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Improving tobacco freezing tolerance by co-transfer of stress-inducible *CbCBF* and *CbICE53* genes

P. LIN[1], C. SHEN[2], H. CHEN[2], X.H. YAO[1]*, and J. LIN[2]*

Research Institute of Subtropical Forestry, Chinese Academy of Forestry, Fuyang, 311400, Zhejiang, P.R. China[1]
Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai, 200438, P.R. China[2]

Abstract

Cold stress is one of the major limitations to crop productivity worldwide. We investigated the effects of multiple gene expression from cold tolerant *Capsella bursa-pastoris* in transgenic tobacco (*Nicotiana tabaccum*) plants. We combined *CblCE53* and *CbCBF* into a reconstruct vector by isocaudomers. Plant overexpression of *CbICE53* under the stress inducible *CbCOR15b* promoter and *CbCBF* under a constitutive promoter showed increased tolerance to both chilling and freezing temperatures in comparison to wild-type plants, according to the electrolyte leakage and relative water content. The expressions of endogenous cold-responsive genes in transgenic tobacco (*NtDREB1*, *NtDREB3*, *NtERD10a* and *NtERD10b*) were obviously upregulated under normal and low temperature conditions. These results suggest that the *CbICE53* + *CbCBF* transgenic plants showed a much greater cold tolerance as well as no dwarfism and delayed flowering. Thus they can be considered as a potential candidate for transgenic engineering for cold tolerant tobacco.

Additional key words: *Capsella bursa-pastoris*, cold inducible genes, *Nicotiana tabaccum*, stress inducible promoter, transgenic plants.

Introduction

Low temperature is a major environmental factor affecting plant growth, development, geographical distribution, and crop yield. Plants show a broad range of ability to acclimate and to survive exposure to low temperature (LT) and freezing conditions; traits that are of the great ecological and economic importance. As a consequence, the physiology and genetics of LT tolerance are areas of intense study (Park *et al*. 2015). Most temperate plants, such as winter wheat (Fowler *et al.* 2016) and winter rape (Urban *et al.* 2013), are able to tolerate both chilling and freezing temperatures. In contrast, species from tropical regions, such as tobacco (Hu *et al.* 2016) and rice (Almeida *et al*. 2016), are unable to tolerate freezing temperatures and even suffer chilling injury when exposed to temperatures in the range of 0 - 12 °C. The mechanical injuries caused by the conformation changes of the cytoplasmic membranes and the loss of water may lead to the cell or the plant death (Steponkus *et al*. 1993, Uemura *et al.* 1997). Many plants improved cold tolerance after exposure to non-freezing temperatures for some period of time and this is called cold acclimation (Thomashow 2010). Under cold stress, a lot of biochemical activities occur to ameliorate the cold damages such as the enhancement of various osmolytes, cytoskeleton reorganization, and so on (Uemura *et al.* 1995, Hughes and Dunn 1996). All these changes are correlated with the activation and expression changes of the respective genes (Xin and Browse 2000). Plant cells can also respond to LT stresses by precise regulation of the expression of transcription factors and gene effectors. The LT signaling pathways include transcriptional, post-transcriptional, translational, and post-translational regulators of LT induced expression of the functional genes. The C-repeat binding factor (CBF) is considered as one of the most important factors in the cold responsive pathway in higher plants, and so it has been a hotspot in cold resistance

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Abbreviations: CBF - C-repeat binding factor; CDKA - cyclin-dependent kinase A; COR - cold-responsive; CRT/DRE - C-repeat/dehydration responsive element; CYCB - cyclin B; CYCD - cyclin D; DREB - dehydration responsive element binding protein; EV - empty vector control; GFP - green fluorescent protein; GUS - β-glucuronidase; ICE - inducer of CBF expression; LT - low temperature; LTRD - low temperature responsive elements; RT-qPCR - reverse transcription quantitative polymerase chain reaction; WT - wild type.
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* Corresponding authors: fax: (+86) 21 51630536, e-mail: yaoxh168@163.com; linjuan@fudan.edu.cn

research (Thomashow 2010, Zhou *et al*. 2011). The CBF transcription factors play an important role by regulating the expression of downstream genes, such as the cold-responsive (*COR*) genes, and ultimately leading to an increase in freezing tolerance (Jaglo-Ottosen *et al*. 1998). In *Arabidopsis thaliana*, there are four *CBF* genes, *CBF1*, *CBF2*, *CBF3* (Gilmour *et al*. 2004), and *CBF4* (Haake *et al*. 2002), also known as dehydration responsive element binding *DREB1a*, *DREB1b*, *DREB1c*, and *DREB1d*, respectively (Liu *et al.* 1998, Sakuma *et al.* 2002). In addition to *Arabidopsis*, *DREB1*/*CBF* exists in a wide range of crops, including rape (Jaglo-Ottosen *et al.* 2001), tomato (Zhang *et al*. 2004), barley (Xue 2003), rice (Dubouzet *et al*. 2003), and wheat (Shen *et al.* 2003). Hence the *CBF*-like genes are considered as excellent candidates for engineering cold tolerant crops. Several different gene transfer approaches have been employed to improve the stress tolerance of plants (Holmberg and Bulow 1998). The ability of ectopic *CBF* transgene activity to increase plant freezing tolerance has been demonstrated in many plant systems, including species of agricultural importance such as peanut (Bhatnagar-Mathur *et al.* 2014), rice (Lee *et al.* 2004), tobacco (Zhou *et al.* 2014), and poplar (Benedict *et al.* 2006). But, the constitutive expression of *CBFs* causes not only increased cold tolerance, but also results in obvious growth retardation including dwarfing (Zhou *et al.* 2014), delayed flowering and prostrate growth habits (Kasuga *et al.* 1999, Gilmour *et al.* 2000), reduction in fruit set and seed number (Hsieh *et al*. 2002a), and inhibition of tuber formation in potato (Pino *et al*. 2007), even under normal growth conditions. Transgenes, whose overexpression causes dwarfism and delayed flowering, include *AtCBF1-3*, *OsDREB1A*, and *OsDREB2A* in *Arabidopsis* (Jaglo-Ottosen *et al*. 1998, Kasuga *et al*. 1999, Gilmour *et al*. 2000, Dubouzet *et al*. 2003), *OsDREB1A* and *OsDREB2A* in rice (Ito *et al.* 2006), *AtCBF3*, *GhDREB1*, and *CbCBF* in tobacco (Kasuga *et al*. 2004, Shan *et al.* 2007, Zhou *et al.* 2014), and *AtCBF1* and *AtCBF3* in tomato (Hsieh *et al.* 2002a,b). The elimination of this kind of growth repression is crucial for utilization of *CBF*-like genes in plant breeding. In preliminary studies, we have observed that constitutive overexpression of *CbCBF* from *Capsella bursa-pastoris* leading to increased freezing tolerance, is also associated with strong growth retardation in tobacco (Zhou *et al.* 2014). In addition, a single gene transfer often leads only to partial improvement in the cold resistance ability. Expressions of *Arabidopsis* COR15a protein, wheat WCS19 protein, and WCOR15-GFP fusion protein improve the freezing tolerance of transgenic plants only under limited conditions (NDong *et al*. 2002, Shimamura *et al.* 2006). The current efforts to improve plant cold stress tolerance by gene transformation have resulted in important achievements; however, the nature of the genetically complex mechanisms of cold stress tolerance, and the potential detrimental side effects, make this task extremely difficult. Some cold tolerance-related traits in crops are controlled by many genes, transcription using multi-gene can effectively improve the cold resistance of crop. Here, we use the stress-inducible *CbCOR15b* promoter to drive the expression of *CbICE53*, design is aimed at reducing the negative effects by the use of CaMV 35S promoter. At the same time, we transferred two stress inducible transcription factors that regulate many genes involved in stress tolerance in tobacco.

Materials and methods

Plants and treatments: *Capsella bursa-pastoris* (L.) Medik. and *Nicotiana tabacum* L. were grown in pots in a greenhouse at a temperature of 28 °C, a 16-h photoperiod, an irradiance of 100 μmol m^{-2} s^{-1}, and a relative humidity of 60 %. For cold treatment, three sets of two-week-old tobacco seedlings, each containing more than 30 plants, were transferred to 4 °C for 12 or 24 h. Then, these seedlings were transferred to a -4 °C incubator for 1 h. After the cold treatments, the samples were immediately frozen in liquid nitrogen and stored at -80 °C until the use. The data were presented as the average of three replicates for each treatment.

Cloning of *CbCBF* and *CbICE53* genes and *CbCOR15b* promoter from *C. bursa-pastoris*: The total RNA was extracted from *C. bursa-pastoris* leaves using the plant RNA mini kit (*Aidlab*, Beijing, China). The *CbICE53* or *CbCBF* gene coding regions were amplified using cDNA as template with Pfu DNA polymerase (*Promega*, Madison, USA) and gene-specific primers: CbICE53-P1 and CbICE53-P2 or CbCBF-P1 and CbCBF-P2, respectively. Two gene specific primer pairs were designed according to the cDNA sequence of *CbICE53* or *CbCBF* described in Zhou *et al*. (2012b, 2014) and listed in the *DDBJ*, *EMBL*, and *GenBank* databases under accession number AY506804 or AY391121. The total genomic DNA of *C. bursa-pastoris* was isolated by the cetyltrimethyl ammonium bromide method (Murray and Thompson 1980). The *CbCOR15b* promoter region was amplified with DNA polymerase (*Promega*) and gene-specific primers CbCOR15b-P1 and CbCOR15b-P2, designed according to the sequence of *CbCOR15b* described in Wu *et al.* (2012). The PCR product was purified, cloned into a pMD19-T vector (*TaKaRa*, Dalian China), and sequenced (*Sangon*, Shanghai, China).

Vector construction for plant transformation: The amplified *CbICE53* fragment was digested with *Nco*I/*Bst*EII and ligated into the corresponding sites of pCAMBIA1304 vector (*CAMBIA*, Canberra, Australia) by replacing *GUS*. This led to the production of a plasmid p35S::*CbICE53*, in which the CaMV 35S promoter drived the expression of *CbICE53* cDNA sequence. Similarly, the

amplified *CbCOR15b* promoter fragment was digested with *Pst*I/*Nco*I and was cloned into p35S::*CbICE53* vector by replacing CaMV 35S promoter using the same restriction sites. To construct p*CbCOR15b*::*CbICE53*, in which the *CbCOR15b* promoter drives the expression of *CbICE53* cDNA sequence. The amplified *CbCBF* fragment was digested with *Nco*I/*Bst*EII and ligated into pCAMBIA1304 vector by replacing *GUS* using the same restriction. This produced plasmid p35S::*CbCBF*, in which the CaMV 35S promoter drives expression of *CbCBF* cDNA sequence. To facilitate the insertion of 35S-CbCBF-Nos into the vector p*CbCOR15b*::*CbICE53*, a forward primer p1304F was designed in p1304 closed to the multiple cloning sites containing an *Eco*RI site (Table 1 Suppl.), whereas a *Spe*I site was flanked with a reverse primer NosR-SpeI (Table 1 Suppl.). The PCR amplification of the fragment p35S-CbCBF-Nos was carried out using the primers p1304F and NosR-SpeI with *KOD plus* DNA polymerase (*Toyobo,* Osaka, Japan). The amplified fragment was digested with *Xba*I and *Spe*I, similarly, the pCbCOR15b::*CbICE53* vector was treated with *Xba*I in 37 °C water for 4 h and purified on agar gel. Then the purified pCbCOR15b-*CbICE53* segment was treated with CIAP for 1 h at a temperature of 37 °C and directly purified on agar gel with PCR clean kit (*TaKaRa*). The two treated fragments were ligated and transformed into DH5α for reconstructed vector and extracted the plasmids. So, the plasmid pCbCOR15b::*CbICE53*+p35S::*CbCBF*, in which the *CbCOR15b* promoter drived the expression of *CbICE53* cDNA and CaMV 35S promoter driven expression of *CbCBF* cDNA was generated (Fig. 1*A)*. The fusion constructs (pCbCOR15b::*CbICE53*+p35S::*CbCBF*) and control (p35S::*GUS*) vector were introduced into *Agrobacterium tumefaciens* strain EHA105.

Tobacco transformation and its confirmation: We used the extracted reconstructed vector plasmid for EHA105 transformation. *CbCOR15b* promoter, *CbICE53* and *CbCBF* genes were identified by DNA-PCR to ensure the transformation. The transformation of tobacco leaf discs was performed as described by Zhou *et al.* (2014). Transformants were selected using 30 mg dm^{-3} hygromycin in the T_0 generation and the transgenic plants were verified with *CbICE53* gene specific primers CbICE-F and CbICE-R to amplify a 1 500 bp fragment, *CbCBF* gene specific primer pair CbCBF-P1 and CbCBF-P1 to amplify a 650 bp fragment and *CbCOR15b* promoter primers CbCOR15b-P1 and CbCOR15b-P2 to amplify a 1120 bp fragment. Afterward the plants were shifted to 4 °C for 24 h and -4 °C for 1 h. Four-week-old plant samples were collected for real-time quantitative PCR analysis.

Physiological characteristics of transgenic tobacco plants: Six-week-old seedlings were grown at 28 °C and exposed to 4 °C for 24 h and -4 °C for 1 h then transferred back to 28 °C. The leaf samples from each of the three replicates were collected and rinsed with distilled water to remove possible surface ion contamination. The electrolyte leakage, relative water content and glucose content measurements were carried out according to Zhou *et al*. (2014). At every time spot, 0.1 g leaf tissue was gathered using 2 cm^3 of deionized water for 2 h to test initial conductivity (IC), followed by 121 °C treatment for 15 min to measure final conductivity (FC). The electrolyte leakage was calculated as IC/FC. Conductivity of the leachate was determined with a *DDS-11A* conductivity detector (*Shanghai SUOSHEN Electrical Equipment Co., Shanghai*, China). Relative water content (RWC) in the leaves was measured as [(FM - DM)/(WSM - DM)] × 100, where FM is fresh mass, DM is dry mass, and WSM is water saturated mass determined after soaking in water for 2 h at room temperature. Glucose content was assayed with a glucose assay kit (*Sigma*, St. Louis, MO, USA). Collected leaves were incubated in 1.5 cm^3 of distilled water and the absorbance of NADH was measured at 340 nm using a *BioPhotometer Plus* (*Eppendorf*, Hamburg, Germany). All measurements were repeated three times, and Student's *t*-test was used for statistical analysis.

Relative mRNA expression analysis using real time quantitative PCR: To remove contaminating genomic DNA, extracted RNAs were treated with *DNase I* (*Promega*) according to the manufacturer's instructions. DNase-treated RNA samples (1 μg) were reverse-transcribed by *PrimeScript RT Master Mix* (*TaKaRa*) in volume of 0.02 cm^3 according to the manufacturer's instructions. Real-time qPCR was carried out using *SYBR Premix Ex Taq*^{TM} *II* (*TaKa*Ra) in a volume of 0.02 cm^3 according to manufacturer's instructions. The reactions were performed in three technical replicates per sample for each run, and each sample was tested on three biological replicates. The specific primers used for these genes (*CbCBF*, *CbICE53*, *NtCDKA*; *4*, *NtCYCD2*; *NtCYCB1*; *NtDREB1*, *NtDREB3*, *NtERD10a*, *NtERD10b*, and *NtTSI1*) were designed using *Primer Express* software following the manufacturer's guidelines (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and their sequences are listed in Table 1 Suppl. Values "Ct" obtained for all genes were normalized to that of an internal control of *actin* gene from tobacco (AJ133422). For spatial expression analysis, the expression level of *CbCBF* or *CbICE53* gene were relative to *actin*, calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). For expression analysis of *NtCDKA*; *4*, *NtCYCD2*; *NtCYCB1*; *NtDREB1*, *NtDREB3*, *NtERD10a*, *NtERD10b*, and *NtTSI1* in response to cold stresses, transcript amount was determined using $2^{-\Delta\Delta Ct}$ method. The transcription of each gene in wild type (WT) plant under 28 °C was indicated as 1. Statistical significances (α = 0.05) of differences between the treated and control plants were determined by one-way *ANOVA* and Tukey test. In order to test the specificity of the primers for *CbCBF*, *CBICE53*, *NtTSI1*, and *NtDREB3* genes sharing a high sequence identity, the real-time PCR products were sequenced (*Sangon*).

Results and discussion

By using the PCR cloning technology, we successfully amplified *CbICE53* gene, *CbCBF* gene, and *CbCOR15b* promoter sequence from *C. bursa pastoris*. The length of the specific fragments were 1 476, 657, and 1 120 bp, respectively (Fig. 1 Suppl.). And then the co-transformation pCbCOR15b::*CbICE53*+p35S::*CbCBF* vector was introduced into tobacco. A total of 27 independent transgenic lines were generated and *CbCBF*, *CbICE53*, and *CbCOR15bP* positive lines were confirmed with PCR using gene specific primers. Fig. 1*B* shows the results of positive transgenic plant line #1 (L1) and line *#6* (L6). The *CbCBF* expression varied among 10 PCR positive lines of T_1 generation with the highest expression in pCbCOR15b::*CbICE53*+p35S::*CbCBF* line #1 (L1) and with intermediate and low expression in lines *#6* (L6) and *#15* (L15) (Fig. 1*C*). The lines L1, L6, and L15 were chosen for the further research.

Since *CbICE53* and *CbCBF* expression is regulated by

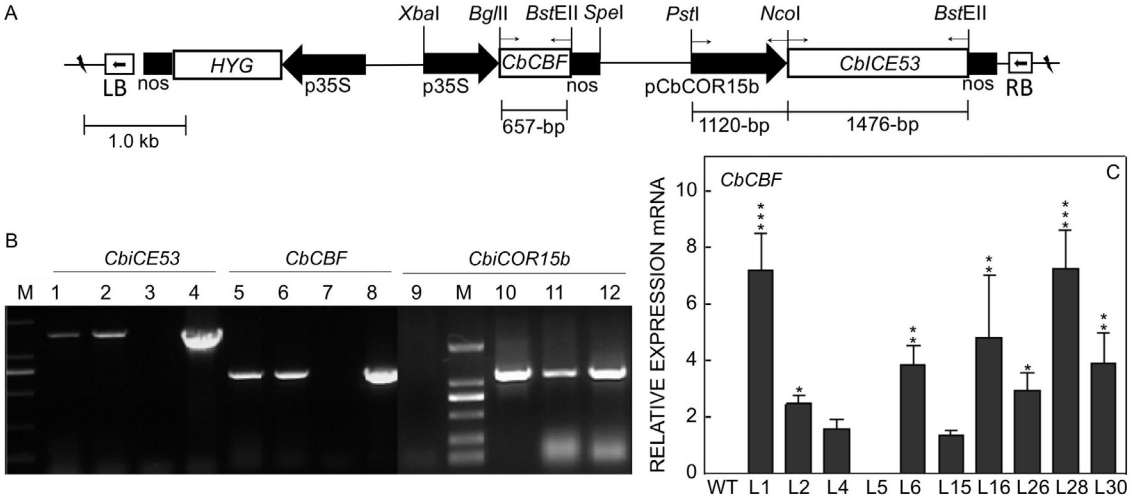

Fig. 1 The T-DNA regions of the binary vector and molecular characterization of transgenic tobacco plants. *A* - T-DNA consists of the hygromycin phosphatase gene (*HYG*) regulated by the CaMV 35S promoter, *CbCBF* gene under control of the CaMV 35S promoter, and *CbICE53* gene under control of the CbCOR15b promoter. nos - NOS terminator; LB - left T-DNA border; RB - right T-DNA border; *arrows* mark the locations of forward and reverse primers. *B* - Verification of transformants from T_3-generation regenerated tobacco lines 1 (L1) and lines 6 (L6) by PCR amplification from genomic DNA. *Lane* M - DL2000 DNA size marker; *lanes* 4, 8, and 10: plasmid control; *lanes* 3, 7, and 9 - WT control; *lanes* 1, 5, and 11 - transgenic tobacco line 1 (L1); *lanes* 2, 6, and 12 - transgenic tobacco line 6 (L6). *C* - The relative expression of *CbCBF* in ten transgenic plant lines ($n = 3$). * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

cold stress, we produced three pCbCOR15b::*CbICE53* + p35S::*CbCBF* lines in *N. tabacum* to gain further insight into the function of *CbICE53* and *CbCBF*. The relative expressions of the *CbICE53* and *CbCBF* genes in three transgenic progeny plants were examined by real time qPCR using gene-specific primers. As shown in Fig. 2*A,B*, the transgenic lines (L1, L6, and L15) displayed high *CbCBF* expression, while *CbCBF* transcripts were not detected in the empty vector control (EV) and WT plants under normal temperature (28 °C). The *CbICE53* gene was slightly expressed under the control conditions, but after exposure to low temperatures, the expression of *CbICE53* gene was considerably elevated, while the transcription of *CbCBF* gene was not upregulated in L1, L6, L15, as well as EV in WT plants. The transcriptions of *CbCBF* gene among L1, L6, L15, EV, and WT plants were not obviously different in contrast to expressions of *CbICE53* gene. The results showed that CbCOR15b promoter regulated the expression of *CbICE53* under low temperature. Subsequently, cold tolerance of the transgenic L1, L6, and L15 lines seedlings was tested. When the 6-week-old seedlings were grown under normal temperature, the phenotypes of the transgenic lines were the same as those of EV and WT (Fig. 2*C*). By contrast, when 6-week-old seedlings were transferred and cultured at low temperature of 4 °C for 12 or 24 h, EV and WT plants displayed severe damage and especially when they were exposed to -4 °C for 1 h, whereas the transgenic lines showed only a slight change (Fig. 2*C*). All transgenic plants survived after recovering at 28 °C for 4 d, while the control plants were very weak with most leaves withered, implicating that the transgenic plants overexpressing *CbICE53* and *CbCBF* showed higher cold tolerance than the EV and WT control plants.

To further evaluate the effect of the *CbICE53* and *CbCBF* overexpression to cold stress, the electrolyte leakage, RWC, and glucose content, which are important indicators of low temperature adjustment in plant cells, were conducted using the leaves of L1, L6, L15, EV, and WT. The values showed no significant disparity between the WT and EV plants, demonstrating that the empty vector had no effect on the cold resistance of plants.

Comparing the transgenic plants with WT and EV under 28 °C, 4 °C for 12 h, and -4 °C for 1 h, we found that the electrolyte leakages in L1, L6, and L15 were significantly lower than in both EV and WT, suggesting that overexpression of *CbICE53* and *CbCBF* supported a better protection of the plasma membrane under cold stress (Fig. 2*D*). The RWC in L1, L6, and L15 lines plants descended definitely less than in EV and WT (Fig. 2*E*). Moreover, L1, L6, and L15 lines accumulated much more glucose, which is considered to be a cryoprotectant of membranes ($P < 0.01$). These results indicated that overexpression of *CbICE53* and *CbCBF* prevented from damage of active cellular components in tobacco plants. There was no obvious disparity in these physiological indices between three transgenic lines, which was consistent with the expression of cold responsive genes.

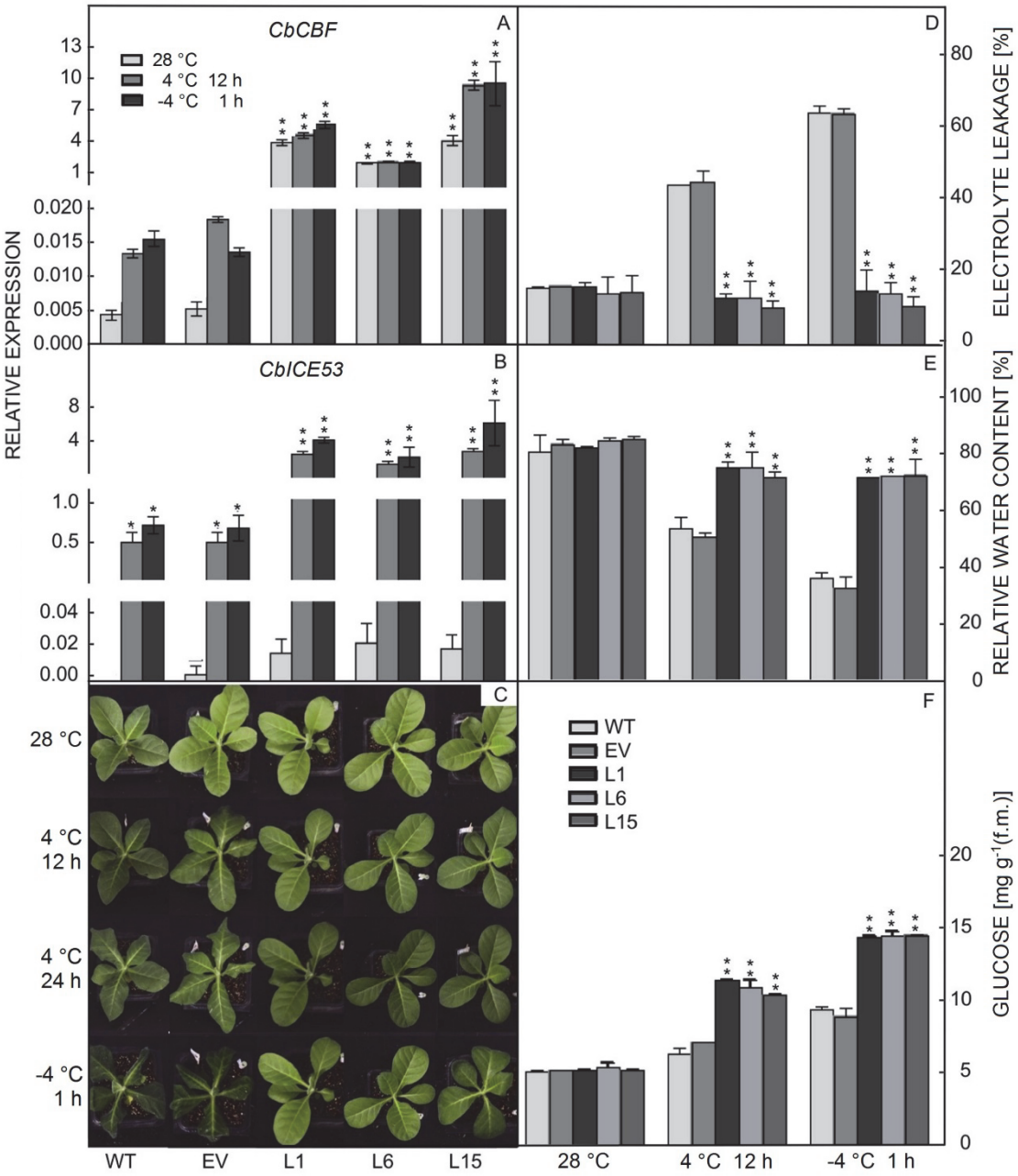

Fig. 2 Differences between transgenic (L1, L6, L15), wild type (WT), and transgenic empty vector (EV) tobacco plants in phenotype characterization and physiological indices for cold tolerance. *A, B* - The relative expression of *CbCBF* and *CbICE53* in transgenic plant (L1, L6, and L15) leaves collected at 28 °C, after 12 h at 4 °C, and after 1 h at -4 °C ($n = 3$). *C* - appearance of different plant types (WT, EV, L1, L6, and L15) under control conditions and low temperature stresses. Electrolyte leakages (*D*), relative water content (*E*), glucose content (*F*) in WT, EV, L1, L6, and L15 plants in control conditions and under low temperature stress. Means ± SDs, $n = 39$, * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

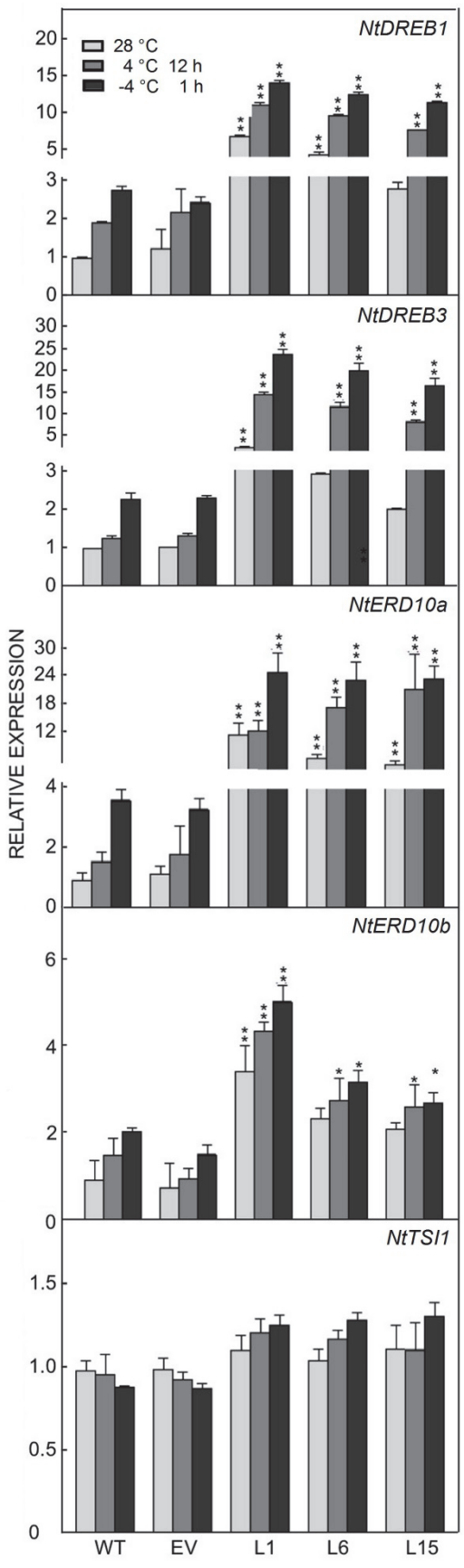

Fig. 3 The relative expression of cold responsive genes (*NtDREB1*, *NtDREB3*, *NtERD10a*, *NtERD10b*, *NtTSI1*) in WT, EV, L1, L6, and L15 plants. The tobacco *actin* gene was used as internal control. The expression of the WT control was set as 1. Means ± SDs from three biological replicates, * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

Our previous results showed that *35S::CbCBF* tobacco plants exhibited smaller plant size, slower growth rate, and delayed flowering. The leaf cells of *CbCBF* over-expressing tobacco lines attained smaller sizes and underwent delayed cell division with reduced expression of cyclin D genes (Zhou *et al.* 2014). In this study, we examined the expression of three endogenous cell cycle regulation genes in tobacco using real time qPCR method such as CDK genes *NtCDKA*; *4* (AF289467) (Yu *et al.* 2003), D-type cyclin genes *NtCYCD2*; *1* (AJ011892) (Sorrell *et al.* 1999) and a B-type cyclin gene *NtCYCB1*; *2* (AF289466) (Sorrell *et al.* 2001). In normal (28 °C) and low temperature (4 °C), no significant difference in their expression (data not shown) was found between WT and *CbICE53* and *CbCBF* co-transformants.

Previously, we reported that *CbICE53* and *CbCBF* serving as transcriptional activators can cause altered expression of downstream genes (Zhou *et al.* 2012b, 2014). In this study, we examined the expression of five endogenous cold-responsive genes in the control and transgenic tobacco plants using real time qPCR. *NtDREB1* (EU727155), *NtDREB3* (EU727157) (Zhou *et al.* 2012b), and *NtTSI1* (AF058827) (Park *et al.* 2001) are *DREB*/*CBF*-like genes encoding AP_2/EREBP-type trans-acting factors. *NtERD10a* (AB049335) and *NtERD10b* (AB049336) are target stress-inducible genes of *DREB*/*CBFs* (Kasuga *et al.* 2004). In the transgenic tobacco plants, the *NtDREB1*, *NtDREB3*, *NtERD10a*, and *NtERD10b* genes except for *NtTSI1* were highly expressed even under normal growth conditions in comparison with the WT plants, and the expression of these genes was significantly increased under low temperature stress (Fig. 3). These results indicate that the *NtDREB1*, *NtDREB3*, *NtERD10a*, and *NtERD10b* genes are target stress-inducible genes of *CbICE53* and *CbCBF* in tobacco and are probably responsible for the stress tolerance of the transgenic tobacco. This result is in agreement with the study of 35S::*CbICE53* transgenic plant (Zhou *et al.* 2012a). Among the four genes, the *NtERD10b* gene showed the lowest expression (Fig. 3). In *Arabidopsis,* this gene is not induced by low temperature while the other genes like the *ICE* and *CBF* target genes are induced by low temperature. However, we detected increased expression of the four genes under a low temperature, which indicated that they were induced by low temperature. Therefore, we think that *NtDREB1*, *NtDREB3*, *NtERD10a,* and *NtERD10b* are the target genes of *CbICE53* and *CbCBF* in tobacco.

Transgenic approaches, which can strictly realize precise modulation of efficient changes in gene expression, have been continually pursued for improvement of crop tolerance to environmental stresses (Kasuga *et al.* 1999).

ICE-like genes are transcription factor genes. The ICE-CBF-COR signaling pathway has been found generally in higher plants, playing a key role in cold response (Chinnusamy *et al.* 2007). Many key genes involved in the regulatory networks under cold stress have been identified (Seki *et al.* 2007). Probably the best studied group of transcription factor genes involved in cold as well as drought stress are the *CBF*/*DREB1* genes (Sakuma *et al.* 2006, Qin *et al.* 2007, Gutha and Reddy 2008). A number of studies have revealed the constitutive expression of the *CBF* genes resulting in stress tolerant transgenic plants. The *DREB1A* gene seems to be effective in different plants; overexpression of the *AtDREB1A* in tobacco (Kasuga *et al.* 2004), wheat (Pellegrineschi *et al.* 2004) and peanut (Bhatnagar-Mathur *et al.* 2014) has been shown to lead to increased drought tolerance. Overexpression of the *Arabidopsis DREB1A* in transgenic rice increased the tolerance to drought and salinity but not to low temperature (Oh *et al*. 2005). But transgenic rice overexpressing *OsDREB1A*, DREB gene orthologue, showed improved tolerance to drought, high salt, and low temperature stresses (Ito *et al.* 2006). Furthermore, *HvCBF4* from barley appears to be more efficient than *DREB1A* from *Arabidopsis* in conferring stress tolerance to transgenic rice (Oh *et al.* 2007). These data suggest functional differences between members of the DREB/CBF family and highlights the variation in stress tolerance between transgenic plant species. These functional differences could be due to complexity and nature of the target genes that are present in the genome and the capacity of the transcription factor to activate or repress each target gene. *C. bursa-pastoris* belonging to *Brassicaceae* family similarly as *A. thaliana* grows in diverse habitats (Han *et al.* 2015) and it is well adapted to different environments, especially low temperatures. Its seeds germinate in autumn with seedling rosettes overwintering, or germinate soon after the ground thaws in spring (Tao and Wang 2012). In the previous work, our lab cloned four genes of ICE-CBF-COR signaling pathway from *C. bursa-pastoris.* The *CBF*-like gene *CbCBF* (Wang *et al*. 2004), *CbICE53* (Zhou *et al.* 2012b), *CbCOR15a* (Zhou *et al.* 2012a) and *CbCOR15b* (Wu *et al.* 2012) with a highly similar sequence to that of *CBF3, ICE1, COR15a*, and *COR15b* from *A. thaliana*. The expression of these genes is obviously responsive to chilling and freezing and *CbCOR15a* and *CbCOR15b* are expressed not only in leaves but also in stems and roots. At the same time, we found that transgenic tobacco plants expressing *CbICE53*, *CbCBF*, or *CbCOR15a/b* showed greater cold tolerance under 4 and 0 °C (Wu *et al.* 2012, Zhou *et al*. 2012a,b, 2014). Particularly, *CbCBF* overexpressing plants exhibited suppressed growth including smaller plant size, slower growth rate, and delayed flowering (Zhou *et al.* 2014). These studies demonstrate the important role of cold induction genes in the acquisition of stress tolerance, which may ultimately contribute to agricultural and environmental practices. Although plant transformation with stress responsive and regulation genes can alter the expression of some downstream stress-associated multiple genes, it may also activate additional non-stress genes that adversely affect the normal agronomic characteristics of a crop. These negative effects can be partially prevented by the use of stress-inducible promoters that control the expression of these genes (Kasuga *et al.* 1999) or by the transfer of several genes that are either involved in signaling or regulatory pathways (Wang *et al.* 2003). In previous studies, some scientists used a stress-inducible promoter replacing the 35S CaMV promoter. They demonstrated that use of the abiotic stress inducible *rd29A* promoter to drive *AtCBF* or *ZmDREB* transgene expression can minimize the negative effects on plant growth (Kasuga *et al.* 2004, Pino *et al.* 2007). The *rd29A* promoter includes both DRE and ABRE elements, where dehydration, high salinity, and low temperatures induce the gene. The DRE motif is recognized by CBF/DREB1 transcription factors (Stockinger *et al.* 1997, Liu *et al.* 1998). Apart from *rd29A* promoters, *COR15* and *COR78* gene promoters induced by cold stress and water stress, respectively, have also been studied. The *COR15* and *COR78* promoter sequences contain also low temperature responsive elements (LTRD) with a consensus sequence of A/GCCGAC. In the 5' upstream region of CbCOR15b, two CRT/DRE core motifs were identified, similar to AtCOR15b (Wu *et al.* 2013). Transgenic plants overexpressing *DREB1a* transcription factor gene under the control of stress inducible promoter CbCOR15a showed a better growth than those obtained using the constitutive CaMV 35S promoter (Shen *et al*. 2014). The *COR15* genes differ in their cold sensitivity: *COR15* from *C. bursa-pastoris* being more sensitive to cold than *COR15* from *A. thaliana; COR15a* from *C. bursa-pastoris* being more sensitive to cold than *COR15b.* Since freezing tolerance is a multigenic trait (Thomashow 2001), transformation of a single functional gene like COR15a appears to have a limited effect on crop freezing tolerance (Artus *et al.* 1996). Because many aspects of cold adaptation process are under transcriptional control, many transcription regulatory factors were chosen as one of the best targets for engineering crops to achieve enhanced cold tolerance.

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