acid sequences of *CBF*1, 2 and 3 in *B. stricta* were used as query sequences for searching homologue *DREB*1/*CBF* genes in *Brassicaceae*. The survey was conducted against the GenBank (http://www.ncbi.nlm.nih.gov/blast/) or Brassica Database (http://brassicadb.org), and sequences were aligned using the MAFFT program (http://mafft.cbrc.jp/). The initial alignments were improved manually and saved in FASTA or NEXUS formats. Find Model (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was used to identify the best base-substitution models for distance analysis and reconstructing gene phylogenies. Bayesian inference method as implemented in MrBayes (v3.1.2) was utilized to construct gene trees and estimate clade support (Ronquist and Huelsenbeck, 2003).

Gene Expression Analysis of *CBF* and *COR* Genes in *Boechera* and *Arabidopsis*

The expression of *CBF* genes in *B. stricta* and *A. thaliana* during exposure of low temperature was evaluated using reverse transcription-quantitative real-time PCR analysis (RT-PCR). Total RNA was isolated from frozen samples with the RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's instructions, and treated with RNA-free DNAase I to remove genomic DNA. The quality and concentration were measured using a Nano-drop and then cDNA was synthesized with oligod $(T)_{18}$ primer

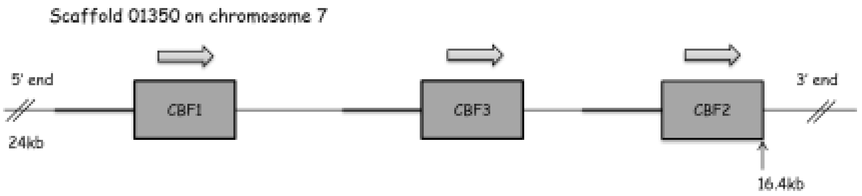

Fig. 1: Genomic map of CBFs in *B. stricta*

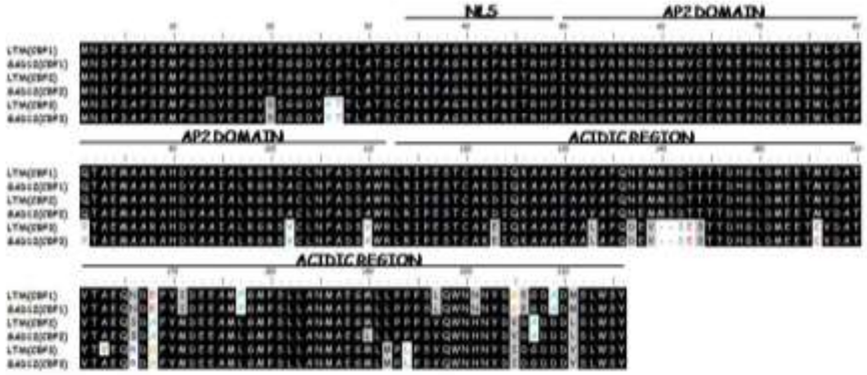

Fig. 2: Alignment of the inferred amino acid sequences of CBFs in two genotypes of *B. stricta*

and SuperScript® III Reverse Transcriptase (Life Technologies, USA) from 5 µg of total RNA. Subsequently, the cDNA was utilized to conduct real time PCR using gene-specific primers of *CBF*1, 2 and3genes in *B. stricta* and *A. thaliana*. Specific primers for *B. stricta* were designed based on the conserved regions within genotypes, whereas for *A. thaliana* they were adapted from earlier studies. In addition, we tested the potential regulatory effects of the three *BsCBF* genes by analysing the transcript levels of several down-stream, cold stress-responsive genes including *COR*15A, *COR*15B, *COR*47, and *COR*78, as known hallmarks of freezing stress adaptation in plants (Shinozaki and Yamaguchi-Shinozaki, 1996). Gene specific primers for investigating their gene expressions were generated using the draft genome of LTM. 1 µL of cDNA template was amplified using the Platinum SYBR Green qPCR supermix-UDG (Invitrogen, the Netherlands) in a 20 µL qPCR reaction according to the manufacturer's protocol. The samples were amplified with PCR as follows: 3 min 50°C, 5 min 95°C, 40 cycles of 15 sec at 95°C followed by 1 min 60°C. Melting curve analyses were performed on the PCR products. Actin2 was used as the reference gene to calculate relative expression levels, using the ΔΔCt method (Livak and Schmittgen, 2001). Three RT-PCR runs were performed per genotype/treatment combination.

Results

In order to identify *CBF*1, 2 and 3 genes in *B. stricta,* we blasted the draft genome information of LTM against the *Arabidopsis* genome database. The *CBF*1, 2 and 3genes in *B. stricta* are physically organized in a tandem array, as is the case in *A. thaliana* (Fig. 1). The complete coding sequences of the three *CBF* genes was inferred from cDNA synthesized from the LTM RNA of leaves exposed to cold, using gene specific primers. The full-length cDNAs of *CBF*1, 2 and 3 in LTM were 651, 651 and 645 bp, encoding 217, 217 and 215 amino acids, respectively. The cDNA sequence alignment of LTM *with CBF*1, 2 and 3 genomic DNA sequences indicated that the *CBF*1, 2 and 3 genes in *B. stricta* included no intron. Specific primer pairs for these genes were also used to amplify the corresponding genes from cold treated leaves of SAD12. The *CBF* cDNA amplicons of SAD12 all had the same length as the corresponding LTM. The pairwise sequence alignment of the isolated *CBF*1 gene from SAD12 revealed that the *CBF*1 gene of SAD12 was identical to LTM. Three SNPs were present in the *CBF*2 gene of SAD12 at positions 54, 567 and 615. A variant of C/G and C/T at positions 54 and 615 led to synonymous mutations, whereas a nucleotide transition from A to T at position 567 led to a non-synonymous mutation from methionine to leucine. In SAD12 *CBF*3, a single T/C variant was observed at position 482, which led to a non-synonymous mutation from valine to alanine. In conclusion, there were only minor differences in *CBF* genes between two genotypes of *B. stricta*.

Analyses of the predicted amino acid sequences of *CBF*1, 2 and 3 in *B. stricta* revealed that they consist of a putative nuclear localization, an AP2 DNA binding domain and a putative acidic activation domain, and have two *CBF* signature sequences, PKKR/PAGR and DSAWR (Fig. 2). When compared to the *Brassicaceae DREB*1/*CBF* amino acid sequences, pairwise amino acid comparison showed that the AP2 DNA binding domain shared remarkably high

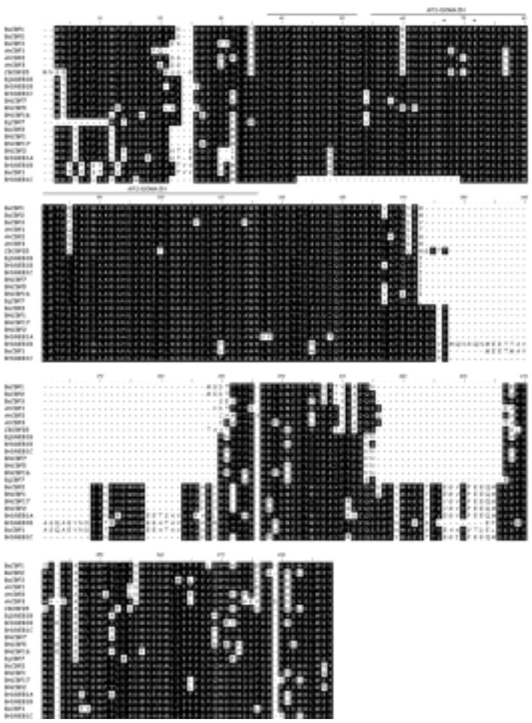

Fig. 3: Alignment of the inferred amino acid sequences of *Boechera* CBFs (LTM) with *Brassicaceae* CBFs. At: *Arabidopsis thaliana*, BJ: *Brassica juncea*, BN: *Brassica napus*, BR: *Brassica rapa*, BO: *Brassica oleracea*, Bs: *Boechera stricta*, CB: *Capsella bursa-pastoris*

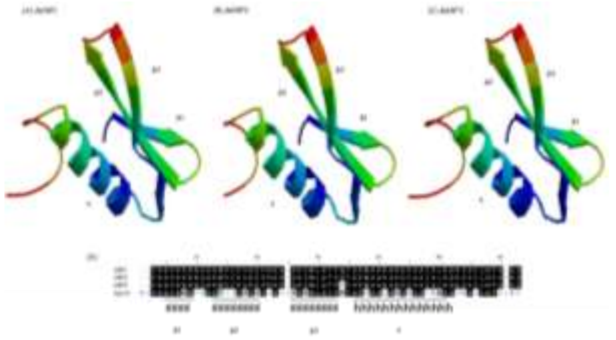

Fig. 4: Molecular models of the AP2 DNA binding domain of *Bs*CBF1 (a), 2 (b) and 3 (c) that modeled by SWISS MODEL and alignment analysis (D) of the AP2 DNA binding domain sequence of *At*ERF1 (PDB ID: 1gccA), *Bs*CBF1, *Bs*CBF2 and *Bs*CBF3

degree of sequence identity with CBFs in *A. thaliana* (Fig. 3). In addition, secondary structure analysis revealed that AP2 DNA binding domains of *CBF*1, 2 and 3 in *B. stricta* contained three-stranded, antiparallel β-sheets and an α-helix (Fig. 4). To further understand the evolution and origin of *CBF*-type genes isolated from *B. stricta*, phylogenetic relationships were investigated using 19 *CBF* aligned amino acid sequences from various *Brassicaceae* species (Fig. 5).

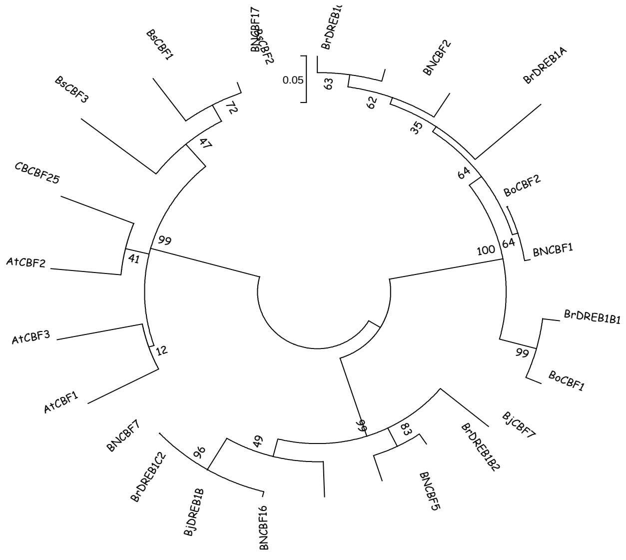

Fig. 5: An un-rooted phylogenetic tree of the DBEB1/CBF transcription factors of *Brassicaceae*. The amino acid sequences of full length of 22 *Brassicaceae* DREB1/CBF proteins were aligned by MAFFT, and the phylogenetic tree was constructed using MrBayes (v3.1.2). Bootstrap values from 1000 replicates were used to assess the robustness of the trees. Branch lengths indicate genetic distance. The Genbank accession numbers or BRAD gene ID of the different genes used for this analysis are:*AtCBF1* (NM118681), *AtCBF2* (NM118679), *AtCBF3* (NM118680), *BjCBF7* (AY887137), *BjDREB1B* (EU136731), *BnCBF1* (AF370733), *BnCBF2* (AF370734), *BnCBF5* (AF499031), *BnCBF16* (AF499033), *BnCBF17* (AF499034), *BoCBF1* (AF370731), *BoCBF2* (AF370732), BrDREB1A (Bra010461), BrDREB1B1 (Bra010460), BrDREB1B2 (Bra022770), BrDREB1C1 (Bra010463), BrDREB1C2 (Bra028290), CBCBF25 (AY491498). At: *Arabidopsis thaliana*, Bj: *Brassica juncea*, Bn: *Brassica napus*, Br: *Brassica rapa*, Bo: *Brassica oleracea*, Bs: *Boechera stricta*, Cb: *Capsella bursa-pastoris*

Our results indicated that *CBF*1, 2 and 3 genes in *B. stricta* were closely related to *AtCBF*1, 2 and 3 genes, and the *Capsella-bursa pastoris CBF*25 gene, although their precise relationship could not be resolved from comparing amino acid sequences.

Our *CBF* gene expression data showed that all *CBF* genes in *B. stricta* were transiently induced by cold treatment, and the expression kinetics were similar to those of *CBF*1, 2 and 3 transcripts in *A. thaliana* (Fig. 6 and 7). The *CBF*1, 2, and 3 transcripts in *B. stricta* were almost undetectable under the control condition, but they reached the highest level at 3 h after exposure to low temperature in both genotypes and then showed a gradual decrease approaching 12 h. Interestingly, expression of the *CBF*2 and *CBF*3 genes at 3 h and 8 h, respectively after cold treatment was significantly higher in LTM than in SAD12. Moreover,

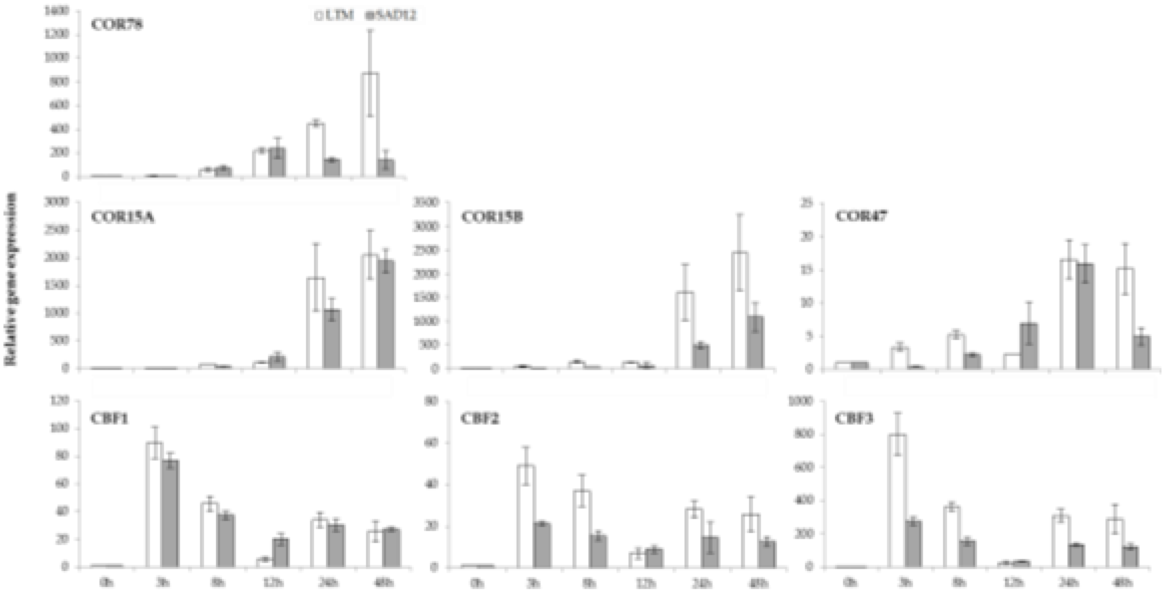

Fig. 6: Time course expression profile for *Bs*CBF and cold responsive genes in leaves of plants shiftedto4°C at LTM verse SAD12. Actin 2 was used as the reference gene for two genotypes. Values are expression relative to the T0 time points (control) for each gene at LTM or SAD12

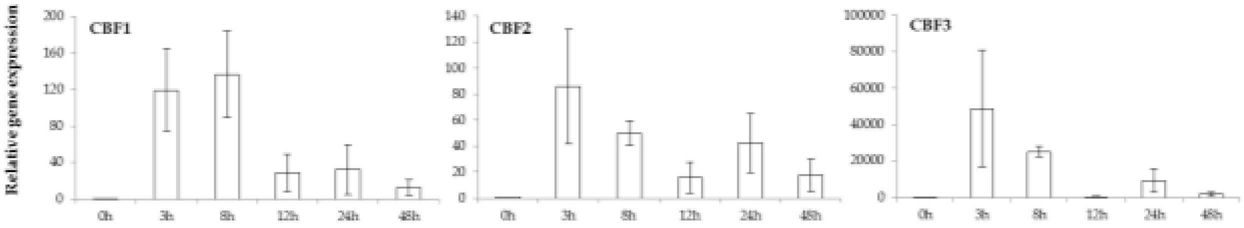

Fig. 7: Time course expression profile for *AtCBF* genes in leaves of plants shifted to 4°C at LTM verse SAD12. Actin 2 was used as the reference gene for two genotypes. Values are expression relative to the T0 time points (control) for each gene

expression after 24 h and 48 h remained higher than the initial control levels, especially for LTM. Previous research showed that contrasting regulation of *CBF* genes resulted in the activation and the differential expression of downstream target genes. Hence, we further examined the expression patterns of five cold stress-responsive target genes. Under cold condition, the activation of selected cold stress-responsive genes was also observed in two genotypes of *B. stricta* (Fig. 6). Although the expression of all of these genes was detected within 3 h, most of cold stress-responsive genes, except for *COR*47, showed the highest levels in expression after 2 days of cold treatment. The expression levels of *COR*15B, *COR*47 and *COR*78 in the LTM were gradually higher than in SAD12 during the cold treatments.

Discussion

In the previous study, we identified the QTLs that determine the genotype differences in freezing tolerances by mapping a population of *B. stricta* and confirming a QTL corresponding to a syntenic region containing *CBF* genes in *A. thaliana* that explained a major effect on genotype differences in freezing tolerance. In *A. thaliana*, the major freezing tolerance QTL was also associated with three tandem-repeated *CBF* genes, and *CBF* genes have been characterized as an important regulator for development of freezing tolerance among various plant species (Alonso-Blanco *et al.*, 2005; Miura and Furumoto, 2013). Based on the possibility that tandem-repeated *CBF* genes are involved in the development of freezing tolerance in *B. stricta*, we isolated three *CBF* genes and characterized their structure and expression patterns. Our bioinformatics analysis revealed that *CBF* genes in *B. stricta* have motifs typical of the *CBF* transcription factors in plants (Medina *et al.*, 2011). Motif represents the common pattern to a set of nucleic or amino acid sequences that share some biological property (Bailey and Elkan, 1995). Hence, the compositions and distributions of the motif among a set of nucleic or amino acid sequences indicate, to a certain extent, the structural and functional similarity. We found that PKKP/RAGR signature sequences bordering the AP2 DNA binding domain were 100% identical with those of the *AtCBF*s. The

PKKP/RAGR motif located immediately upstream of the AP2 DNA binding domain might function as a NLS and has recently turned out to be essential for transcriptional activity of *AtCBF* proteins (El-Kayal *et al.,* 2006; Canella *et al.,* 2010). We also observed that the secondary structure of AP2 DNA binding domains in *BsCBF*s had three-stranded antiparallel β-sheets connected by loops and an α-helix and, participated in interactions with DNA and other transcriptional factors, resembling *CBF* genes in *A. thaliana*. Our results additionally showed that *CBF* genes in *B. stricta* had Valine-14 and Glutamic-19 amino acid residues. They are distinct from alanine and aspartic acid of ERF protein and are essential for its binding specificity (Sakuma *et al*., 2002). Because *BsCBFs* contained the same conserved V14 and E19 at these two positions, it indicated that it might have similar binding patterns as *CBF*s of *Arabidopsis* to DRE/CRT motif in the promoter of some downstream stress-induced genes. Furthermore, our phylogenetic analysis demonstrated that *CBF* genes in *B. stricta* have a close evolutionary relationship with *CBF* genes in *A. thaliana*. Further evidence found in bioinformatics analyses strongly implies that *CBF*s in *B. stricta* may have the same DNA binding specificity as *CBF*s in *A. thaliana*.

In this study, we also examined the relative expression levels of *CBF*1, 2 and 3 in *B. stricta* to investigate whether *CBF* genes in *B. stricta* are induced in response to cold, and if expression levels differ between the two genotypes. The gene expression level in leaves was quickly upregulated and reached peak level at 3 h, and *CBF* genes in *B. stricta* had similar expression behaviors as *CBF*s in other plant species. In addition, our gene expression study clearly exhibited differential expression at the molecular level in *CBF*2 and 3 between two genotypes of *B. stricta*, and differences in expression of *COR* genes existed. Interestingly, expression of these genes was consistently higher in LTM than in SAD12. These results indicated that *CBF* genes are involved in the cold acclimation process in *B. stricta* and can be an important factor underlying the differential freezing tolerance of *B. stricta*. Over-expression of *CBF* and *COR* genes results in accumulation of metabolites and enzymes for sugar metabolism and fatty acid desaturation, components that are essential for winter survival in plants (Cook *et al.,* 2004; Maruyama *et al.,* 2009). Increased levels of expression of *CBF* and *COR* genes have already been shown to correlate with enhanced freezing tolerance during cold acclimation in *A. thaliana* (Jaglo-Ottosen *et al.,* 1998; Kasuga *et al.,* 1999; Zuther *et al.,* 2012). Similarly, we also observed that LTM developed much higher levels of freezing tolerance than SAD12 during seedling as well as adult stages of cold acclimation, based on previous studies (Heo *et al.,* 2014). Hence, differential regulation patterns in two genotype of *B. stricta* with contrasting freezing tolerance strongly suggest that the signal transduction of *CBF* genes based on genetic background is one of the central pathways in the development of freezing tolerance in *B. stricta*.

Although we found significant differences in the expression of *BsCBF*2 and 3 genes, we only found minor genotypic sequence differences in *BsCBF*2 and *CBF*3. These results imply that even though *BsCBF* genes have similar structures, their binding specificity to the CRT/DRE element can be different. Responses leading to different freezing tolerances can thus be due to presence/absence of binding sites in the promoter region (Dubouzet *et al*., 2003) rather than differences in the *CBF* proteins. To confirm this, it is necessary to study promoter regions in the future.

Conclusion

Three *CBF* genes were isolated from two genotypes of *B. stricta*, and their structure and expression patterns were characterized. Three *CBF* genes were successfully sequenced, but only minor differences on sequences of three *CBF* genes were found between two genotypes of *B. stricta*. However, gene expression analysis showed that a genotype difference between LTM and SAD12 was obvious in the levels of *CBF* genes and *COR* genes. A three dimensional structural model indicated that *CBF* genes in *B. stricta* contained highly conserved AP2 DNA binding domains that have crucial roles in DNA binding and the activation of cold responsive genes for development of freezing tolerance in *AtCBF* genes. It therefore, is likely that the *CBF* genes are one of important genetic components driving differential freezing tolerance in *B. stricta*.

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