Vol.56, n.6: pp. 895-903, November-December 2013
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Cloning of *Ammopiptanthus mongolicus C-repeat-binding factor* Gene and Its Cold-induced Tolerance in Transgenic Tobacco

Lijiang Gu, Han Zhang, Huiming Guo and Hongmei Cheng*
Biotechnology Research Institute; Chinese Academy of Agricultural Sciences; Beij; PR China

ABSTRACT

C-repeat-binding factors (CBFs) are a type of important regulon in stress-related signal transduction pathways that control plant tolerance of abiotic stress. Ammopiptanthus mongolicus *is the only evergreen broadleaf shrub in the northwest desert of China. The species shows strong resistance to environmental stress, especially to cold stress. An* A. mongolicus *CBF1 gene (AmCBF1) was cloned and transformed into tobacco. Expression of AmCBF1 could be detected in* A. mongolicus *shortly after exposure to low temperature of 4°C. Analysis on ratio of electrolytic leakage, soluble sugar content, free proline content, malondialdehyde (MDA) content and peroxidase (POD) activity before and after cold treatment (4°C) for 24 h indicated AmCBF1 conferred higher cold tolerance to AmCBF1 transgenic tobacco compared with the wild type and empty vector transformed tobacco.*

Key words: *Ammopiptanthus mongolicus*, *C-repeat-binding factor*, Gene transformation, Physiological index detection

INTRODUCTION

After a period of low-temperature exposure, plants are better able to tolerate freezing temperatures (Hughes and Dunn 1996). This phenomenon is referred to as cold acclimation (Thomashow 1998). Exposure to low temperature triggers a highly complex regulatory program that results in extensive reorganization of the transcriptome. These changes include up- and down-regulation of hundreds of genes when plants are incubated at low temperature (Kilian et al. 2007, Robinson and Parkin 2008). The best-characterized genetic control of the cold acclimation process is executed by the C-repeat-binding factor/dehydration-responsive element binding factor (CBF/DREB1) cold-response pathway in *Arabidopsis* (Thomashow 2001, Shinozaki et al. 2006). *CBF* genes are widespread in higher plants, but their expression patterns vary among different species. Cold acclimation in *Arabidopsis* involves the action of the CBF cold-response pathway. In *Arabidopsis*, three cold-inducible *CBF* genes, *CBF1*, *CBF2*, and *CBF3*, also referred to as *DREB1b*, *DREB1c*, and *DREB1a*, respectively, are encoded (Liu et al. 1998). These transcription factors bind the cold- and dehydration-responsive DNA regulatory element (CRT/DRE) present in the promoters of cold-inducible genes, including *COR15a* and *COR78/RD29a* (Stockinger et al. 1997), and stimulate their transcription.

As a relictual evergreen xerophytic shrub and a peculiar species in the Tengger Desert in western China, *Ammopiptanthus mongolicus (Maxim.)*

*Author for correspondence: chenghongmei@caas.cn

Cheng f. shows strong resistance to drought, cold, heat, solar radiation stress and sterile soil (Wei et al. 2011) and can survive winter temperatures even less than −30°C. Previous studies on *A. mongolicus* have focused mainly on antifreeze proteins, genetic diversity, spatial heterogeneity of soil, and the cold-induced *AmCIP* gene (Ge 2005; Jia 2006; Liu 2006).

Freezing temperatures create adverse environmental conditions that limit the geographical distribution of plants and account for significant reductions in the yields of important crops. Consequently, considerable efforts have been directed at determining the nature of freezing injury and the protection mechanisms of plants to increase tolerance of environmental stresses. In the present study, a *CBF1* gene designated *AmCBF1* from *A. mongolicus* was cloned and the gene was transferred to tobacco via an *Agrobacterium*-mediated method. The effect of *AmCBF1* on the cold tolerance of transgenic tobacco plants was evaluated.

MATERIALS AND METHODS

Plant Material

Nicotiana tabacum L. cv. NC89 was maintained in the laboratory. *A. mongolicus* seeds were collected from a wild population in Gansu province, China. Seeds of *A. mongolicus* were sown in the pots in greenhouse in a 16 h/8 h day/night cycle. After more than a month, true leaves of seedlings were harvested for immediate genomic DNA and RNA extraction.

Extraction of Genomic DNA and Total RNA of *A. mongolicus* and Reverse Transcription of Total RNA

Genomic DNA of *A. mongolicus* was extracted using the method of Zur et al. (1999), and a buffer that comprised 3% cetyl trimethyl ammonium bromide (CTAB), 1.4 M NaCl, 20 mm EDTA, 100 mm Tris–HCl pH 8.0, and 0.2% 2-mercaptoethanol. Total RNA was isolated by an improved method based on the phenol extraction method of Huang (2007). First-strand cDNA synthesis was conducted using the ReverTra Ace-α-® kit (Toyobo). The quality and quantity of RNA and DNA were assessed by agarose gel electrophoresis and ultraviolet spectroscopy.

Amplification of *AmCBF1* Core Sequence

The degenerate primers *Am*jbL and *Am*jbR (Table 1) were designed according to conserved regions of several known *CBF1* mRNA core sequences. The polymerase chain reaction (PCR) amplification protocol was as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. The PCR products were inserted in the pMD18-T vector (TaKaRa) for sequencing.

Rapid Amplification of 3'/ 5' cDNA Ends (3'- and 5'-RACE)

Specific primers for 3'-/5'-RACE were designed according to the core sequence of *AmCBF1*. The primers used were as follows: 3'-*Am*OP was used as the 3'-RACE outer primer, and 3'-*Am*IP was used as the 3'-RACE inner primer; 5'-*Am*OP was used as the 5'-RACE outer primer, and 5'-*Am*IP was used as the 5'-RACE inner primer (Table 1). 3'- and 5'-RACE were carried out using the GeneRacer RLM-RACE Kit (Invitrogen, USA). The pMD18-T vector (TaKaRa) was used for sequencing.

Expression of *AmCBF1* Induced by Cold

Semi-quantitative reverse transcription-PCR (RT-PCR) and Real-Time PCR were carried out to detect the cold induced expression of *AmCBF1* in the leaves of *A. mongolicus*. Leaf RNA was extracted by cold treatment of 0, 15 and 30 min, 1, 2, 4, 8, 16 and 24 h. The leaf RNA was reverse transcripted into first strand cDNA, which was used as template for semi-quantitative RT-PCR and Real-Time PCR (FastStart Universal SYBR Green Master (ROX), Roche). For semi-quantitative RT-PCR, primers of *AmCBF1*-R and *AmCBF1*-F were used to amplify part of *AmCBF1*; primers of *β-actin*-F and *β-actin*-R were used for part of *β-actin* to homogenize the sample volume. The PCR amplification protocol for both pairs of primers was as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 40 s; and 72°C for 10 min. For Real-Time PCR, primers of RealL and RealR were used for part of *AmCBF1*; primers of *β-actin*-F and *β-actin*-R were used for part of *β-actin*. The Real-Time PCR amplification protocol was as follows: 50°C 2 min; 95°C 10 min; 40 cycles of 95°C 15 s, 60°C 30 s. Primers used here are listed in Table 1.

Table 1 - List of primers used in the study.

Primers	For short	Sequence(5'—3')
Degenerate primers for core sequence of *AmCBF1*	*Am*jbL	AAGTTTCG(AT)GA(GA)AC(AT)CG(TAG)CA(CT)CC(AG)
	*Am*jbR	(CAT)GA(AG)TCAG(CA)(GA)AA(AG)TT(CG)A(AG)ACA(AGT)GC
Specific primers for 3'-RACE of *AmCBF1* cDNA	3'-*Am*OP	AGTGGGTTTGTGAAGTGAGGGAGC
	3'-*Am*IP	GACAAGGATCTGGCTCGGGACTTT
Specific primers for 5'-RACE of *AmCBF1* cDNA	5'-*Am*OP	GTGAGCCCTTGCCGCCATTTCC
	5'-*Am*IP	GGACCTACCCCTAAGAGCAATCG
Specific primers for full-length of *AmCBF1* gene (with restriction site)	*Am*L	TGCTCTAGAATGTTTTCCTTCAATCATTT
	*Am*R	CGCGGATCCTCAAATTGAATAACTCCACA
Specific primers for Real-Time PCR	RealL	CAACAAGAAGACCAGGATTTGG
	RealR	CGTCAGCACCACCATAGTCA
Specific primers for part of *AmCBF1* gene	*AmCBF1*-F	CGGGAGAAAGAAGTTCAGGG
	AmCBF1-R	CGTCAGCACCACCATAGTCAG
Specific primers for part of *nptII* gene	*nptII*-F	ATGATTGAACAAGATGGATTGC
	nptII-R	TCAGAAGAACTCGTCAAGAAG
Specific primers for part of *A. mongolicus β-actin* gene	*β-actin*-F	TCCATGCTCAATGGGATACT
	β-actin-R	TTCAACCCCTTGTCTGTGAT
Specific primers for part of tobacco *β-actin* gene	*NtActin*-F	TCCATGCTCAATGGGATACT
	NtActin-R	TTCAACCCCTTGTCTGTGAT

Construction of Plant Expression Vector

The full-length of *AmCBF1* gene was amplified using the cDNA and genomic DNA as template. Restriction sites of *Xba*I and *Bam*HI were incorporated in the specific primers of *Am*L and *Am*R (Table 1). The PCR amplification protocol was as follows: 94°C for 5 min; 28 cycles of 94°C for 30 s, 61°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. The pMD18-T vector (TaKaRa) was used for sequencing. The full-length of *AmCBF1* gene was linked to the *Cauliflower mosaic virus* (CaMV) 35S strong promoter and was constructed in the pBI121 vector (designated pBI121-*AmCBF1*) with the screenable marker gene npt*II* (Fig. 1). The construct was transferred into *Agrobacterium tumefaciens* strain LBA4404 (designated LBA4404-*AmCBF1*) for *Agrobacterium*-mediated transformation.

***Agrobacterium*-Mediated Transformation of Tobacco**

LBA4404-*AmCBF1* was used for *Agrobacterium*-mediated transformation to transfer the *AmCBF1* gene into tobacco NC89. Axenic leaves of tobacco were immersed in the bacterial suspension for 10–15 min. For co-cultivation, Murashige and Skoog (MS) solid medium was supplemented with 0.2 mg/L and 2 mg/L 6-BA, incubated at 25 ± 1°C for three days in the dark. For selective cultivation, MS medium was supplemented with 0.2 mg/L, 2 mg/L, 100 mg/L Kanamycin (Kan) and 500 mg/L Carbenicillin (Carb) and incubated under a 16 h/8 h day/night cycle. After 2–3 weeks, the resistant shoots were transferred to MS medium with 100 mg/L Kan and 500 mg/L Carb to induce root development.

Detection of PCR and GUS Staining to Identify Transgenic Tobacco

PCR reaction of genome was used to detect *AmCBF1* in T_0 and T_n transgenic tobacco. For *AmCBF1* transgenic tobacco (designated Trans-*AmCBF1*), the specific primers of *AmCBF1*-F and *AmCBF1*-R (Table 1) were used to amplify the part of *AmCBF1*. The empty pBI121 vector was transferred into LBA4404 (designated LBA4404-CK) previously, which was transferred to NC89 via *Agrobacterium*-mediated transformation. The transgenic strains were designated as Trans-control. Seeds of Trans-control were conserved in the laboratory and the specific primers npt*II*-F and npt*II*-R (Table 1) were used to amplify the part of npt*II*. Trans-control was used to assess the effect caused by insertion of alien gene. GUS staining was also conducted for T_0 transgenic tobacco. Tissues were lysed in a staining solution that contained 75% 0.1 M NaH_2PO_4 pH 7.0, 1% 5 mm $K_3[Fe(CN)_6]$, 1% 5 mm $K_4[Fe(CN)_6]$ $3H_2O$, 2% 0.5 mm EDTA, 20% methanol, 0.1% Triton X-100, and 1% 0.1mg/μL -Gluc. After staining in 28~37°C overnight, tissues were rinsed in 70% ethanol for 5 min twice. After several generations of selection, the pure strains of Trans-*AmCBF1* and Trans-control were obtained.

Cultivation of Transgenic Tobacco and Its Identification by PCR and Semi-Quantitative RT-PCR

Seeds of NC98 and T_n transgenic tobacco were cultivated on MS medium. For transgenic tobacco, MS was supplemented with 100 mg/L Kan. After growth for three or more weeks, seedlings were transplanted in the pots for growth in the greenhouse and were watered every three days. PCR amplification of genomic DNA and semi-quantitative RT-PCR of mRNA were applied to transgenic tobaccos for identification. For PCR amplification, primers of *AmCBF1*-F and *AmCBF1*-R were used for primary detection of the *AmCBF1* transgenic lines. For semi-quantitative RT-PCR, primers of *AmCBF1*-F and *AmCBF1*-R were used for part of *AmCBF1*; primers of *NtActin*-F and *NtActin*-R were used for part of *β-actin* to homogenize the sample volume. The PCR amplification protocol was similar to the protocol in the method of expression of *AmCBF1* induced by cold. Primers used here are listed in Table 1.

Physiological Analysis of Tobaccos Exposed to Cold Stress

Physiological analysis was performed when tobacco strains of NC89, Trans-*AmCBF1* and Trans-control were about 25 cm tall. For cold treatment, all the plants were exposed to 4°C for 24 h. Electrolytic leakage, free proline content, soluble sugar content, malondialdehyde (MDA) content, and peroxidase (POD) activity were analyzed for each plant before and after the cold treatment. Plants of the same growth stage were selected and 18 individuals were taken at each strain, considering each strain as an individual group. Samples of leaf discs were collected by hole puncher from the leaves of each plant at the same part. Three replicates were taken for all the cases, representing mean values were used for statistical analysis by SAS GLM procedure using the T-Test (SAS Institute, Inc., Cary, NC, USA).

Membrane leakage was determined by the measurement of electrolyte leaked from leaves. Five leaf discs were immersed in 5.0 mL high purity water at room temperature with gentle shaking for 3 h, and the solution was measured for conductivity by a conductivity meter (DDS-11A, Shanghai, China). The total conductivity was determined after boiling the sample for 10 min. The ratio of electrolytic leakage is expressed as the percentage of the initial conductivity over the total conductivity (Nanjo et al. 1999).

For free proline determination, fresh samples were extracted with 3% sulfosalicyclic acid, placed in a boiling water bath for 10 min and centrifuged at 10,000×g for 15 min at 4°C. Two milliliter of the extract was added to 6.0 mL (final volume) assay medium containing 2.0 mL acld-ninhdrin (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid) and 2.0 mL glacial acetic acid and incubated for 30 min at 100°C, and then cooled on ice. The colored product was extracted with 4.0 mL toluene by shaking and the absorbance of resultant organic layer was measured at 520 nm using toluene for a blank. The free proline concentration was determined from a standard curve (Bates et al. 1973).

Soluble sugar content was determined by the anthrone method using sucrose as the standard. Fresh samples were placed in distilled water and boiled for 30 min. Reaction mixture of 7.5 mL contained 2.0 mL extracts, 0.5 mL mixed reagent (1.0 g anthrone in 50 mL ethyl acetate) and 5.0 mL H_2SO_4 (98%). The mixture was boiled for 1 min and absorbance was read at 630 nm.

For MDA content, fresh tissue was ground in 10% TCA and centrifuged at 10,000 × g for 10 min at 4°C. Two milliliter TBA was added to 2.0 mL supernatant and the mixture was heated at 100°C for 15 min and then quickly cooled on ice. The absorbance of the supernatant at 600, 532 and 450 nm was read.

POD was extracted from 0.1 g fresh leaves with 0.1 M phosphate buffer (pH 6.0). The extract was centrifuged at 10,000 × g for 15 min at 4°C, and the supernatant was used to determine the activity of POD. The reaction was initiated by the addition of enzyme extract in reaction mixture (70 μL guaiacol and 19 μL of 30% H_2O_2 in 50 mL phosphate buffer). The absorbance was read at 470 nm at 0.5 min, 1 min, 1.5 min, 2.0 min 2.5 min and 3.0 min.

RESULTS

Cloning and Sequence Analysis of *AmCBF1* Full-Length

Because most *CBF1* genes are known to have no intron, degenerate primers were designed based on known *CBF1* mRNA sequences of different plants and genomic DNA of *A. mongolicus* was used as template for amplification of *AmCBF1*core sequence. 3’ and 5’ ends were obtained by 3’- and 5’-RACE. The 5’ end and 3’ end were spliced to

core sequence to obtain the full-length of *AmCBF1* sequence. Analysis by DNAMAN software showed the longest open reading frame of *AmCBF1* mRNA (GenBank accession No. EU840990.1) was 666 bp with an initiation codon of ATG and a stop codon of TGA and encoded a protein of 221 amino acids. AmCBF1 protein contained a specific CBF protein signature of PKK/RPAGRxKFxETRHP and DSAWR (Fig. 2).

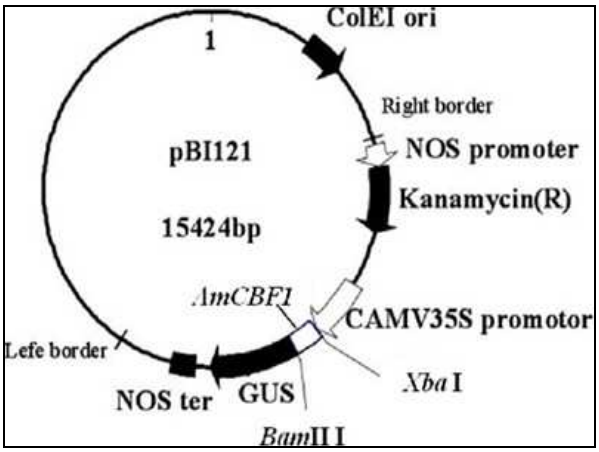

Figure 1 - Plant expression vector of pBI121-*AmCBF1*.

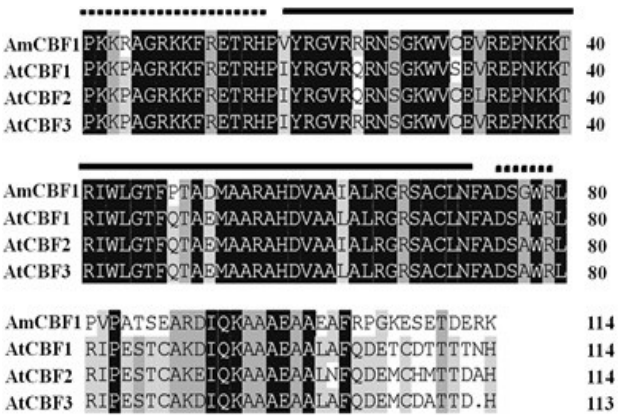

Figure 2 - Comparison of identity between CBF conserved protein sequences. Dots indicate the CBF conserved protein sequence; lines indicate the AP2 domain.

This result indicated that *AmCBF1* belonged to the *CBF* family of *A. mongolicus*. The full-length of *AmCBF1* was amplified with cDNA and genomic DNA as two different templates. Both the cDNA and genomic DNA yielded the same sequencing result, i.e., *AmCBF1* did not contain an intron.

AmCBF1's Cold Induced Expression in Leaves

Expression of *AmCBF1* gene was detected after 1 h of 4 °C and reached a peak at 2 h, then declined at 4 h till to a low level at 24 h (Fig. 3).

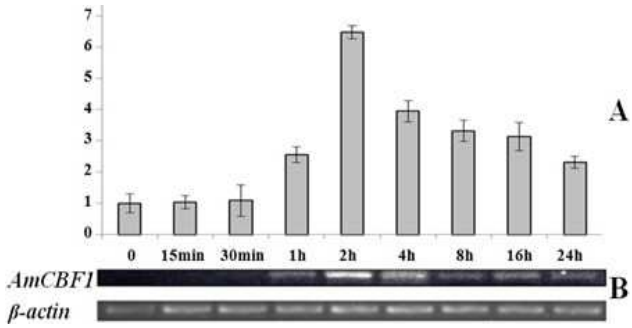

Figure 3 - *AmCBF1*'s cold induced expression in leaves. A: Real-Time PCR result; B: semi-quantitative RT-PCR result.

Identification of Transgenic Tobacco

PCR analysis and GUS staining showed the presence of four positive Trans-*AmCBF1* tobacco strains. PCR analysis of T_0 *AmCBF1*-transgenic tobacco amplified a 470 bp fragment which was part of the *AmCBF1* sequence, except for the wild type and water control, and the Trans-control only carried npt*II* (data not shown). Analysis of the GUS staining showed that the tissues of 4 T_0 Trans-*AmCBF1* tobacco strains had been dyed blue and the wild type was white (Fig. 4). This confirmed that the alien genes had been integrated into the genome of four tobacco plants. PCR and semi-quantitative RT-PCR results further demonstrated that the T_n progeny inherited and transcribed the *AmCBF1* gene (Figs. 5 and 6).

Physiological Analysis of Tobaccos Exposed to Cold Stress

Before the cold treatment, there was no significant difference in the ratio of electrolytic leakage between the wild type and two transgenic tobacco groups of Trans-control and Trans-*AmCBF1*. After the cold treatment for 24 h, each group showed significantly increased ratio of electrolyte leakage compared with the pretreatment level, the wild type and Trans-control were both significantly higher (1.2-fold, $p < 0.0001$ for both) than Trans-*AmCBF1*, whereas little difference was observed between the wild type and Trans-control (Fig. 7).

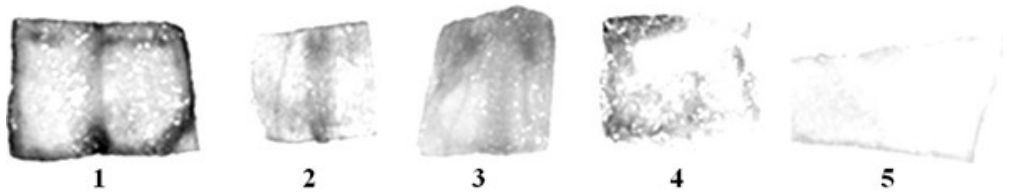

Figure 4 - GUS assay. 1 to 4: T_0 transgenic plants of *AmCBF1*; 5: wild type tobacco plant of NC89 as negative control.

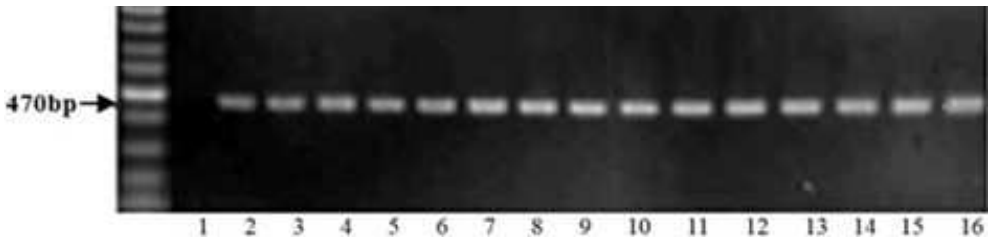

Figure 5 - PCR analysis of genome. Lane 1: wild type tobacco plant of NC89; lanes 2–16: T_n transgenic plants of *AmCBF1*.

Figure 6 - Part result of semi-quantitative RT-PCR analysis of total RNA. Lane 1: wild type tobacco plant of NC89; Lane 2-7: T_n transgenic plants of *AmCBF1*.

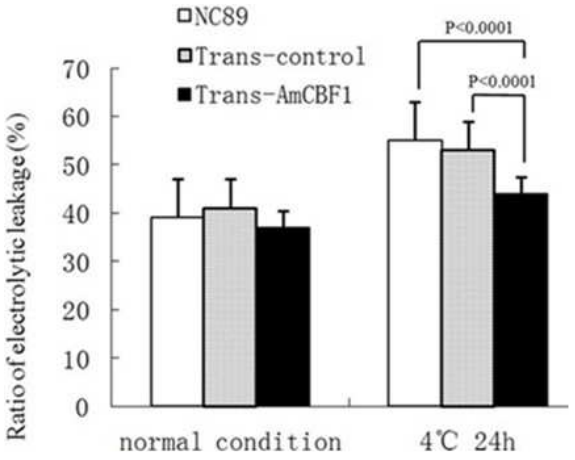

Figure 7 - Ratio of electrolytic leakage in leaves of NC89, Trans-control and Trans-*AmCBF1* tobacco.

The free proline content of Trans-*AmCBF1* was 1.33-fold of the wild type and Trans-control before the cold treatment, but the difference among the three groups was not significant. After the cold treatment, the proline content increased significantly in each group, and Trans-*AmCBF1* was significantly higher than the wild type and Trans-control (1.38-fold and p = 0.0009, 1.24-fold and p = 0.0164; Fig. 8), the wild type and Trans-control did not differ significantly.

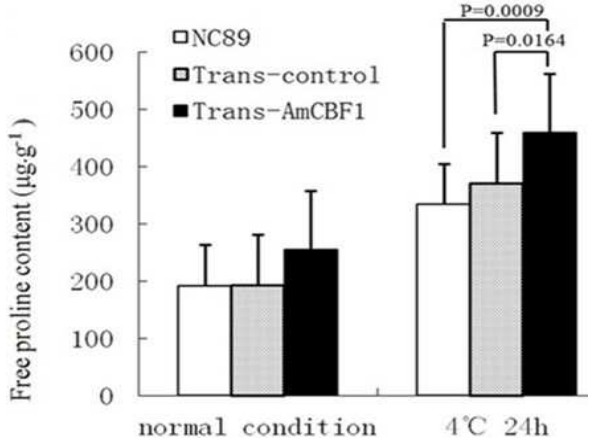

Figure 8 - Free proline content in leaves of NC89, Trans-control and Trans-*AmCBF1* tobacco.

The soluble sugar content of Trans-*AmCBF1* was 1.44-fold of the wild type ($p = 0.0003$) and 1.42-fold of Trans-control ($p = 0.0004$) before the cold treatment. After the treatment, the soluble sugar content of each group increased significantly and Trans-*AmCBF1* was 1.46-fold of the wild type ($p < 0.0001$) and 1.44-fold of Trans-control ($p < 0.0001$; Fig. 9). Trans-*AmCBF1* was significantly higher than the wild type and Trans-control both before and after the cold treatment, whereas the wild type and Trans-control did not differ significantly.

Although each group showed significantly increased MDA contents in response to cold exposure (Fig. 10), no significant differences among the three groups either before, or after the cold treatment were observed.

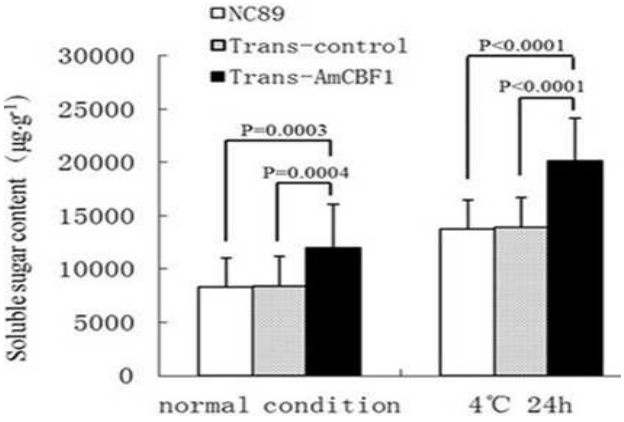

Figure 9 - Soluble sugar content in leaves of NC89, Trans-control and Trans-*AmCBF1* tobacco.

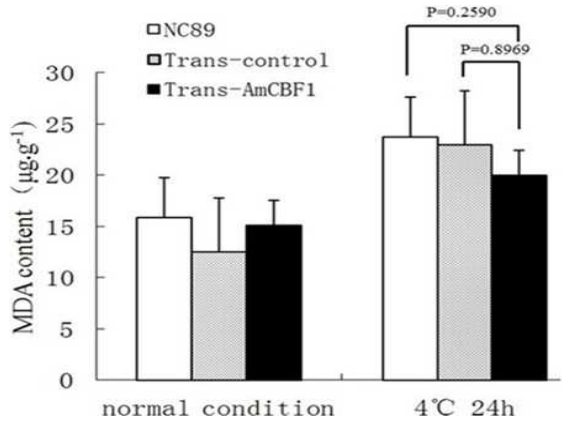

Figure 10 - MDA content in leaves of NC89, Trans-control and Trans-*AmCBF1* tobacco.

Before the cold treatment, POD activity of Trans-*AmCBF1* was 1.92-fold of the wild type and 1.62-fold of Trans-control ($p < 0.0001$ for both), and in both comparisons, the differences were significant. After the cold treatment, POD activity in each group was reduced significantly, but Trans-*AmCBF1* was still significantly higher in the other two groups (1.93-fold of the wild type and $p < 0.0001$, 1.53-fold of Trans-control and $p = 0.0023$; Fig. 11). No significant differences in POD activity between the wild type and Trans-control were observed before, or after the cold treatment.

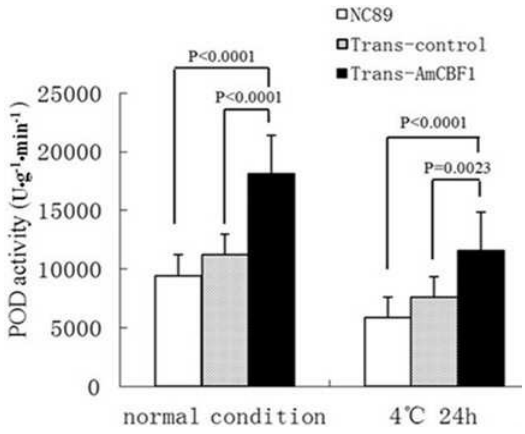

Figure 11 - POD activity in leaves of NC89, Trans-control and Trans-*AmCBF1* tobacco.

DISCUSSION

CBFs are EREBP transcription factors and belong to the AP2/EREBP family. CBFs contain the AP2/EREBP domain and can bind the core sequence of DRE elements, which leads to a regulatory role in resistance to low temperature, drought and other stresses. CBF transcription factors are proven to significantly improve plants' resistance to drought, low temperature, salinity and other abiotic stresses. Many *CBF* genes are known in the plants; for example, six *CBF* genes are present in *Arabidopsis* (Sakuma 2002), three in tomato (Zhang 2004), three in soy (Chen 2007), and more than 10 are known in barley (Dubcovsky 2001). The genes of *CBF1*, *CBF2*, and *CBF3* in *Arabidopsis* are strongly expressed after exposure to 4°C for 15 min, and expression of *LeCBF1* in tomato is detectable after 15 min of cold treatment (Liu et al. 1998). In perennial ryegrass, expression of *LpCBF1* was detected after 1 h of low temperature and peaked at 4 h (Xiong et al. 2006).

Analysis of *AmCBF1* showed that it contained the CBF protein signature of PKK/RPAGRx

KFxETRHP and DSAWR and belonged to the *CBF* family of *A. mongolicus*. Thus, *AmCBF1* might take part in the cold signal transduction pathways of *A. mongolicus.*

Previous studies have shown that electrolytic leakage rate, free proline content, soluble sugar content, MDA content and POD activity could be used as markers to measure plant stress resistance (Si et al. 2009; zhao et al. 2011). Electrolytic leakage rate and MDA level are negatively correlated with the stress resistance of the plants. Free proline content, soluble sugar content, and POD activity were positively correlated. In *CBF*-transgenic *Arabidopsis*, over-expression of alien *CBF* leads to many physiological and biochemical changes, including generation of LEA (late embryogenesis abundant) proteins, or hydrophilic peptides, and accumulation of free proline and a variety of soluble sugars, which have been proved to play an important role in plants' stress tolerance (Zhang et al. 2009). Hsieh et al. (2002) demonstrated when the *CBF1* gene was linked to the CaMV 35S promoter and NOS terminator in transgenic tomato, the cold and drought tolerance of the transgenic T_1 and T_2 generations was improved compared with the wild type. Ito et al. (2006) transferred *OsCBF* into rice and contents of free proline and soluble sugar indicated that the tolerance to cold, drought and salt of transgenic rice were greatly improved. The response reaction of the plant in abiotic stress is a complex network of signal transduction pathways, which are relatively independent and share some crossover nods among themselves. So the *CBFs* usually can be induced by several different abiotic stresses. Likewise, *AmCBF1* may probably be induced by not only cold, but also by salt and drought, and even some other plant hormones such as ethylene and abscisic acid (ABA). In the present study we cloned *AmCBF1* and introduced the gene into tobacco to make it express constitutively. Analysis of electrolytic leakage, contents of soluble sugar and free proline, and POD activity indicated *AmCBF1* endowed tobacco with an enhanced level of cold tolerance. Expression of *AmCBF1* gene was detected shortly after 1 h of 4 °C. Therefore, apparently *AmCBF1* played a very important role in the development of cold tolerance in tobacco. Results proved the cold tolerance of *AmCBF1* and apparently *AmCBF1* could also be induced by some other, or plant hormones, which, however, would require further studies.

CONCLUSIONS

Genetic engineering is an important focus of crop genetic transformation to improve the crop stress tolerance. Discovery and application of *CBF* genes provide an effective means to improve the stress resistance of crop plants. In this study, *AmCBF1* was cloned, characterized and introduced into tobacco, and physiological analyses markers of stress resistance indicated the cold tolerance of transgenic tobacco was improved. This study laid the foundation for further investigation of the physiological function of *CBF* genes. Successful introduction of *CBF* genes into tobacco would be helpful for its development of enhanced levels of stress tolerance.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (no. 31171905) and a 973 projects grant (no. 2009CB119201-4) from the Ministry of Science and Technology of China.

REFERENCES

Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. *Plant Soil*. 1973; 39(1): 205-207.

Chen M, Wang QY, Cheng XG, Xu ZS, Li LC, Ye XG, et al. CmDREB2, a soybean DRE-binding transcription factor conferred drought and high-salt tolerance in transgenic plants. *Biochem Biophys Res Commun*. 2007; 353(2): 299-305.

Dubcovsky J, Ramakrishna W, SanMiguel PJ, Busso CS, Yan L, Shiloff BA, et al. Comparative sequence analysis of colinear barley and rice bacterial artificial chromosomes. *Plant Physiol*. 2001; 125(3): 1342-1353.

Ge XJ, Yu Y, Yuan YM, Huang HW, Yan C. Genetic diversity andgeographic differentiation in endangered *Ammopiptanthus* (Leguminosae) populations in desert regions of northwest China as revealed by ISSR analysis. *Ann Botany*. 2005; 95(5): 843-851.

Huang WK, Cheng HM, Guo JY, Gao BD, Wan FH. Method of RNA extraction from different tissues of invasive alien weed *Eupatorium adenophorum*. *Biotechnol Bull*. 2007; 2: 147-150.

Hughes MA, Dunn MA. The molecular biology of plant acclimation to low temperature. *J Exp Bot*. 1996; 47(3): 291-305.

Hsieh TH, Lee JT, Charng YY, Chan MT. Tomato plants ectopically expressing *Arabidopsis CBF1* show enhanced resistance to water deficit stress. *Plant Physiol*. 2002(2); 130: 618-626.

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, et al. Functional analysis of rice DREB1/CBF2 type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol*. 2006; 47(1): 141-153.

Jia XH, Li XR, Zhang JG, Zhang ZS, Wang XP, Tan HJ, et al. Spatial heterogeneity analysis of fractaldimension of soil particle for *Ammopiptanhus mongolicus* shrub. *Acta Ecol Sin*. 2006; 26(9): 2827-2833.

Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, et al. The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J*. 2007; 50(2): 347-363.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, et al. 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*. 1998; 10(8): 1391-1406.

Liu MQ, Lu CF, Shen X, Yin WL. Characterization and function analysis of a cold-induced AmCIP gene encoding a dehydrin-like protein in *Ammopiptanthus mongolicus*. *DNA Seq*. 2006; 17(5): 342-349.

Nanjo T, Kobayashi M, Yoshiba Y, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K. Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. *FEBS Lett*. 1999; 461(3): 205-210.

Robinson SJ, Parkin IAP. Differential SAGE analysis in *Arabidopsis* uncovers increased transcriptome complexity in response to low temperature. *BMC Genom*. 2008; 9: 434.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K. DNA binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration and cold inducible gene expression. *Biochem Biophys Res Comm*. 2002; 290(3): 998-1009.

Shinozaki KY, Shinozaki K. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu Rev Plant Biol.* 2006; 57: 781-803.

Si J, Wang JH, Zhang LJ, Zhang H, Liu YJ, An LZ. *CbCOR15*, A Cold-Regulated Gene from Alpine *Chorispora bungeana*, Confers Cold Tolerance in Transgenic Tobacco. *J Plant Biol*. 2009; 52(6): 593-601.

Stockinger EJ, Gilmour SJ, Thomashow MF. *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator thatbinds to the C-repeat/DRE, a cis-acting DNA regulatory element thatstimulates transcription in response to low temperature and water deficit. *PNAS*. 1997; 94: 1035-1040.

Thomashow MF. Role of Cold-Responsive Genes in Plant Freezing Tolerance. *Plant Physiol*. 1998; 118(1): 1-8.

Thomashow MF. So what's new in the field of plant cold acclimation? Lots! *Plant Physiol*. 2001; 125(1): 89-93.

Xiong YW, Fei SZ. Functional and phylogenetic analysis of a DREB/CBF-like gene in *perennial ryegrass* (*Lolium perenne* L.). *Planta*. 2006; 224(4): 878-888.

Wei Q, Guo YJ, Cao HM, Kuai BK. Cloning and characterization of an AtNHX2-like Na^+/H^+ antiporter gene from *Ammopiptanthus mongolicus* (Leguminosae) and its ectopic expression enhanced drought and salt tolerance in *Arabidopsis thaliana*. *Plant cell tiss org*. 2011; 105(3): 309-316.

Zhao X, Zhan LP, Zou XZ. Improvement of cold tolerance of the half-high bush Northland blueberry by transformation with the LEA gene from *Tamarix androssowii*. *Plant Growth Regul.* 2011; 63(1): 13-22.

Zhang X, Fowler SG, Cheng HM, Lou YG, Rhee SY, Stockinger EJ, et al. Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant *Arabidopsis*. *Plant J*. 2004; 39(6): 905-919.

Zhang G, Chen M, Li L, Xu Z, Chen X, Guo J, et al. Overexpression of the soybean GmERF3 gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. *J Exp Bot*. 2009; 60(13): 3781-3796

Zur NT, Abbo S, Mylaboski D, Mizrahi Y. Modified CTAB procedure for DNA isolation from epiphytic cacti of the Genera *Hylocereus* and *Selenicereus* (Cacataceae). *Plant Mol Biol Rep*. 1999; 17(3): 249-254.

Received: May 17, 2012;
Accepted: September 12, 2013.