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Transgenic tobacco plants over expressing cold regulated protein CbCOR15b from *Capsella bursa-pastoris* exhibit enhanced cold tolerance

Lihua Wu, Mingqi Zhou, Chen Shen, Jing Liang, Juan Lin*

State Key Laboratory of Genetic Engineering, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200433, China

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ABSTRACT

Low temperature is among the most significant abiotic stresses, restricting the habitats of sessile plants and reducing crop productivity. Cold regulated (COR) genes are low temperature-responsive genes expressing under regulation of a specific signal transduction pathway, which is designated C-repeat-binding-factor (CBF) signaling pathway. In the present article, cold bioassay showed that the transcript level of cold regulated gene *CbCOR15b* from shepherd's purse (*Capsella bursa-pastoris*) was obviously elevated under cold treatments. Reverse transcription-PCR (RT-PCR) and GUS report system revealed that unlike *AtCOR15b*, *CbCOR15b* expressed not only in leaves but also in stems and maturation zone of roots. When transgenic tobacco plants ectopically expressing CbCOR15b were exposed to chilling and freezing temperatures, they displayed more cold tolerance compared to control plants. According to the electrolyte leakage, the relative water content, the glucose content and the phenotype observation, *CbCOR15b* transformants suffered less damage under cold stress. Further investigation of the subcellular localization of CbCOR15b by transient expression of fusion protein CbCOR15b–GFP revealed that it was localized exclusively in the chloroplasts of tobacco mesophyll cells and in the cytoplasm of onion epidermal cells. It can be concluded that CbCOR15b which located in the chloroplasts and in the cytoplasm of cells without chloroplasts was involved in cold response of *C. bursa-pastoris* and conferred enhanced cold tolerance in transgenic tobacco plants.

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Introduction

Temperature is one of the key environmental factors that affect the distribution of vegetation and the productivity of crops. In temperate regions, plants gain greater cold tolerance after adapting to non-freezing temperature for a period of time, which is designated cold acclimation (Thomashow, 2010). During cold acclimation, the composition of lipids in the cytoplasmic membrane, the soluble osmotic regulating substances in the cytoplasm and various cryoprotectants go through complex and extraordinarily delicate changes, which correlates with changes in gene expression (Uemura et al., 1995; Hughes and Dunn, 1996; Buskirk and Thomashow, 2006). The C-repeat-binding-factor (CBF) signaling pathway is considered to be the most important cold responsive pathway in higher plants and has drawn great attention from researchers in recent days. A large number of key genes in this pathway have been cloned and characterized (Thomashow, 2010; Zhou et al., 2011a), among which there are a group of genes named cold responsive genes (*COR*) genes regulated by CBF transcription factors by binding to the C-repeat-element/dehydration-responsive-elements (CRT/DRE) in their promoter regions. Promoter sequence deletion analysis of *COR* genes from *A. thaliana* and other species indicated that some of them contained a highly conserved sequence CCGAC (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Thomashow, 1999; Wang and Hua, 2009; Li et al., 2010; Chen et al., 2011). In *A. thaliana*, five groups of *COR* genes have been characterized, including *COR6.6*, *COR47*, *COR78*, *COR15* and *COR413* (Hajela et al., 1990). Each group consist of two genes, which locate in tandem with each other on the same chromosome, such as *COR6.6* (*KIN1* and *KIN2*), *COR78* (*RD29a* and *RD29b*) and *COR15* (*COR15a* and *COR15b*) (Thomashow et al., 2001). It has been reported that regulation of these genes from each group differs from each other. Transcripts for *KIN1* and *KIN2* accumulate in response to low temperature and ABA but only those for *KIN2* accumulate in response to drought, which is the same as the expression pattern of *Arabidopsis COR15a* and *COR15b* (Wilhelm and Thomashow, 1993).

Constitutive expression of COR genes from *A. thaliana*, using the cauliflower mosaic virus (CaMV) 35S promoter, enhances freezing tolerance in *A. thaliana*. The over expression of AtCOR15a enhances

Abbreviations: ABA, abscisic acid; COR, cold-responsive; EL, electrolyte leakage; EV, empty pCAMBIA 1304 or pCAMBIA 1301 vector control; GA_3, gibberellic acid; GC, glucose content; GFP, green fluorescence protein; IAA, indole-3-acetic acid; MeJA, methyl jasmonate; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; RWC, relative water content; SA, salicylic acid; WT, wild type; X-Gluc, 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid.

* Corresponding author. Fax: +86 21 65642949.
E-mail address: linjuan@fudan.edu.cn (J. Lin).

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the freezing tolerance of both chloroplasts and protoplasts from non-acclimated plants (Artus et al., 1996). The mature form of AtCOR15a, AtCOR15am functions as a cryoprotective protein which forms oligomers in the chloroplast stroma to prevent the formation of hexagonal II-phase lipids (Steponkus et al., 1998; Nakayama et al., 2007). The homologue of AtCOR15am, AtCOR15bm may be targeted to the chloroplast as the same as AtCOR15am to protect the chloroplast from freezing injury (Wilhelm and Thomashow, 1993). Many homologous genes of *AtCOR15a* have been characterized from other species (Weretilnyk et al., 1993; Crosatti et al., 1999; Liu et al., 2004; Si et al., 2009; Chen et al., 2011). A novel cold-regulated gene *CsCOR1* from *Camellia sinensis* enhances salt- and dehydration-tolerance in tobacco (Li et al., 2010). The *BnCOR25* from *Brassica napus*, the expression of which is reported to be mediated by ABA-dependent pathway, is significantly induced by cold and osmotic stress treatments (Chen et al., 2011). The cold-regulated barley gene *HvCOR14b*, the protein product of which is localized in the chloroplast, is controlled by the interaction between cold and light (Crosatti et al., 1999). Citrus dehydrin CuCOR19, which is localized predominantly in mitochondria and the soluble fraction, greatly enhanced the cold tolerance when over expressed in transgenic tobacco (Hara et al., 2003). A member of the wheat *COR/LEA* gene family, *WCOR15* was fused with the *GFP* report gene and transferred into tobacco plants, and the transgenic plants showed abundant accumulation of WCOR15 in the stromal compartment of the chloroplasts and significantly improved cold tolerance (Shimamura et al., 2006). Likewise, *CbCOR15* from *Chorispora bungeana* expressing in the leaves but not in the roots of cold-acclimated plants confers greater cold tolerance in transgenic tobacco plants (Si et al., 2009). The cosmopolitan plant species *Capsella bursa-pastoris*, the closest relative of *A. thaliana*, is also an important model system for developmental and evolutional study (Ceplitis et al., 2005). According to the results of a cold assay conducted in our lab on plants after exposure to chilling (4 °C) or freezing (−5 °C) temperatures (data not published), the cold tolerance of *C. bursa-pastoris* is higher than that of *A. thaliana*. The mechanism responsible for this greater cold resistance is not yet clear and needs intensive study. Additionally, it is valuable to identify cold responsive genes in the candidate species of *C. bursa-pastoris* for transgenic breeding research for improvement of crop cold resistance. Previous research identified that transcripts of *AtCOR15a* accumulated in the leaves but not in the roots (Lin and Thomashow, 1992), but transcripts of *CbCOR15a* from *C. bursa-pastoris*, a homologue of *AtCOR15a*, accumulated not only in the leaves but also in the roots and stems under cold treatments (Zhou et al., 2012), indicating that CbCOR genes might have different functions compared with *AtCOR* genes. *CbCOR15b* from *C. bursa-pastoris*, the homologue of *CbCOR15a*, was first cloned and identified to be greatly induced under cold treatments (Liu et al., 2004). The predicted protein product of *CbCOR15b* was found to have a potential chloroplast-targeting signal peptide. Study of the differential expression patterns of *CbCOR15b* and *CbCOR15a* and its possible cryoprotective functions requires the knowledge of its promoter activity and the subcellular localization of CbCOR15b. In the present article, we report that no transcript of CbCOR15b was detected in normal temperature, which was different from the previous results that CbCOR15a had a weak but testable expression at 26 °C. Meanwhile, CbCOR15b had a significantly induced expression pattern in the leaves and stems and weaker induction in the roots under both cold acclimation and cold induction test. The histochemical staining assay in transgenic tobacco plants over expressing CbCOR15bP::GUS displayed no GUS activity at 26 °C and then strong activity both in leaves and the maturation zone of roots under cold condition. Investigation of the subcellular localization of CbCOR15b indicated its localization in the chloroplast in mesophyll cells and in the cytoplasm of cells without chloroplasts. Transgenic tobacco plants over expressing *CbCOR15b* showed enhanced cold tolerance under chilling (4 °C) and freezing (−4 °C) temperatures according to the values of the three physiological indexes, including the electrolyte leakage, the relative water content and the glucose content. Based on our results, potential cryoprotective roles of CbCOR15b in the chloroplast of mesophyll cells and the cytoplasm of cells without chloroplasts as well as its potential application in gene engineering is discussed.

Materials and methods

Analysis of CbCOR15b expression characteristic

The seeds of *Capsella bursa-pastoris* cleansed with 75% ethanol for 30 s and subsequently with 18% sodium hypochlorite for 8 min were placed on Murashige and Skoog solid medium in controlled-environment chamber at 26 °C with a 16 h photoperiod from cool-white fluorescent illumination (140–150 μmol m^{-2} s^{-1}). For the cold treatments, 3 different 4-week-old seedlings were transferred to 12 °C for 4 d, followed by 4 °C for 4 d, 0 °C for 2 h and −4 °C for 2 h. Another set 3 seedlings were exposed to 4 °C, and different tissue samples (leaf, root and stem) were collected at 1, 3, 6 and 24 h and used for RNA extraction and RT-PCR analysis. Total RNA was isolated from approximately 200 mg leaf, root or stem tissue using the Plant RNA Mini Kit (Watson Biotechnologies, Inc., China) and treated with the DNAase I (Promega, Madison, WI, USA) to remove any DNA contamination following the manufacturer's instructions. The total RNA (1 μg) was reversely transcribed with the PrimeScript® RT Master Mix Perfect Real Time kit (TaKaRa, China). First strand cDNA was used as template for semi-quantitative RT-PCR amplifications using the gene-specific primers CbCOR15b-F: 5′-ATGTCTTTCTCAGGAGCTGT-3′ and CbCOR15b-R: 5′-GCTTTCTTTGCTTCCTCTGTCGCCG-3′ and the primers Cb18S-F: 5′-ATGATAACTCGACGGATCGC-3′ and Cb18S-R: 5′-CTTGGATGTG GTAGCCGTTT-3′ from 18S rRNA gene (AY662285) were used as the internal control.

Isolation and analysis of the CbCOR15b promoter

The total genomic DNA of *C. bursa-pastoris* was isolated by the CTAB method (Murray and Thompson, 1980). The Genome Walker DNA libraries were constructed using the Universal Genome Walker™ Kit (Clontech, USA). The 5′ upstream promoter region of *CbCOR15b* was used to conduct two PCR amplifications per library. The primary PCR used gene specific primers CbCOR15b-F1: 5′-GTCATCGAGGATGTTGCCGTCACCT-3′ designed according to the full-length cDNA sequence of *CbCOR15b* gene (AY437888) and AP1: 5′-GTAATACGACTCACTATAGGGC-3′ (kit provided adaptor primer), and the product was afterward diluted and used as the template in the secondary PCR using primer pairs of CbCOR15b-F2: 5′-TACTCCGCTGTGGAAAGAAGAACCC-3′ and AP2: 5′-ACTATAGGGCACGC GTGGT-3′ (kit provided adaptor primer). Subsequently the resulting fragment was cloned into the pMD18-T simple vector (TaKaRa, China) and sequenced by Shanghai Majorbio Biotechnology Company. The transcription start site was analyzed using Neural Network Promoter Prediction (http://fruitfly.org:9005/seq_tools/promoter.html). The promoter nucleotide sequence analysis was carried out in the PLANTCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Construction of the plant expression vectors and tobacco transformation

The CbCOR15b cDNA coding sequence of *C. bursa-pastoris* was reported in previous research (Liu et al., 2004). This fragment

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