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Characterization of *BdCBF* genes and genome-wide transcriptome profiling of *BdCBF3*-dependent and -independent cold stress responses in *Brachypodium distachyon*

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ABSTRACT

Freezing stress substantially reduces crop yields and limits plant distribution. The identification of genes critical for cold acclimation is thus of great importance. C-repeat binding factors (CBFs) are transcription factors that play key regulatory roles in the cold acclimation process, which dramatically increases freezing tolerance in plants. We report here that *B. distachyon* can successfully cold acclimate and we identified a *CBF* gene family consisting of eight genes in a tandem array and are designated as *BdCBF1-8*. Expression analysis indicated that all the eight *BdCBF* genes are induced by cold. Freezing tolerance experiments showed that the knockdown of *BdCBF3* gene in RNAi *cbf3* mutant plants results in a significant reduction in survival after an exposure to freezing temperatures. RNA-seq transcriptomic analysis was conducted using the wild type and *cbf3* mutant plants under both normal and cold conditions. We identified 460, 3213, 2839 and 1871 differentially expressed genes exhibiting different expression levels by pairwise comparisons of *cbf3* (23 °C) vs. WT (23 °C), WT (23 °C) vs. WT (4 °C), *cbf3* (23 °C) vs. *cbf3* (4 °C), and *cbf3* (4 °C) vs. WT (4 °C), respectively. These differentially expressed genes were enriched in several biological pathways. Combined analyses of differentially expressed genes in some of the enriched pathways provide insights into mechanisms of plant response to cold in the *BdCBF3*-dependent, –independent or –compensatory categories.

1. Introduction

Low temperature is one of the environmental stresses that adversely affect plant growth, development, distribution and productivity. Significant crop damages casued by freeze has resulted in an estimated of up to one billion dollars annual losses in the U.S. Plants grown in temperate regions in particular need to undergo the transition period between warm and cold seasons during which the temperatures can change drastically. Most temperate plants such as wheat (*Triticum aestivum* L.), canola (*Brassica napus* L.), and *Arabidopsis* have evolved the ability to tolerate chilling and freezing temperatures with a period of exposure to low, but nonfreezing temperatures, which is known as cold acclimation, an adaptive process [1–3]. Without cold acclimation, winter rye (*Secale cereale* L.), for example, is killed by freezing at about −5 °C but it can survive freezing below −20 °C following a period of cold acclimation [4]. Multiple physiological and biochemical changes are involved during cold acclimation. These include modifications in lipid compositions such as increases of unsaturated fatty acid content in cell membranes to maintain membrane fluidity; changes in protein and carbohydrate composition; accumulations of anti-freezing and anti-oxidative substances [5,6]. Most of these changes are the results of transcriptional regulations of a large class of cold-regulated (*COR*) genes, whose expression is induced by cold acclimation and play critical roles in protecting plants against cold stress [3]. Most of the *COR* genes have copies of a C-repeat/dehydration-responsive element (CRT/DRE) in their promoter regions, which contains the core motif of G/ACCGAC [7,8] and is responsible for the low temperature-responsiveness of these genes.

The CRT-binding factors/DRE-binding proteins 1 (CBF/DREB1) function as transcription factors and bind to the CRT/DRE *cis*-acting element present in *COR* genes [9,10]. *CBF/DREB1* genes have been isolated from a wide range of plants and are involved in cold

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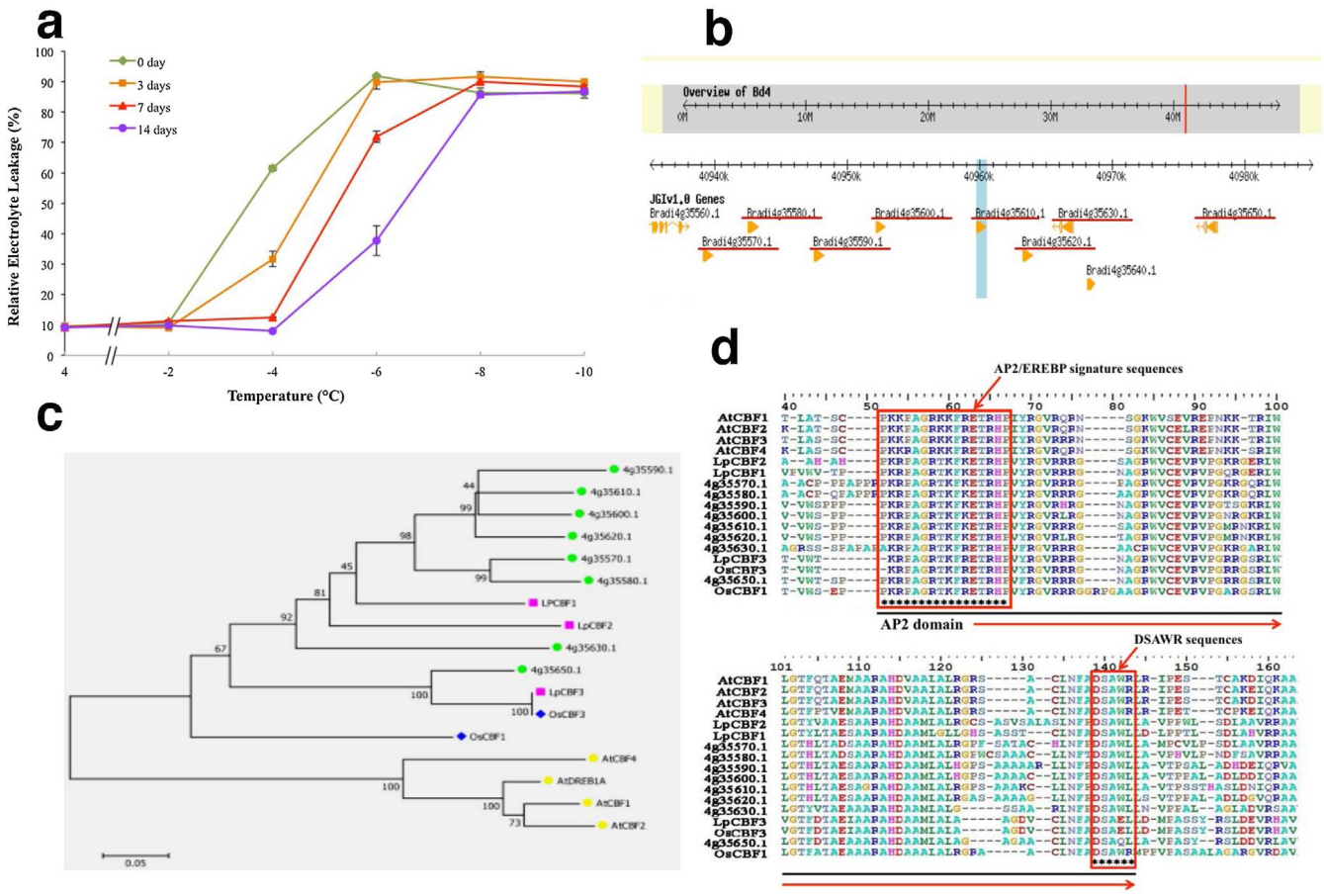

Fig. 1. Cold acclimation assay and phylogenetic analysis. (a) Cold acclimation ability of B. distachyon was tested by electrolyte leakage assay. Three-week-old B. distachyon plants were either maintained in a growth chamber at 23 °C as the non-cold acclimation control or exposed to 4 °C for 3, 7, or 14 days. The electrolyte leakage was measured at 4, −2, −4, −6, −8, and −10 °C. Data are means from three independent experiments. Error bars indicate standard deviations. (b) Eight BdCBF homologs are present in a tandem array on chromosome 4. BLAST search was conducted against the B. distachyon database using CBF3 gene of Lolium perenne as a query. (c) Phylogenetic analysis among Arabidopsis, B. distachyon, and Lolium perenne CBF genes. The phylogenetic tree was derived from an alignment of CBF proteins from Arabidopsis, B. distachyon, and Lolium perenne. The neighbor-joining tree was obtained based on the alignment results. Numbers at the nodes are bootstrap values that are percentages of the tree obtained from 100 re-samplings of the data. (d) Amino acid sequences alignment of CBF proteins. The amino acid sequences shown are for: Lp, Lolium perenne; 4g35570.1-4g35650.1, B. distachyon; Os, Oryza sativa; and At, Arabidopsis. The AP2/ERF domain is indicated by an underline and the signature sequences PKK/RPAGRxKFxETRHP and DSAWR are indicated by black asterisks (*). Alignment results are for alignment scale from position 40–163 only.

acclimation in many species, suggesting that the CBF cold-responsive pathway regulating the expression of many stress-inducible genes is broadly conserved in plants [9].

CBF genes often exist as a multi-gene family. For example, in *Arabidopsis*, three *CBF/DREB1* genes are present in tandem on chromosome 4 in the order of *CBF1/DREB1B*, *CBF3/DREB1A*, and *CBF2/DREB1C* [11,12]. It has been demonstrated that overexpression of *CBF1/DREB1B* and *CBF3/DREB1A* in many plants such as *Arabidopsis*, tomato (*Solanum lycopersicum*), and tobacco (*Nicotiana tabacum*) can significantly improve the cold tolerance of transgenic plants, but this is not the case for *CBF2/DREB1C* [13–15]. Novillo et al. [16] reported that *CBF2/DREB1C* functions as a negative regulator of *CBF1/DREB1B* and *CBF3/DREB1A* expression in *Arabidopsis*. They also demonstrated that increased expression of *CBF1/DREB1B* and *CBF3/DREB1A* in the *cbf2* null mutant conferred increased freezing tolerance [17]. Overexpression of *AtCBF2* in potato (*Solanum tuberosum* L.) failed to confer increased freezing tolerance [18]. These results indicate that *CBF1* and *CBF3* play a different role than *CBF2* in both constitutive freezing tolerance and freezing tolerance acquired through cold acclimation.

Homologs of *Arabidopsis CBF/DREB1* genes have been identified in many other plant species including wheat, rye (*Secale cereale*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), maize (*Zea mays*), tomato, perennial ryegrass and *Brachypodium distachyon* [9,19–27]. The CBF/DREB1 proteins belong to the AP2/ERF (APETALA2/ethylene-responsive element binding factor) family that has a highly conserved AP2/ERF domain. The AP2/ERF domain has been identified as a DNA-binding motif of about 60 amino acids [28]. In *Arabidopsis*, a common feature of CBF proteins that distinguish them from other AP2/ERF proteins is the presence of a set of conserved CBF signature sequence motifs (PKK/RPAGRxKFxETRHP and DSAWR) directly flanking the AP2 domain [9]. Overexpression of the *Arabidopsis CBF/DREB1* genes in transgenic *B. napus*, tobacco or maize plants can effectively improve the freezing tolerance of the transgenic plants [9,22,29]. It has been confirmed that these regulatory systems are highly conserved in monocots as well as in dicots [30,31].

The primary goal of this research is to determine the cold acclimation capacity of *B. distachyon* and to isolate and study the function of *CBF* genes in *B. distachyon* during cold acclimation. *B. distachyon* is an established temperate grass model plant that is phyologenetically close to many cool-season forage grasses, turfgrasses and cereal crops [32–34]. In the present study, we demonstrated that *B. distachyon* has the ability to cold acclimate. We isolated a *CBF*-like gene family consisting of eight homologous genes that are designated as *BdCBF1-8*, which are present in tandem on chromosome 4. Expression analysis indicated that all eight genes are induced by cold. Transgenic plants of *cbf1* and *cbf3* RNAi lines were generated and evaluated for freezing tolerance. Pairwise comparisons of RNA-seq data between the wild type (WT) and *cbf3* RNAi mutant plants under either normal or cold