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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 CBFs 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 (CBFs) (Alonso-Blanco et al., 2005; Tayeh et al., 2013). In A. thaliana, three CBFs occur in a tandem array (CBF1, CBF2 and CBF3) in the following order: CBF1 ->CBF3->CBF2. 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 CBF1 and CBF3 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). ACBF 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.

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, BsCBF1, 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 \(\mu\)mol 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 CBF1, 2 and 3 genes from two genotypes of B. stricta. The scaffold containing CBF1, 2 and 3 genes in B. stricta was provided from Mitchell-Olds laboratory at Duke University. Primers for isolating genomic DNAs of CBF1, 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 DNAeasy 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 CBF1, 2 and 3 in B. stricta were used as query sequences for searching homologue DREB1/CFB 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/findmo del.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 DNAse 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
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 CBF1, 2 and 3 genes 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 downstream, cold stress-responsive genes including COR15A, COR15B, COR47, and COR78, 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 CBF1, 2 and 3 genes in B. stricta, we blasted the draft genome information of LTM against the Arabidopsis genome database. The CBF1, 2 and 3 genes 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 CBF1, 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 CBF1, 2 and 3 genomic DNA sequences indicated that the CBF1, 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 CBF1 gene from SAD12 revealed that the CBF1 gene of SAD12 was identical to LTM. Three SNPs were present in the CBF2 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 CBF3, 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 CBF1, 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 DREB1/CBF amino acid sequences, pairwise amino acid comparison showed that the AP2 DNA binding domain shared remarkably high
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degree of sequence identity with CBFs in A. thaliana (Fig. 3). In addition, secondary structure analysis revealed that AP2 DNA binding domains of CBF1, 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).

Our results indicated that CBF1, 2 and 3 genes in B. stricta were closely related to AtCBF1, 2 and 3 genes, and the Capsella bursa-pastoris CBF25 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 CBF1, 2 and 3 transcripts in A. thaliana (Fig. 6 and 7). The CBF1, 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 CBF2 and CBF3 genes at 3 h and 8 h, respectively after cold treatment was significantly higher in LTM than in SAD12. Moreover,
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 COR47, showed the highest levels in expression after 2 days of cold treatment. The expression levels of COR15B, COR47 and COR78 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 AtCBFs. 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 BsCBFs 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 CBFs 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 CBFs in *B. stricta* may have the same DNA binding specificity as CBFs in *A. thaliana*.

In this study, we also examined the relative expression levels of CBF1, 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 CBFs in other plant species. In addition, our gene expression study clearly exhibited differential expression at the molecular level in CBF2 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 BsCBF2 and 3 genes, we only found minor genotypic sequence differences in BsCBF2 and BsCBF3. 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*.

**References**


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