J. Plant Biol. (2009) 52:593–601
DOI 10.1007/s12374-009-9077-z

ORIGINAL RESEARCH

CbCOR15, A Cold-Regulated Gene from Alpine *Chorispora bungeana*, Confers Cold Tolerance in Transgenic Tobacco

Jing Si · Jian-hui Wang · Li-jing Zhang · Hua Zhang · Ya-jie Liu · Li-zhe An

Received: 19 January 2009 / Revised: 13 August 2009 / Accepted: 14 August 2009 / Published online: 27 October 2009

Abstract Rapid amplification of cDNA ends was performed to isolate cold-regulated *CbCOR15* (EF208112) from *Chorispora bungeana*. This alpine species is distributed in subnival areas. Transcripts were detected in the leaves, but not the roots, of plants acclimated to cold temperatures. Expression was induced at high levels at both 4°C and −4°C. In comparing its deduced protein sequence to that of *AtCOR15a* (cold-regulated *15a* in *Arabidopsis thaliana*), the N terminus had less homology than the C terminus while still containing a region analogous to the chloroplast-targeted signal peptide of *AtCOR15a.* We also introduced *CbCOR15*, with the *CaMV* 35S promoter, into tobacco. Second-generation (T1) plants had significantly increased tolerance to chilling, as determined by their electrolyte leakage, chlorophyll content, and relative water content. Further freezing-stress experiments showed that the tolerance of transgenic lines was significantly greater than that of the nontransgenics. Although the degree of chilling and freezing tolerance in the transgenic plants was not directly correlated with the accumulated levels of *CbCOR15*, we could conclude that this gene confers cold tolerance.

Keywords Chilling stress · *Chorispora bungeana* · Cold acclimation · *COR15* gene · Freezing stress · Transgenic tobacco

J. Si · H. Zhang · Y.-j. Liu · L.-z. An (✉)
Key Laboratory of Arid and Grassland Agroecology of Ministry of Education, School of Life Sciences, Lanzhou University, Lanzhou 730000, China
e-mail: lizhean@lzu.edu.cn

J.-h. Wang
Cold and Arid Regions Environmental and Engineering Research Institute, Chinese Academy of Sciences, Lanzhou 730000, China

L.-j. Zhang
College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730000, China

Cold or freezing temperatures are among the most significant abiotic stresses, restricting the habitats of sessile plants and reducing crop productivity. Acclimating plants to the cold via gradual exposure to low but nonfreezing temperatures elicits complex physiological and molecular responses that allow for survival (Sakai and Larcher 1987). Many adaptive mechanisms are regulated by alterations in gene expression (Guy 1990; Thomashow 1993, 2001). Of the numerous low temperature-responsive genes that have been isolated and characterized, the most well-characterized gene family has been designated as cold-responsive or cold-regulated (COR). These COR genes are highly induced during cold acclimation, and their expression is regulated by a specific signal transduction pathway (Thomashow 1998, 1999). A functional cis-acting element occurs in the *Arabidopsis* COR gene, i.e., a CCGAC core motif known as a C repeat/dehydration responsive element (CRT/DRE) sequence (Baker et al. 1994; Yamaguchi-Shinozaki and Shinozaki 1994). Constitutive expression of CRT/DRE binding protein genes (CBF/DREB1) is induced within 15 min of exposure to low, nonfreezing temperatures, along with the expression of CRT/DRE-controlled COR genes (Gilmour et al. 1998; Jaglo-Ottosen et al. 1998; Liu et al. 1998; Dhanaraj et al. 2005). Among these, *AtCOR15a* encodes a chloroplast-targeted *Cor15a* protein and is processed to the mature polypeptide during import, *Cor15am*. This decreases the incidence of freeze-induced lamellar-to-hexagonal II phase transitions by altering the

intrinsic curvature of the inner membrane of the chloroplast envelope (Steponkus et al. 1998). Constitutive expression of *AtCOR15a*, using the cauliflower mosaic virus (*CaMV*) 35S promoter, enhances freezing tolerance in *Arabidopsis* (Artus et al. 1996). Likewise, induced expression of the wheat *Wcor15* gene positively contributes to the development of such tolerance in plants (Takumi et al. 2003; Shimamura et al. 2006).

Chorispora bungeana is a representative alpine subnival perennial herb in the Brassicaceae family. Growing in the freeze–thaw tundra at glacier borders where other flowering plants cannot, it survives thin air, high radiation, strong wind, frequent temperature fluctuations, and freezing (Ayitu et al. 1998; An et al. 2000). Such alpine plants, however, do not have special morphological characteristics that ensure survival. Therefore, molecular mechanisms presumably account for their adaptation to a freezing environment. As a candidate species, *C. bungeana* is valuable to the identification of stress-related genes for future cloning and research that can elaborate the mechanism for cold resistance.

Although cloning, molecular characterization, and expression of COR have not been studied previously in any alpine subnival plant, similar genes have been isolated and studied in *Arabidopsis* (Gilmour et al. 1992; Nordin et al. 1993; Thomashow 1990, 1993), wheat, and barley (Cattivelli and Bartels 1990; Chauvin et al. 1993; Tsvetanov et al. 2000). Here, we isolated a novel COR gene from *C. bungeana* and employed a transgenic approach to investigate the functioning of *CbCOR15* in protection against cold stress.

Materials and Methods

Cloning of *CbCOR15* from *C. bungeana*

Total RNA was extracted with TRIzol reagent (Invitrogen) from the leaves of *C. bungeana* after plants were treated at 2°C for 1 day (cold stress). Two degenerate primers, P1 (5′-GYTCGTCGYCGTYKCTCARCGC-3′) and P2 (5′-CCCTCYRCGAACTCSGCCGC-3′) (where Y is C and T, K is G and T, R is A and G, and S is G and C), were used in a reverse transcription polymerase chain reaction (RT-PCR) to obtain a fragment of partial *CbCOR15*. Those primers correspond to the highly conserved amino acid sequences for *COR15a* of *Arabidopsis* (NM180040), *Capsella bursa* (AY437888), and *Brassica napus* (S68726). Amplifications were performed at 94°C for 4 min, followed by 30 cycles of amplification (94°C for 40 s, 45°C for 40 s, and 72°C for 40 s). Afterward, 5′ and 3′ rapid amplification of cDNA ends were conducted with a Gene Racer kit (Invitrogen) to produce full-length *CbCOR15* cDNA. *CbCOR15* was sequenced for both strands on an ABI 377 Sequencer (Perkin–Elmer, USA). These sequence data were then compared with known sequences in NCBI databases using the BLAST search website (Altschul et al. 1997). Amino acid sequence analysis and multiple alignments were performed with DNAMAN software. Signal sequence predictions used the IpSORT Program (Bannai et al. 2002; http://hc.ims.u-tokyo.ac.jp/iPSORT/). Predictions of hydropathy profiles and secondary structure were carried out by ProtScale and SOPMA, respectively. Those bioinformatics tools are found at www.expasy.org (Walker and Croteau 2000; Jaakola et al. 2001).

Semiquantitative RT-PCR Analysis

To investigate tissue-specific expression patterns of *CbCOR15*, total RNA was extracted from leaves and roots after plants were exposed to 2°C for 1 day. Cold stress-related expression was examined from leaves sampled daily from plants treated over a 7-day period at either 4°C or −4°C. All extractions were done with TRIzol reagent (Invitrogen). For RT-PCR, the specific primers P3 (5′-GAGAAAGATGGCAACATCCTCG-3′) and P4 (5′-TGGTGGCATCCTTAGCATCTC-3′) were used to obtain a fragment of partial *CbCOR15*. Primers P5 (5′-GGAGCT GAGAGATTCCGTTGC-3′) and P6 (5′-GAAGCATTTC CTGTGGACAATCGA-3′), designed according to the conserved regions of the *C. bungeana* housekeeping gene (*ACTIN*; Accession No. AY825363), were used as an internal control to verify equal loading of RNA. Pre-amplification (three runs per sample) was performed at 94°C for 4 min, followed by 25 cycles of amplification (94°C for 40 s, 52°C for 40 s, and 72°C for 60 s). PCR products were separated on 1% agarose gels stained with ethidium bromide. Band intensity of the PCR products was quantified by Quantity One-4.6, Gene Tools software (Bio-Rad). Relative mRNA expression was calculated as the amount of *CbCOR15*/amount of *ACTIN*.

Tobacco Nuclear Transformation and Confirmation

The *CbCOR15* cDNA coding region was amplified with Pfu DNA polymerase (Promega) and gene-specific primers containing the *XbaI* (5′-GCGTCTAGAATGGCGATGT CATTT-3′) or *SacI* (5′-GCTGAGCTCCTACTTGGTGGC-3′) restriction sites. Conditions were 94°C for 4 min; then 30 cycles of 94°C for 1 min, 47°C for 1 min, and 72°C for 1 min; followed by a final extension at 72°C for 10 min. The amplified *CbCOR15* fragment was digested with *XbaI*/*SacI* and ligated into the corresponding sites of pBI121 by replacing *GUS*. This produced plasmid pBI-*CbCOR15*, in which the *CaMV* 35S promoter drives expression of *CbCOR15* cDNA.

The plasmid pBI-*CbCOR15* was introduced into the tobacco genome by *Agrobacterium*-infection method. This

plasmid was first introduced into *Agrobacterium tumefaciens* LBA4404 by direct transformation (Hofgen and Willmitzer 1988). Afterward, leaf discs of tobacco (*Nicotiana tabacum* cv. Xanthi) infected with LBA4404 (Maniatis et al. 1982) were transferred to a shoot-induction medium (MS (Murashige and Skoog 1962) plus 0.01 mg L^{-1} NAA and 2.0 mg L^{-1} 6-BA) that contained 100 mg L^{-1} kanamycin and 200 mg L^{-1} carbenicillin. These discs were incubated in the dark for 2 days at 25°C before being transferred to a root-induction medium (half-strength MS with 100 mg L^{-1} kanamycin and 200 mg L^{-1} carbenicillin). Putative transgenic plants were placed in greenhouse pots after 2 weeks of hardening, and were self-pollinated to produce a T1 generation.

Transformants were verified with Primers P3 and P4, amplifying total DNA from both transgenic and non-transgenic plants according to the CTAB method (Doyle and Doyle 1990). These reactions consisted of 30 cycles with 40 s at 94°C for denaturing, 40 s at 52°C for annealing, and 40 s at 72°C for extension. PCR products were analyzed by electrophoresis on a 1% agarose gel.

Determining the Expression Content of *CbCOR15* in Transgenic Tobacco by Real-time Quantitative RT-PCR

The expression content of the transferred genes in regenerated tobacco was confirmed by real-time quantitative RT-PCR. Total RNA was extracted from transgenic and nontransgenic plants with TRIzol reagent (Invitrogen). Primers P3 and P4 were used for RT-PCR to obtain a partial fragment of *CbCOR15*. As an internal reference, we used primers P7 (5′-GGAAAGTCCTACCAGCATTG-3′) and P8 (5′-ATCTATTGTCTCCCACGAAG-3′) for amplification of the GAPDH gene (Accession No. AJ133422).

Real-time RT-PCR was conducted three times for each sample with SYBR Premix Ex Taq (TaKaRa) on a Multicolor Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's recommendations.

Preparation of Plant Materials for Chilling- and Freezing-Stress Treatments

Transgenic (T_1) and nontransgenic plants were placed on MS medium in controlled-environment chambers at 25°C, under a 16-h photoperiod from cool-white fluorescent illumination (110 to 120 umol $m^{-2}s^{-1}$). Six-week-old seedlings were used. For our chilling-stress experiments, they were transferred to a cold chamber maintained for 7 days at 2°C. To introduce freezing stress, the seedlings were exposed for 1 h to 0°C, −2°C, −4°C, or −6°C. For trials that monitored the effect of freezing over time, plants were exposed to −2°C, and leaf samples were collected at 0.5, 1, 2, 4, 6, and 8 h. All treatments were conducted under the same conditions of photo-intensity and photoperiod.

Measurement of Electrolyte Leakage

Leaves were rinsed with distilled deionized water to remove possible surface ion contamination. Discs were removed with a 6-mm paper punch (four from each of three replicate samples). They were then incubated for 10 h in a small amount of distilled water. Conductivity of the leachate was measured with a conductivity detector (DDS SJ-308A, China). All the electrolytes were released from these tissues by autoclaving at 121°C for 15 min to completely lyse the plant cell walls and then cooling the samples at room temperature. Electrolyte conductivities of boiled solutions were recorded as "absolute conductivity." The percentage of electrolyte leakage (EL) was calculated as initial conductivity/absolute conductivity. The midpoint in the transition of EL, EL_{50}, was considered the killing temperature, i.e., the temperature at which leakage of 50% of all electrolytes occurred.

Measurement of Chlorophyll Content

Four leaf discs (6 mm diam.) were punched and incubated in 80% chilled acetone for 24 h. Chlorophyll concentration was calculated by the following formula: Chl content $\left(\mathrm{mgL}^{-1}\right) = 20.3\ \mathrm{OD}_{645} - 4.67\mathrm{OD}_{663}$. To prevent light-induced pigment degradation, all procedures were carried out in the dark, using only a dim green light.

Measurement of Relative Water Content

Water status in the leaves was determined as follows: relative water content $= (\mathrm{FW} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW}) \times 100$, where FW is the fresh weight of the leaf discs, TW is the disc turgid weight after soaking in water for 4 h at room temperature, and DW is the disc dry weight after being oven-dried at 85°C for 24 h (Weatherley 1950).

Results

Cloning, Sequencing, and Characterization of *CbCOR15*

The full-length *CbCOR15* cDNA of alpine *C. bungeana* comprises 612–423 bp in the open reading frame (ORF), 83 bp in the 5′-untranslated region (UTR), and 106 bp in the 3′-UTR with a poly (A) tail (Fig. 1a). The ORF encodes a polypeptide of 140 amino acids, for which the calculated isoelectric point and molecular mass are predicted to be 5.20 and 14.9 kDa, respectively (*pI*/Mw Tool at www.expasy.org). Here, the predicted polypeptide, designated

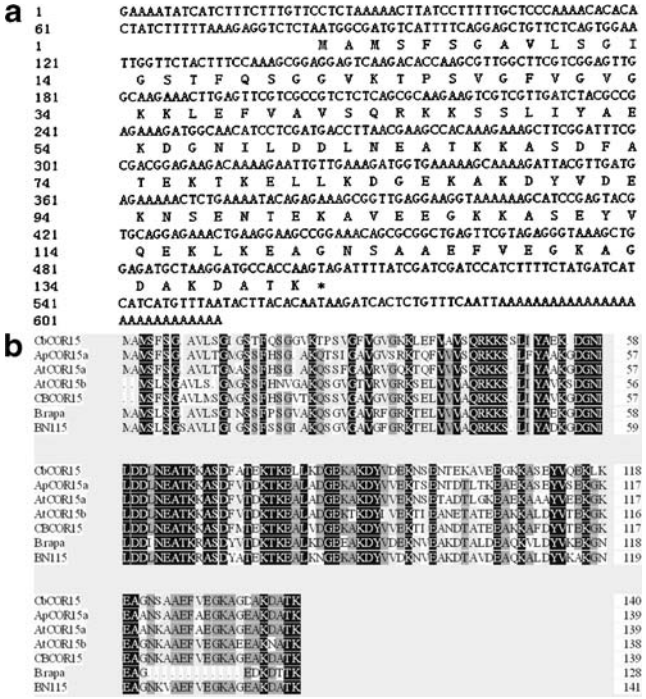

Fig. 1 **a** Full-length cDNA sequence and deduced amino acid sequence of *CbCOR15*. **b** Alignment of *C. bungeana CbCOR15* (GenBank Accession No. EF208112) with COR15s from other plant species. *Boxes in black* represent 100% similarity; *gray*, 86% conserved amino acids. Genes included *AtCOR15a* (NP_181782 *Arabidopsis*), *AtCOR15b* (AAA32774 *Arabidopsis*), *BN115* (AAA66068 *B. napus*), *CBCOR15* (AAR99417 *C. bursa-pastoris*), *ApCOR15a* (AAS98217 *A. pumila*), and ABF60663 (*B. rapa subsp. pekinensis*)

CbCor15, had high lysine (15.71 mol%) and alanine (11.43 mol%) contents but was devoid of cysteine, tryptophan, histidine, and proline residues. The hydropathy profile of the *CbCor15* protein indicated that the N-terminal one third had both hydrophobic and hydrophilic regions but that the remaining two thirds of the polypeptide were primarily hydrophilic (Fig. 2). The predicted secondary structure of that protein (Fig. 3) consisted of 82.86% alpha helix, 8.57% random coil, 2.86% beta turns, and 5.71% extended strands. The random coil- and alpha helix-constituted domain comprised the main part of the secondary structure. Furthermore, the C-terminal two thirds of the polypeptide was entirely alpha helix. Our iPSORT analysis also predicted a putative chloroplastic transit peptide in the N-terminal (Bannai et al. 2002). The sequence of the N-terminal one third had a serine-plus-threonine content of 21.1 mol% but contained glutamic and aspartic acid residues of only 1.9 mol%. These results correspond well with the theory that chloroplast transit peptides generally have a high content of serine and threonine residues and low numbers of acidic amino acid residues (Lin and Thomashow 1992).

These cDNA and predicted amino acid sequences have been submitted to the NCBI GenBank with the Accession Number EF208112. Multiple alignment with other plant species (Fig. 1b) showed that the *CbCor15* protein is most homologous to *Arabis pumila ApCOR15a* (70.71%), *C. bursa-pastoris CBCOR15* (66.67%) (Liu et al. 2004), *Arabidopsis AtCOR15b* (65.25%), *Arabidopsis AtCOR15a* (63.57%), and *B. napus BN115* (61.97%) (White et al. 1994). From the above-sequence analyses, we were able to identify many characteristics that *CbCOR15* has in common with other COR15s, thereby demonstrating that our gene is a novel member of the COR15 family.

Expression Patterns for *CbCOR15* in Different Tissues and under Cold Stress

After 1 day of cold-stress treatment at 2°C, *CbCOR15* expression was detected in *Chorispora* leaves, but not the roots (Fig. 4a). Therefore, we concluded that this gene is leaf-tissue-specific.

We also examined whether *CbCOR15* expression is correlated with tolerance to low temperatures (4°C or −4°C), using a time-course trial and RT-PCR. Although *CbCOR15* transcripts were undetectable at Time Zero, transcript levels at each temperature increased markedly after 1 day of treatment (Fig. 4b, c). At 4°C, transcripts were highest on day 6, while at –4°C, the maximum was reached on day 4. Expression at either temperature was then maintained at elevated levels up to day 7.

Confirmation of Nuclear Transgenic Plants by PCR

We generated transgenic tobacco that overexpressed *CbCOR15* at a normal growing temperature by placing its coding sequence under the control of the *CaMV* 35S promoter. The construct was transformed into plants by the floral dip method. In all, 38 independent transgenic plants were recovered in a kanamycin-containing medium. Using specific primers P3 and P4 and basing our conclusion on the expected 262-bp amplification product, we confirmed that 32 lines were true transformants, whereas gels for nontransgenic plants showed no bands (Fig. 5).

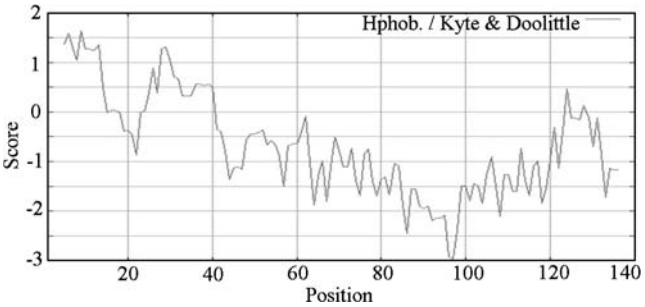

Fig. 2 Hydropathy profile of *CbCOR15*. *Numbers on abscissa* indicate amino acid residues; *those on ordinate*, hydrophobicity values. *Negative values* indicate hydrophilic regions

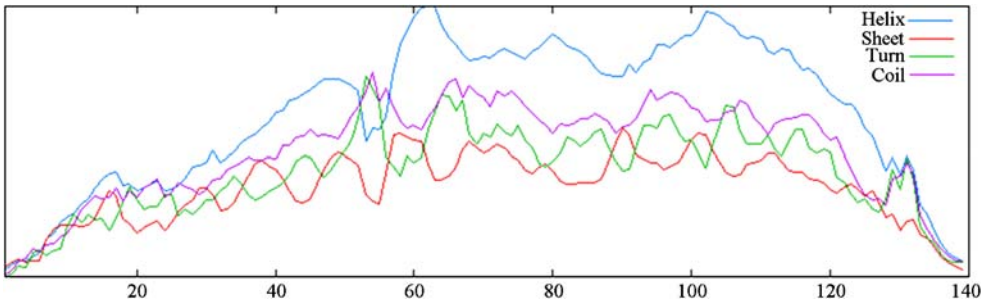

Fig. 3 Prediction of secondary structure for *CbCOR15*. Helix, sheet, turn, and coil are indicated with *blue*, *red*, *green*, and *peachblow lines*, respectively

CbCOR15 Expression in Transgenic Tobacco via Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR was used to analyze *CbCOR15* expression in tobacco leaves. All tested transgenic lines (eight selected from 32 total) had verified expression while the nontransgenics did not. However, although all confirmed plants were kanamycin-resistant, their degree of expression was not uniform. For example, plants in Lines A5, A6, A9, and A11 showed high transcript levels whereas those in Lines A8 and A15 had weak expression. We selected Lines A5 (high) and A15 (weak) for further study (Fig. 6).

Chilling Test

We confirmed that constitutive expression of *CbCOR15* conferred resistance to chilling stress by measuring electrolyte leakage, chlorophyll content, and relative water content in two transgenic lines and one nontransgenic line. After exposure to 2°C for 7 days, EL values increased in all plants, although they were significantly lower in the transgenics ($P<0.05$) (Fig. 7). Leakage from transgenic lines then increased to 21.4% and 23.8% compared with 35.9% from the nontransgenic line. Chlorophyll contents and relative water content (RWC) decreased in all plants, but values for transgenics were significantly higher ($P<0.05$) (Figs. 8, 9). After chilling stress, RWC in the nontransgenic line decreased by 25.5% versus 13.0% and 11.9% for the transgenics. Chlorophyll content in those transgenic plants declined by 18.1% and 21.9%, which was significantly less than the 44.4% drop in nontransgenics. These results suggested that the *CbCOR15* product functions in protecting the plasma membrane, preventing water loss and crystallization of cellular components under chilling stress.

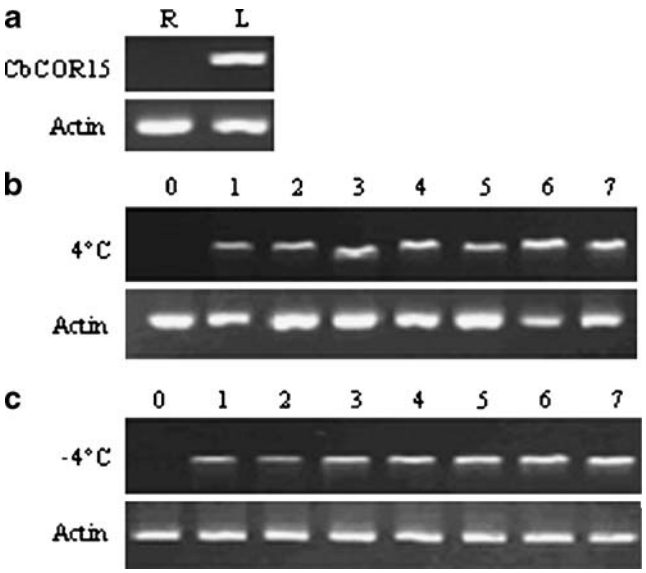

Fig. 4 Expression patterns of *CbCOR15*. **a** Tissue-specific expression after 1 day of treatment at 2°C; leaves (*L*), roots (*R*). **b**, **c** Expression profiles under cold stress. Total RNA was isolated at 0, 1, 2, 3, 4, 5, 6, and 7 days after treatment at 4°C (**b**) or −4°C (**c**). *ACTIN* served as internal control (*lower panel*)

Freezing Test

Nontransgenic lines exposed to −4°C for 1 day did not survive, but transgenics under the same conditions did. To correlate *CbCOR15* expression with freezing tolerance, we measured electrolyte leakage to estimate the cellular damage from freeze-induced membrane lesions in leaves. Plants were treated for 1 h at 0°C, −2°C, −4°C, or −6°C. Leakage increased in all plants afterward, although the extent of that phenomenon differed at each temperature point (Fig. 10). The EL_{50} was approximately −2.1°C for leaves from nontransgenic plants compared with approximately −4°C for transgenic tissues. EL rose to 46.8% at −2°C for the nontransgenic leaves, which was significantly more than in the transgenics, where values increased to 21.4% and 22.4% ($P<0.05$) (Fig. 10a). These results

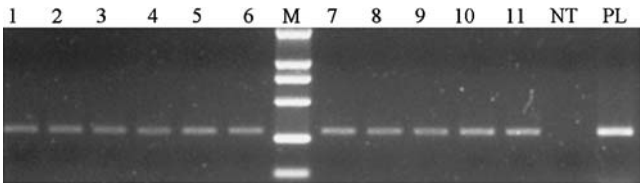

Fig. 5 Verification of transformants from T0-regenerated tobacco plants by PCR. *M* DL2000 Marker; *NT* nontransgenic line (negative control); *PL* plasmid pBI-*CbCOR15* (positive control); *1–11*, transgenic lines

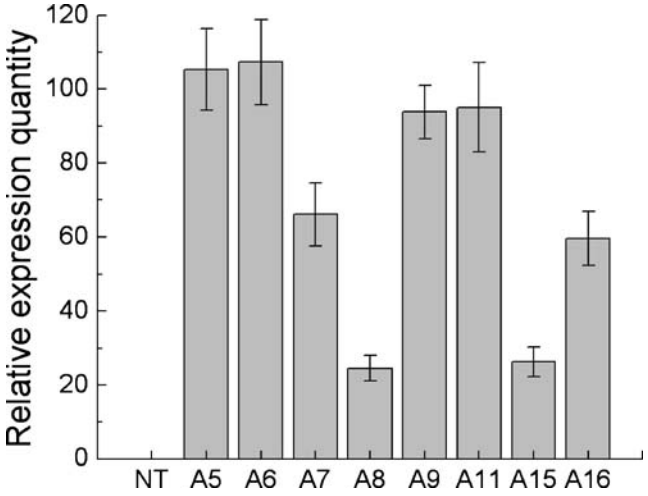

Fig. 6 Real-time quantitative RT-PCR analysis of *CbCOR15* expression in transgenic (*Lines A5*, *A6*, *A7*, *A8*, *A9*, *A11*, *A15*, and *A16*) and nontransgenic (*NT*) plants. Each value represents mean of measurements from three plants; *error bars* indicate SE

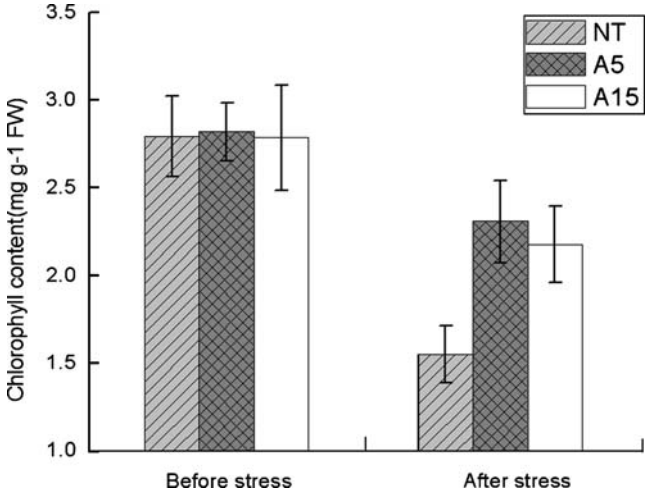

Fig. 8 Chlorophyll content analysis in transgenic (*A5* and *A15*) and nontransgenic (*NT*) tobacco lines before and after chilling stress. Each value represents mean of measurements from three plants; *error bars* indicate SE

demonstrated that nontransgenic plants exhibited severe freezing damage following exposure to −2°C for 1 h; transgenic plants showed only slight damage.

In further testing, leaf samples were collected at different time points during this −2°C stress period. Throughout treatment, transgenic plants had lower EL values (Fig. 10b). Furthermore, the EL of nontransgenics increased significantly, to 46.8%, after only 1 h while that of the transgenic lines rose to 48.5% and 54.3% after 4 h. Altogether, these results indicated that freezing tolerance was greater in the transgenic plants. This is consistent with reports that overexpression of analogous *COR15* genes enhances freezing tolerance in other species (Lin and Thomashow 1992; Steponkus et al. 1998; Shimamura et al. 2006).

Under normal conditions, no difference in EL, chlorophyll content, and RWC was found between transgenic and nontransgenic tobacco. However, when treated with chilling or freezing, those two genetic sources differed significantly. Meanwhile, the difference was only slight between the two transgenic lines with high and weak expressions. Our findings suggested that the degree of chilling or freezing tolerance in transgenic tobacco was not directly correlated with the level of *CbCOR15* accumulation.

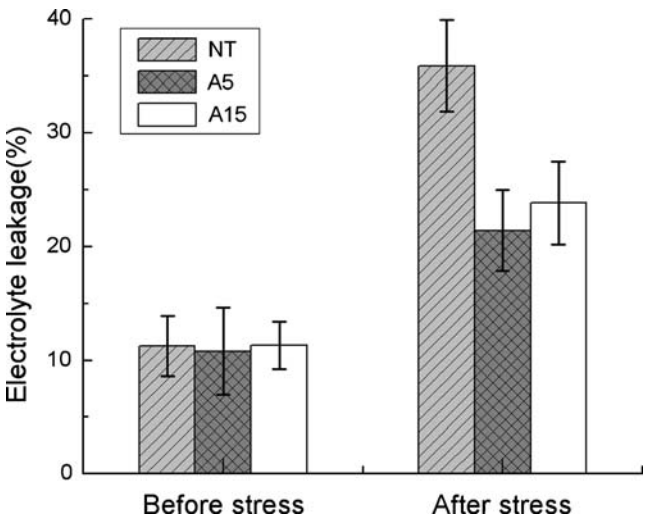

Fig. 7 Electrolyte leakage analysis in transgenic (*A5* and *A15*) and nontransgenic (*NT*) tobacco lines before and after chilling stress. Each value represents mean of measurements from three plants; *error bars* indicate SE

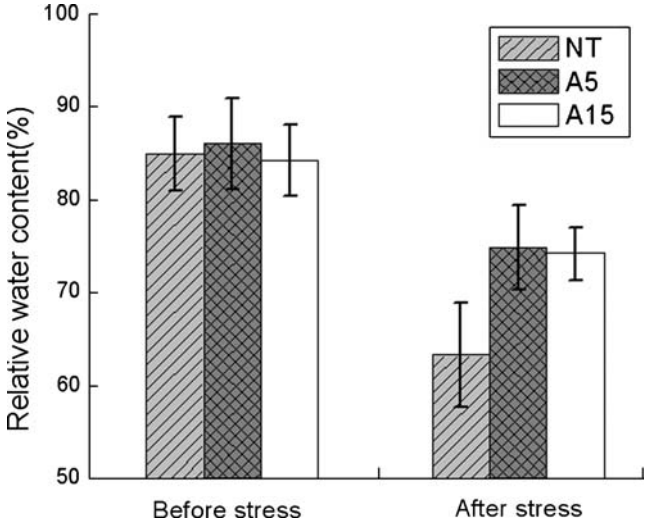

Fig. 9 Relative water content analysis in transgenic (*A5* and *A15*) and nontransgenic (*NT*) tobacco lines before and after chilling stress. Each value represents mean of measurements from three plants; *error bars* indicate SE

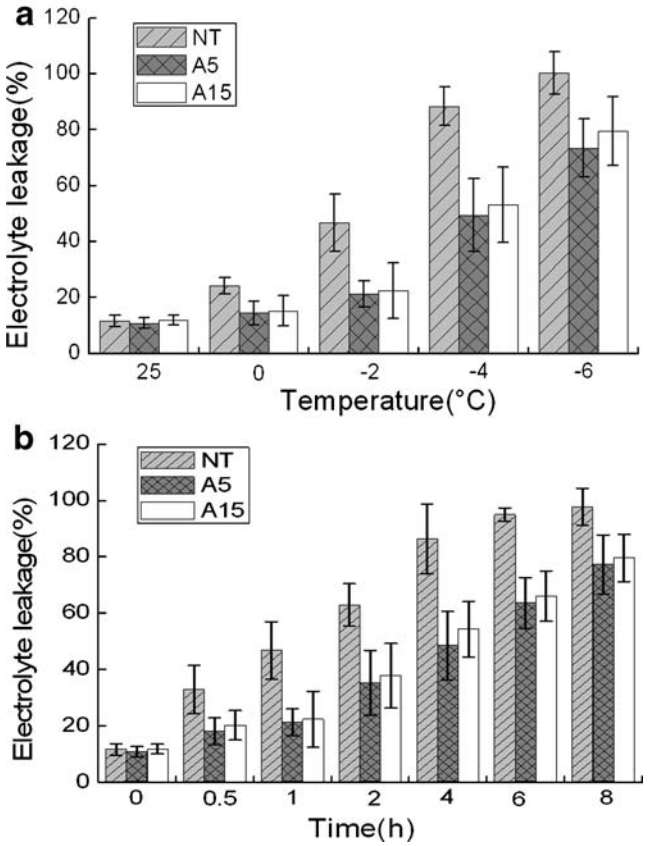

Fig. 10 Freezing tolerance of leaf tissues from transgenic (*A5* and *A15*) and nontransgenic (*NT*) tobacco lines. **a** Electrolyte leakage from plants after incubation at different temperatures for 1 h. **b** Electrolyte leakage from plants after incubation at −2°C for different time periods. Each value represents mean of measurements from three plants; *error bars* indicate SE

Discussion

The COR gene has been described from many plants, but its cloning and molecular characterization have not been reported for any cryophyte species. Here, we isolated a novel cold-responsive gene, *CbCOR15*, from *C. bungeana*, a rare alpine subnival plant and a valuable species for research targeting the mechanism of cold-hardiness and stress-responsive genes (An et al. 2000; Fu et al. 2006). The deduced protein *CbCor15* has an amino acid sequence that is highly conserved with that of *A. pumila ApCOR15a, Arabidopsis AtCOR15a, Arabidopsis AtCOR15b*, *B. napus BN115*, and *C. bursa-pastoris CBCOR15*. This similarity indicates that *CbCOR15* belongs to the COR15 gene family, members of which are frequently expressed in vegetative tissues in response to low-temperature environmental stresses (Artus et al. 1996; Thomashow 1999; Takumi et al. 2003).

The *COR15a* gene encodes a small, plastid-targeted polypeptide that is processed to a mature form, *COR15am*, by cleavage of the N-terminal chloroplast-targeting signal peptide. This form is almost exclusively localized to the chloroplast stroma and increases freezing tolerance by protecting organelle membranes, altering the lamellar-to-hexagonal II phase transition temperature (Steponkus et al. 1998). Overexpression of many COR proteins, such as *Arabidopsis COR15a* or wheat *WCOR15* in *Arabidopsis* or tobacco, improves the freezing tolerance of protoplasts and whole plants (Artus et al. 1996; Shimamura et al. 2006). Our sequence analyses showed that the *CbCor15* protein is highly hydrophilic, especially the C-terminal two thirds of the polypeptide. As with other COR genes, this property may increase tolerance to cold stress by transgenic plants (Thomashow 1998).

In addition, our *CbCor15* amino acid sequence analysis indicated that the N-terminal has several features in common with transit peptides that target proteins to the stromal compartment of chloroplasts. *CbCOR15* expression was detected only in the leaves after 1 day of treatment at 2°C, thereby demonstrating that this gene is leaf-tissue-specific and suggesting that it is localized to the chloroplast stroma just as At*COR15a* is (Nakayama et al. 2007). Expression was induced at both 4°C and −4°C and was maintained at high levels for at least 7 days. We, therefore, can infer that *CbCOR15* is a cold-induced gene, playing an important role in the ability of *C. bungeana* to tolerate freezing stress.

In our bioassay, we transformed *CbCOR15* into the tobacco nuclear genome. All transgenic lines showed greater chilling/freezing tolerance than the nontransgenics. When chilled, the transgenic T_1 plants had less electrolyte leakage, a higher chlorophyll content, and greater relative water content. This suggests that the *CbCOR15* gene product functions in plasma membrane protection prevents water loss and crystallization of cellular components and increases chilling tolerance. Our results also demonstrated that freezing tolerance is significantly increased in transgenic plants, which is consistent with previous reports (Steponkus et al. 1998; Shimamura et al. 2006). Although overexpression leads to greater tolerance, this result differs from those that suggest that transformation of a single functional gene, e.g., *COR15a* appears to have little effect on crop freezing tolerance (Artus et al. 1996). We conclude *CbCOR15* plays an important role in conferring cold/freezing tolerance in transgenic tobacco plants, similar to the functioning of *AtCOR15a* and *Wcor15* (Steponkus et al. 1998; Thomashow 1999; Takumi et al. 2003; Shimamura et al. 2006).

Finally, electrolyte leakage, chlorophyll content, and RWC did not differ between transgenic lines—high versus weak expression—indicating that the extent of tolerance is not directly correlated with *CbCOR15* accumulations. This suggests that freezing tolerance is a multigenic trait, resulting from several factors. Therefore, the effective

threshold for such tolerance is apparently attained only through the collective action and cumulative effect of individual COR/LEA proteins, each with its own limited influence (Artus et al. 1996; Shimamura et al. 2006).

Acknowledgments This study has been supported by the National Outstanding Youth Foundation of China (30625008), the National High Technology Research and Development Program (863 Program) (2007AA021401), and the National Basic Research Program of China (973 Program) (2007CB108902). We thank Dr. Philip Hall for his elaborate reviews.

References

Altschul SF, Madden TL, Schafer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

An LZ, Liu YH, Feng HY, Feng GN, Chen GD (2000) Studies on characteristics of element contents of altifrigetic subnival vegetation at the source area of Urumqi River. Acta Bot Boreal-Occident Sin 20:80–86

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C, Thomashow MF (1996) Constitutive expression of the cold regulated *Arabidopsis thaliana COR15a* gene affects both chloroplast and protoplast freezing tolerance. Proc Natl Acad Sci USA 93:13404–13409

Ayitu R, Dunyan T, Li JZ, Fang Y (1998) The relationship between the structure of vegetative organs in *Chorispora bungeana* and its environment. J Xinjiang Agric Univ 21:273–277

Baker SS, Wilhelm KS, Thomashow MF (1994) The 5′-region of *Arabidopsis thaliana* cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol Biol 24:701–713

Bannai H, Tamada Y, Maruyama O, Nakai K, Miyano S (2002) Extensive feature detection of N-terminal protein sorting signals. Bioinformatics 18:298–305

Cattivelli L, Bartels D (1990) Molecular cloning and characterization of cold-regulated genes in barley. Plant Physiol 93:1504–1510

Chauvin LP, Houde M, Sarhan F (1993) A leaf-specific gene stimulated by light during wheat acclimation to low temperature. Plant Mol Biol 23:255–265

Dhanaraj AL, Slovin JP, Rowland LJ (2005) Isolation of a cDNA clone and characterization of expression of the highly abundant, cold acclimation-associated 14 kDa dehydrin of blueberry. Plant Sci 16:949–957

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Fu XY, Chang JF, An LZ, Zhang MX, Xu SJ, Chen T, Liu YH, Xin H, Wang JH (2006) Association of the cold-hardiness of *Chorispora bungeana* with the distribution and accumulation of calcium in the cells and tissues. Environ Exp Bot 55:282–293

Gilmour SJ, Artus NN, Thomashow MF (1992) cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. Plant Mol Biol 18:13–21

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J 16:433–442

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol 41:187–223

Hofgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res 16:9877

Jaakola L, Pirtila AM, Halonen M, Hohtola A (2001) Isolation of high quality RNA from bilberry (*Vaccinium myrtillus L.*) fruit. Mol Biotechnol 19:203–210

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. Science 280:104–106

Lin C, Thomashow MF (1992) DNA sequence analysis of a complementary DNA for cold-regulated *Arabidopsis* COR15 and characterization of the COR15 polypeptide. Plant Physiol 115:171–180

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10:1391–1406

Liu S, Wang X, Fan Z, Pang Y, Sun X, Wang X, Tang K (2004) Molecular cloning and characterization of a novel cold-regulated gene from *Capsella bursa-pastoris*. DNA Seq 15:262–268

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, p 440

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nakayama K, Okawa K, Kakizaki T, Honma T, Itoh H, Inaba T (2007) *Arabidopsis* Cor15am is a chloroplast stromal protein that has cryoprotective activity and forms oligomers. Plant Physiol 144:513–523

Nordin K, Vahala T, Palva ET (1993) Differential expression of two related, low-temperature-induced genes in *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol 21:641–653

Sakai A, Larcher W (1987) Frost survival of plants. Responses and adaptation to freezing stress. In: Billings WD, Golley F, Lange OL, Olson JS, Remmert H (eds) Ecological studies, vol 62. Springer, Berlin, p 252

Shimamura C, Ohno R, Nakamura C, Takumi S (2006) Improvement of freezing tolerance in tobacco plants expressing a cold-responsive and chloroplast-targeting protein WCOR15 of wheat. J Plant Physiol 163:213–219

Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF (1998) Mode of action of the COR15a gene on the freezing tolerance of *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 95:14570–14575

Takumi S, Koike A, Nakata M, Kume S, Ohno R, Nakamura C (2003) Cold-specific and light-stimulated expression of a wheat (*Triticum aestivum L.*) Cor gene Wcor15 encoding a chloroplast-targeted protein. J Exp Bot 54:2265–2274

Thomashow MF (1990) Molecular genetics of cold acclimation in higher plants. In: Scandalios J (ed) Genomic responses to environmental stress. Academic, San Diego, pp 99–131

Thomashow MF (1993) Genes induced during cold acclimation in higher plants. In: Steponkus PL (ed) Advances in low-temperature biology, vol 2. JAI Press, London, pp 183–210

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol 118:1–8

Thomashow MF (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571–599

Thomashow MF (2001) So what's new in the field of plant cold acclimation? Lots! Plant Physiol 125:89–93

Tsvetanov S, Ohno R, Tsuda K, Takumi S, Mori N, Atanassov A, Nakamura C (2000) A cold-responsive wheat (*Triticum aestivum L.*) gene wcor14 identified in a winter-hardy cultivar `Mironovska 808'. Genes Genet Syst 75:49–57

Walker K, Croteau R (2000) Taxol biosynthesis-molecular cloning of a benzoyl-CoA-taxane 2a-O-benzoyltransferase cDNA from *Taxus* and functional expression in *Escherichia coli*. Proc Natl Acad Sci USA 97:13591–13596

Weatherley PE (1950) Studies in the water relations of cotton plants: 1. The field measurement of water deficit in leaves. New Phytol 49:81–97

White TC, Simmonds D, Donaldson P, Singh J (1994) Regulation of BN115, a low-temperature-responsive gene from winter *Brassica napus*. Plant Physiol 106:917–928

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6:251–264