Cloning and characterization of cold, salt and drought inducible C-repeat binding factor gene from a highly cold adapted ecotype of *Lepidium latifolium* L.

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Abstract The dehydration-responsive element-binding (DREB) protein/C-Repeat Binding Factors (CBFs) belongs to APETALA2 (AP2) family transcription factors that binds to DRE/CRT cis-element in cold-responsive (COR) genes and induce *COR* genes. CBFs have been isolated and characterized from evolutionarily diverse plant species. CBF pathway is conserved by CBF regulon and the size or the number and kind of target genes vary among freezing sensitive and tolerant plants. Hence, cloning of CBFs from highly freezing tolerant plants such as *Lepidium latifolium* L. will be useful in understanding the freezing tolerance of this species. In this study, *LICBF*, a CBF1 family gene from *L. Latifolium* L., was cloned using RT-PCR and RACE-PCR. The full length mRNA of *LICBF* is 948 bp with an open reading frame of 642 bp, encoding a protein of 213 amino acids with a molecular weight of 23.92 kDa and a theoretical isoelectric point of 4.80. Amino acid sequence analysis showed that *LICBF* has an AP2 DNA binding domain, a potential CBF type nuclear localization signal (NLS) and C-terminal acidic domain. Semi-quantitative RT-PCR analysis of *LICBF* revealed that this gene is up-regulated by high salt, dehydration and low temperature stresses. The investigation is therefore successful in cloning of a gene having strong homology with CBF transcription factors and responsive to low temperature, high salt and dehydration conditions.

Keywords *Lepidium latifolium* L. · Cold · Salt and drought inducible · *LICBF* transcription factor

Abbreviations

AP2/EREBP	Apetala2/Ethylene responsive element binding protein
NLS	Nuclear localization signal
DREB/CBF	Dehydration response element binding/C-repeat binding factor
ICE1	Inducer of CBF expression 1
COR	Cold-regulated
HOS1	High expression of osmotically responsive genes 1
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase PCR
RACE	Rapid amplification of cDNA ends
ORF	Open reading frame
EST	Expressed sequence tag

Introduction

The geographical distribution of the crops is determined by many environmental conditions, low temperature being one of the important factors. In order to adapt to low temperature condition, many plants have ability to acquire freezing tolerance by prior exposure to a period of low but non-freezing temperatures, a process known as cold acclimation (Thomashow 1999; Agarwal et al. 2006a). Adaptation processes include an array of biochemical and physiological alterations such as changes in lipid composition from saturated to unsaturated form, increased sugar content and other osmolytes, as well as protein contents and enzyme activities (Guy 1990; Thomashow 1999). Specific proteins are induced in compliance to low temperature that help cells to cope up with chilling and freezing stress (Thomashow 1999; Knight et al. 1999; Tahtiharju and Palva 2001; Gong et al.

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2002; Hsieh et al. 2002). The functions of cold stress-induced proteins include metabolism of carbohydrates, lipids, and phenylpropanoids, as antioxidants, molecular chaperones, mRNA binding proteins, proteinase inhibitors and antifreeze proteins, and also tolerance to cellular dehydration caused by extra-cellular freezing (Mohapatra et al. 1989; Guy 1990; Thomashow 1999).

In plants, cold signalling pathways have been best elucidated in *Arabidopsis*. The cDNA microarray studies of 7,000 full length genes in *Arabidopsis* indicated that 299 genes are drought-inducible, 213 are high-salt stress inducible, 54 are cold-inducible and 254 are ABA inducible (Seki et al. 2002; Shinozaki et al. 2003). Of these, many genes are regulatory in nature and regulate the expression of target downstream genes via binding to specific cis-element, and finally enhance the tolerance to various abiotic stresses in plants (Zhu 2002; Kirch et al. 2005; Vinocur and Altman 2005; Lee et al. 2006). These regulatory genes are transcription factors and belong to several families, such as AP2/EREBP, bZIP, NAC, MYB, MYC, Cys2His2 zinc-finger, and WRKY (Umezawa et al. 2006). *Arabidopsis* genome encodes 145 AP2/EREBP transcription factors that are grouped into five subfamilies, including DREB (dehydration-responsive element-binding protein)/CBF (C-Repeat Binding Factor), ERF (ethylene-responsive transcription factor), AP2 (APE-TALA 2), RAV (related to ABI3/VP1), and one very specific gene, AL079349, based on the similarities of their DNA-binding domain and number AP2/ERF domain. Genes belonging to the CBF/DREB subfamily are thought to be important switches to regulate expression of many stress-inducible genes. DREB1/CBF like genes, belonging to the A-1 subgroup, are induced by low temperature stress but not by drought or high salt stresses, and therefore activates the expression of many cold stress-regulated genes. DREB2-like genes, belong to the A-2 subgroup, are mainly involved in regulation of genes responsive to osmotic stress (Sakuma et al. 2002; Nakashima and Yamaguchi-Shinozaki 2006). *Arabidopsis* homologs of DREB1/CBF genes have been isolated and characterised in evolutionarily diverse plants with different levels of adaptation to cold stress such as, common wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), rye (*Secale cereale* L.), maize (*Zea mays* L.), canola (*Brassica napus*), etc., and overexpression of DREB1/CBF genes in transgenic plants increases tolerance to drought, high salt, and freezing stresses (Dubouzet et al. 2003; Shen et al. 2003; Qin et al. 2004; Zhang et al. 2004a; Hong and Kim 2005; Benedict et al. 2006; Zhao et al. 2006; Chinnusamy et al. 2010). So, CBF genes may be useful for improving the stress tolerance of crops (Yamaguchi-Shinozaki and Shinozaki 2006). In *Arabidopsis* upstream regulators of CBF expression has been isolated by using targeted forward genetic

analysis. The inducer of CBF1 Expression 1 (ICE1) encodes a MYC-like basic helix-loop-helix (bHLH) protein that binds specifically to MYC cis-elements (CANNTG) in the DREB1A/CBF3 promoter and induces the expression of CBF3, which, in turn, activates expression of many downstream genes, leading to a significantly enhanced tolerance to chilling and freezing (Chinnusamy et al. 2003). The signalling components that transduce the cold stress signal to ICE1 remain to be identified. It is also unclear whether ICE1 also functions in other abiotic stress response pathways (Chinnusamy et al. 2004). Overexpression of *Arabidopsis* ICE1 enhances chilling tolerance of transgenic rice (Xiang et al. 2008) and cucumber (Liu et al. 2010). Over-expression of the wheat ICE genes enhanced freezing tolerance (FT) in the heterologous *Arabidopsis* system (Badawi et al. 2008). These studies suggest that ICE1-CBF regulon is conserved in diverse plant species. The regulon size or the number and kind of target genes vary among freezing sensitive and tolerant plants (Zhang et al. 2004b). The calmodulin binding transcription activator (CAMTA) family proteins act as transcriptional regulators of CBF2 expression in *Arabidopsis*. The CAMTA3 protein binds to CBF2 promoter and induces CBF2 expression. Since CAMTA proteins can interact with calmodulins, cold-induced calcium signals may regulate CBFs expression through CAMTA proteins (Doherty et al. 2009). A cold adapted plant *Lepidium latifolium* L., thriving well on dry slopes as perennial herb at higher elevation varied in altitude from 3,260 to 3,650 m in Leh, Suru and Zaskar valleys of Jammu and Kashmir, India was explored in the present investigation for cloning of cold stress-regulated genes (Chaurasia et al. 2007; Aslam et al. 2009). This species probably has evolved effective stress tolerance mechanisms that permit its growth, development and reproduction in such environments. However, only limited efforts have been made to unravel the molecular basis of stress tolerance in *L. latifolium* L. for utilization in crop improvement. The present piece of work is an effort to explore, clone and characterize CBF transcription factor from freezing tolerant *L. latifolium* L. species.

Materials and methods

Plant material and stress treatment

Seeds of *L. latifolium* L., ecotype 'Ladakh' were obtained from Defence Institute of Hill Agriculture (earlier known as Field Research Laboratory) Leh, India (located at 3,500 m altitude). The small pots (diameter 16.0 cm, height 15.0 cm) filled with equal mixture of peatmoss, vermiculite and soil (v/v) were used to grow the seeds of *Lepidium latifolium* L.

Germinated seedlings were maintained under white light at 25 °C with 16 h light and 8 h dark cycles.

To induce expression of the target gene, abiotic stress treatments were applied to 2-month-old seedlings for 12 h unless otherwise stated. Low-temperature treatments were applied by transferring plants to a growth chamber set to 4 °C. Drought and salinity treatments were applied by submerging the roots of the plants in an aqueous solution of 15 % PEG6000 and 250 mM NaCl, respectively. After each treatment, the leaves were harvested, frozen in liquid nitrogen, and stored at –70 °C for further analysis.

RNA isolation and first strand cDNA synthesis

Total RNA was extracted from the leaves using Trizol reagent (Invitrogen Corporation) in combination with RNase-free DNase treatment (Qiagen) to remove contaminated DNA. The first-strand cDNA was synthesized with 2.5 µg total RNA in 20 µl reaction volume using first strand cDNA synthesis Kit according to the manufacturer's instruction (Fermentas Life sciences).

RT-PCR for *CBF* fragment amplification

Information on cDNA/EST available in other dicot plants such as *Brassica*, *Arabidopsis*, cucumber, tomato etc. on different abiotic stress transcription factors were aligned using CLUSTALW (www.ebi.ac.uk/clustalw/). Based on homology and conserved region, the following pair of degenerate primer was designed: Primer pairs are 5'AGT TTC GDG AGA CKC GTCACC3' (sense) and 5'AGC CTC MTG SAW CGT CTC CTC3' (anti-sense). The RT-PCR was performed using 2 µl of the first-strand cDNA as template in 50 µl volume containing 36 µl H₂O, 5 µl 10X PCR buffer, 3 µl 25 mM MgCl₂, 1 µl 10 mM dNTP mix, 1 µl each 10 mM sense and anti-sense primer, 1 µl (1 unit/µl) Taq DNA polymerase (Fermentas Life Sciences). The PCR temperature program was 1 cycle of 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C, and finally 1 cycle of 10 min at 72 °C. The resulting ~300 bp PCR product was separated on 1 % agarose gel and purified using PCR purification kit (Qiagen), then cloned into pGEM T-Easy vector (Promega, Madison, WI, USA), and finally transformed into *Escherichia coli* DH5α. The positive clones were get sequenced (DNA Sequencing Facility, South Campus, Delhi University, India). Molecular cloning experiments in this study were performed according to Sambrook et al. (1989).

Isolation of 3' and 5' end by RACE-PCR

The 5' and 3' ends of the cDNA sequence were amplified using the 'RACE System for Rapid Amplification of cDNA

Ends, version 2' (Invitrogen Corporation, USA) according to the manufacturer's protocol. In order to perform 3'RACE, two internal gene specific forward primer GSPF1 5'GTC AAC TTG CCC AAA GGA TAT TCA G3' and GSPF2 5'GGC TTT TCA AGA TGA AAT GAC GAC3' were designed from *L. latifolium* L. partial cDNA of *CBF* cloned by us. Abridge Universal Amplification Primer (AUAP), 5'GGC CAC GCG TCG ACT AGT AC3' were used as anti-sense primer. PCR reaction was carried out in a total volume of 50 µl including 36.5 µl autoclaved distilled water, 5 µl 10X PCR buffer, 3 µl 25 mM MgCl₂, 1 µl 10 mM dNTP mix, 1 µl 10 µM forward gene specific primer, 1 µl 10 µM AUAP, 2 unit Taq DNA polymerase, 2 µl cDNA. PCR conditions were as follows: 94 °C for 3 min, 35 cycles of three steps, 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, and final extension was performed at 72 °C for 10 min. The 5'RACE were carried out using four gene specific internal reverse primers, designed from partial *L. latifolium* L. *CBF* cDNA of 338 bp. First strand cDNA were synthesized using GSPR1 5' GCC ACG TAG AGC TAG AGC AG3'. First round of PCR were carried out by GSPR2 5'GTG AGC ACG AGC TGC CAT TTC AG3' and AUAP, in a total volume of 50 µl reaction. PCR condition and composition were same as 3'RACE. Subsequently, PCR product of the first round PCR was diluted 100-times and used as template in the nested PCR amplifications using GSPR3 5'CTT GTT TGG CTC TCT CAC TTC AC3' and GSPR4 5'GAC GTA CTC CTC TAT AAA TTG GG3'. The resulting PCR products were separated on 1 % agarose gels. The purified fragments were cloned in to pGEMT-Easy vector and transformed in to DH5α strain of *E. coli*. Finally, positive clones were sequenced.

In-silico generation of full length gene

All three fragment of *CBF* cloned was assembled manually. An entire open reading frame (ORF) was found using ORF finder programme of NCBI (www.ncbi.nlm.nih.gov/).

Sequence analysis

BLAST program in National Centre for Biotechnology Information Server (<http://www.ncbi.nlm.nih.gov/>) was used to validate the nucleotide as well as protein homology. Multiple protein sequence alignment was performed using CLUSTALW program of Jalview 2.3 version. The phylogenetic tree of proteins was constructed using the Neighbour-Joining algorithm (www.genome.jp/tools/clustalw/). Theoretical isoelectric point and mass values for the protein was predicted using ExPASy ProtParam tool (<http://us.expasy.org/tools/protparam.html>). The conserved domains were searched at the NCBI web site <http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi>.

Semi quantitative RT-PCR

To analyse the expression levels of cloned cDNA fragment of *L. latifolium* L. under different stresses and duration of stresses using RT-PCR, the same set of specific primers which were used for cloning full length cDNA, was used. *Lepidium* 26SrRNA gene was selected as endogenous internal standard, because it is a house keeping gene and expressed at all stages and in all the tissues. The internal control primers used were 5'CAC AAT GAT AGG AAG AGC CGA C3' as forward primer and 5'CAA GGG AAC GGG CTT GGC AGA AT3' as reverse primer. cDNAs were exponentially amplified using Taq Polymerase (Fermentas Life Sciences) according to manufacturer's instruction. Densitometry analysis was done with the help of Gene Profiler software (Alpha Innotech Corporation USA). Briefly, individual gels were scored by placing the cursor over individual band and recording the relative densitometry values.

Results

Nucleotide sequence and predicted amino acid sequence analysis

A partial cDNA fragment of 338 base pair (bp), with high similarity to CBF genes was cloned from *L. latifolium* L. using degenerate primers. Subsequently, RACE PCR was performed to clone the 3' and 5' ends of the sequence. The *L. latifolium* L. cDNA encodes a 948-bp mRNA with an ORF of 642 bp with a 172-bp 5' Un-Translation Region (UTR) and a 134-bp 3' UTR. The *LICBF* peptide consists of 213 amino acids with a calculated molecular weight of 23.92 KDa and theoretical isoelectric point of 4.80 (Fig. 1).

BLAST analyses of the amino acids sequence revealed homology with *Arabidopsis* CBF3 (81 %), *Capsella bursa-pastoris* DREB1A (81 %), *Nicotiana tabacum* DREB1A (81 %), *Chorispora bungeana* C-repeat binding factor-like protein (77 %), *Eutrema salsugineum* DREB1 (78 %), *Oli-marabidopsis pumila* CBF1 (79 %) and *Thlaspi arvense* cold responsive transcription factor (80 %) (Fig. 2). The *LICBF* cDNA showed higher homology with CBF3 proteins of *Brassica* family probably because *L. latifolium* L. also belongs to the same family, but had minimum similarity with monocot DREB1A proteins like ZmDREB1 and OsDREB1 except conserved AP2 domain. The cloned cDNA fragment of *L. latifolium* L. was therefore named as *LICBF* (GenBank accession number JN561591) and considered as ortholog of CBF subfamily.

Bioinformatics validation revealed that *LICBF* sequence had typical structural characteristics of an AP2/ERF functional domain. The *LICBF* also had putative nuclear localizing consensus signal sequence PKKPAGRKKFRETRHP

before the AP2 domain, which is characteristics feature of DREB1 type proteins (Agarwal et al. 2006b). This suggested that the *LICBF* is an AP2 type transcription factor than can be localized into nucleus for transcriptional regulation. An acidic amino acid region was identified in the C-terminal region that may act as a transcriptional regulatory domain. The DSAW motif at the end of the AP2/ERF domain and LWSY motif at the end of the C-terminal are conserved in most of the DREB1-type proteins (Agarwal et al. 2006b). In the present work, *LICBF* shares a similar DSAW domain at the end of AP2 domain, but having LWN Y motif instead of LWSY at C-terminal region, distinguishing it from other DREB1 type proteins reported so far. Two CBF1 homologs, *Triticum aestivum* L. CBF1 (accession number AAL37944) and *Lycopersicon esculentum* CBF1 (accession number AAK57551) cloned earlier also had 'N' in place of 'S', indicating that LWN Y may be a functional equivalent of LWSY.

Secondary and three-dimensional structural analyses

We examined the tertiary structure of *LICBF*'s AP2 domain in reference to *AtERF*, the only protein belonging to the AP2 family whose crystallographic structure is known (Allen et al. 1998). The tertiary structure of *LICBF* exhibits classic ERF fold consisting of three β -sheets connected by loops and a C-terminal α -helix packed approximate parallel to β -sheets. The two amino acids in β -sheet 14th valine and 19th glutamic acid play crucial role in the determination of DNA-binding specificity were found to be completely conserved (Liu et al. 1998; Cao et al. 2001; Sakuma et al. 2002). The amino acid residues Arg6, Arg8, Trp10, Glu16, Arg18, Arg25 and Trp27 of AP2 domain that directly make contact with DNA for DNA-binding activity were also completely conserved except Trp10, which was substituted by amino acid serine (Allen et al. 1998). Wang and He (2007) also reported serine in place of Trp10 in *AtCBF3* (accession number NP 567720).

The YRG and RAYD elements are found in all known AP2 domains (Fig. 2). The RAYD element contains a conserved core region that is predicted to form an amphipathic α -helix. One hypothesis for the function of this α -helical structure is that it is involved in DNA binding, perhaps through the interaction of its hydrophobic face with the major groove of DNA (Zubay and Doty 1959). Since identity is only 55 %, other residues which are differing from *AtERF* may lead to differences in recognition and binding of some cis-acting elements (Fig. 3).

To determine the phylogenetic relationships between *LICBF* and other DREB1-like proteins from different plant species, a phylogenetic tree was constructed using Neighbour-Joining algorithm (Fig. 4). *LICBF* was placed entirely on separate clade. It is separated from monocots as

Fig. 1 Nucleotide sequence and deduced amino-acid sequence of *LICBF*. The putative nuclear localization signal (NLS) is singly underlined and AP2 domain is doubly underlined. Conserved motifs are indicated in bold letters

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GTTGAAGAAAGAATCCTTCACCTGAAAAAGAAAAAGAAAGAGAGAGAAATAAAAAAT
ATTTATATCAAAACAGAGATCTTCTCTTTATCCAAAAAGAACAGAGTCTTTACTCTCGAA
TTACTCACTACTTAACGTTATCTGTTATTTCAGAAAGAATTTTGATCA
atggactcttttctgctcttttctgaggtgttagttccgactacgagtctccaatttcc
M D S F S S F S E V F S S D Y E S P I S

tcaggcagtggttattgtccgacgcttgacgaggtgtccgaagaaccagcaggttagg
S G S G Y C P T L A A S C P K K P A G R

aagaagtttcgagagacgcgtcacccaatttatagaggagtacgtctcagaaactctggt
K K F R E T R H P I Y R G V R L R N S G

aatgggtttgtgaagtgagagagccaaacaagaaatctagaatttggttggaactttc
K W V C E V R E P N K K S R I W L G T F

ccaaccgctgaaatggcagctcgtgctcacgacgtcgtgctctagctctacgtggccga
P T A E M A A R A H D V A A L A L R G R

tctgcttgctcaatttcgacagattcagcttggtggcgtcaaaatcccgagtgcaacttgc
S A C L N F A D S A W R L K I P E S T C

ccaaaggatattcagaaagcggcggtgaagctgcttttggttttcaagatgaaatgacg
P K D I Q K A A A E A A L A F Q D E M T

acggaccatgactttgacatggaggagacgttccatgaggctattgtaacaccggaaccg
T D H D F D M E E T F H E A I V T P E P

agtgaagatatgttttatgtagacgaggagcgatgtttggaatgccgaatatgttggt
S E D M F Y V D E E A M F G M P N M L A

aatatggctgaaggtatgcttttgccgccaccgtccgtacaatggaatcacgattatgaa
N M A E G M L L P P P S V Q W N H D Y E

GgggatggtgatgaagatgacctgtcgcttttggaattattaaAACTTTATGTTTCATTTCC
G D G D E D D L S L W N Y -
ATTTTGGGACTATAATTATTTTATTGTTTTCAGTTTTGTGTCTTTTGGATATTTT
TAAAGAGTGGGTGGATGATCTCTTCATTTTAATGGATAAAAAAAAAAAAAAAAAA

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well as other plants like capsicum, *Atriplex hortensis*, soybean which are phylogenetically distant as expected. However, it is interesting to note that *LICBF* is also divergent from CBFs/DREBs from *Arabidopsis*, *Eutrema salsugineum*, and *Capsella bursa-pastoris* and other plants belonging to the same family and had more than 70 % identical amino acid sequence.

Expression pattern analysis of *LICBF* gene

The semi-quantitative RT-PCR method was used to investigate the expression pattern of the *LICBF* gene under various stress treatments. The *Lepidium* 26S rRNA was used as the internal standard control. The results showed that the expression of this gene is induced under low temperature, high salt and dehydration (Fig. 5). However, the expression kinetics was observed to be different under different stress conditions. In low temperature condition, it induced as early as in 30 min and reached highest level of expression at 2 h and then gradually decreased to reach a basal level at 48 h. In response to salt stress, the transcript level increased to a maximum level within 30 min, maintained up to 2 h, and thereafter decreases

to reach a basal level by 16 h of salt stress. Similar results were also observed in case of drought stress.

Discussion

Understanding the mechanisms of tolerance to environmental stresses in plants has the potential to provide novel strategies for genetic improvement of stress tolerance in plants. The transcription factors play a paramount role in controlling various component traits of stress tolerance by regulating an array of genes. Earlier studies demonstrated that the expression of the *CBF* genes in *Arabidopsis* resulted in an increased tolerance to freezing, salt and drought stresses (Liu et al. 1998; Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000). The cDNAs encoding CBF transcription factors have been identified and cloned from various plant species including *Arabidopsis*, rice, wheat, canola, soybean (Liu et al. 1998; Dubouzet et al. 2003; Shen et al. 2003; Li et al. 2005). In the present investigation, we have cloned and characterized the first member of CBF gene

EsDREB1	MNSFSAFAEMFGSEYESPVTVGDDYCPATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
TacoldTF	MNSFSAFAEMFGSEYSPDTIGADYCPATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
AtDREB1C	MNSFSAFSEMFGSDYESPVSDDYSPKATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
CbCBF	MNSFSAFSEMFGSDYESPVSDDYSPKATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
ApCBF	MSSFSAFSEMFGSDYESPVSDDYSPKATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
AtDREB1B	MNSFSAFSEMFGSDYESPVSDDYSPKATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	57
AtDREB1A	MNSFSAFSEMFGSDYESPVSDDYSPKATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
CbDREB1A	MNSFSAFSEMFGSDYESPVSDDYSPKATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
NtDREB1A	MNSFSAFSEMFGSDYESPVSDDYSPKATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
L1CBF	MDSFSSFEVFSDDYSPVSDDYSPKATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
BjDREB1B	MTSFTSTFSEMLGSEYSPVTLGGYCPATLSCPKKPKGRKKFRETRHPIYRGVRRRNSG	60
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EsDREB1	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
TacoldTF	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
AtDREB1C	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
CbCBF	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
ApCBF	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
AtDREB1B	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	117
AtDREB1A	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
CbDREB1A	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
NtDREB1A	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
L1CBF	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
BjDREB1B	KWVCEVREPNNKSRILWGTFTPTAEMAARAHDAALALRGRSACLNFDASAWRLRIPETC	120
* * * : * : * * * : * * * * * : * * * : * * * : * * * : * * * : * * * : *		
EsDREB1	AKDIQKAAAEAAVAFQAE-MSDTMTSDHGLDMEETIVEIVTTEEQSEGFMDEEAMFGM	179
TacoldTF	AKDIQKAAAEAAVAFQAE-MSDTMT- DHGLDMEETIVEIVTTEE- QSGGFYMDDEEAMFGM	177
AtDREB1C	AKDIQKAAAEAAALNFQDE- MCHMTTDAHGLDMEETLVEAIYTPQSQDAFYMDDEEAMFGM	179
CbCBF	AKDIQKAAAEAAALNFQDE- MCHMTTDAHGLDMEETLVEAIYTPQSQDAFYMDDEEAMFGM	179
ApCBF	AKDIQKAAAEAAALNFQDE- MCHMTTDAHGLDMEETLVEAIYTPQSQDAFYMDDEEAMFGM	179
AtDREB1B	AKDIQKAAAEAAALNFQDE- TCDTTTTHHGLDMEETLVEAIYTPQSQDAFYMDDEEAMFGM	176
AtDREB1A	AKDIQKAAAEAAALNFQDE- MCDATT- DHGLDMEETLVEAIYTPQSQDAFYMDDEEAMFGM	178
CbDREB1A	AKDIQKAAAEAAALNFQDE- MCDATT- DHGLDMEETLVEAIYTPQSQDAFYMDDEEAMFGM	178
NtDREB1A	AKDIQKAAAEAAALNFQDE- MCDATT- DHGLDMEETLVEAIYTPQSQDAFYMDDEEAMFGM	178
L1CBF	PKDIQKAAAEAAALNFQDE- - - - - MTDDHDFMEETLVEAIYTPQSQDAFYMDDEEAMFGM	175
BjDREB1B	PKDIQKAAAEAAALNFQAE- - INNTTTHHGLDMEETLVEAIYTPQSQDAFYMDDEEAMFGM	177
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EsDREB1	PRLLANMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	216
TacoldTF	PRLLANMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	214
AtDREB1C	SSLLDNMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	216
CbCBF	SSLLDNMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	216
ApCBF	PSLLANMAEGMMLPPPSVQWGHNYDCGDAD- MPLWSY	216
AtDREB1B	PTLLDNMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	213
AtDREB1A	PSLLANMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	216
CbDREB1A	PSLLANMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	216
NtDREB1A	PSLLANMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	216
L1CBF	PNMLANMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	213
BjDREB1B	PALLANMAEGMMLPPPSVQWGHNYDCGDAD- VSLWSY	214
. : : . * * * * * : * : : . : . * * * : * * * *		

Fig. 2 Multiple amino acid sequence alignment among the **L1CBF** protein with the corresponding protein sequences of other plants: *Eutrema salsugineum* DREB1 protein (GenBank accession number AAS00621), *Thlaspi arvense* cold TF (GenBank accession number ABV82985), *Arabidopsis thaliana* DREB1C (GenBank accession number AB007789), *Chorispora bungeana* CBF (GenBank accession number AAY21899), *Arabis pumila* CBF (GenBank accession number ABA42927), *AtDREB1B* (GenBank accession number AB007788), *AtDREB1A* (GenBank accession number AB007787), *Capsella*

bursa-pastoris DREB1A (GenBank accession number ABM21468), *Nicotiana tabacum* DREB1A (GenBank accession number ABD65969), *Brassica juncea* DREB1B (GenBank accession number ABX00639). “*” indicates positions which have a single, fully conserved residue; “:” indicates highly conserved positions, and “.” indicates the weaker conserved positions. Dashes indicate gaps in the amino acid sequences in order to optimize alignment. A thin line and thick line above amino acid sequence show the location of the YRG and RAYD elements in the AP2 domain, respectively

family, *L1CBF* from *L. latifolium* L., a plant highly tolerant to freezing stress. Cloning and characterization of CBFs genes from such a plant is of prime importance as it will elucidate the mechanisms underlying the CBF-mediated abiotic stress

tolerance and their role in adaptation of this plant to harsh environmental conditions.

The *L1CBF* gene cloned in this study showed high similarity to the previously characterized CBF genes from different

Fig. 3 Comparison of the conserved AP2 DNA binding domain sequences and structures of **L1CBF** and *AtERF1* (pdb ID 1gcca). In the aligned sequences, “*” indicates conservative amino acid residues.

The three β -sheets and a α -helix were marked above the corresponding sequences. The amino acid residues that contact DNA were indicated in bold

Fig. 4 Phylogenetic tree of *LICBF* and other CBF-like proteins: *Nicotiana tabacum* DREB1A (A.N. ABD65969), *AtDREB1C* (A.N. AB007789), *Choripora bungeana* CBF (cbCBF) (A.N. AAY21899), *AtDREB1B* (A.N. AB007788), *AtDREB1A* (A.N. AB007787), *Arabidopsis thaliana* CBF (AtCBF) (A.N. ABA42927), *Eutrema salsugineum* DREB1 (EsDREB1) (A.N. AAS00621), *Thlaspi arvense* cold TF (Tacold) (A.N. ABV82985), *Brassica juncea* DREB1B (BjDREB1B) (A.N. ABX00639), *Raphanus sativus* CBF1 (RsCBF1) (A.N. ACX48435), *Capsicum annuum* DREB (CaDREBLP1) (A.N. AY496155), *Barley* CBF3 (A.N. AF239616), *OsDREB1A* (A.N. AF300970), *OsDREB1B* (A.N. AF300972), *Triticum aestivum* CBF1 (TaCBF1) (A.N. AF376136), *Glycine max* DREBa (GmDREBa) (A.N. AY542886), *Triticum aestivum* DREB1 (TaDREB1) (A.N. AF303376), *Atriplex hortensis* DREB1 (AhDREB1) (A.N. AF274033), *Medicago sativa* DREB1 (MsDREB1) (A.N. ABY78835), *Capsella bursa-pastoris* DREB1A (CbDREB1A) (A.N. ABM21468)

plants. BLAST analysis showed that *LICBF* shared high amino acid identity with CBF 3 protein of *Arabidopsis* (81 %), which is known to regulate the expression of abiotic stress responsive genes. The *LICBF* sequence analysis indicated the presence of AP2 domain, nuclear localization signal sequence and other conserved motifs typical to DREB1/CBF type of transcription factors. Further, sequence comparison showed that *LICBF* contains the conserved 14th valine (V14) and the 19th glutamic acid (E19) similar to other member of DREB1/CBF transcription factors. The PI value of about 3.8 of the C-terminal acidic domain indicates the presence of acidic domain. The structural analysis of AP2 domain indicated typical features of packing of α -helix nearly parallel to the three-stranded anti-parallel β -sheet, which are involved in DNA recognition as well binding specificity of the DREB1 proteins (Sakuma et al. 2002; Allen et al. 1998). Alignment analysis and domain comparison with proteins from different plant species therefore suggest that *LICBF* is homologous to DREB1/CBF type of TFs. Moreover, phylogenetic tree analysis revealed that *LICBF* is genetically divergent from CBF transcription factors reported earlier from other plant species. DREB1/CBF proteins are typically highly conserved in AP2

domain and other signature sequences probably because of substantial selective constraint maintained during the course of evolution to retain the DNA binding properties of DREB1/CBF TFs. However, relaxed constraint in non-DNA binding region during evolutionary process makes DREB1/CBF TFs structurally and functionally divergent even from member of the same family (Chang et al. 2005). Phylogenetically divergent relationship of *LICBF* with other member of the same family may be because of evolutionary modification specifically in non-DNA binding region of the gene to fulfil the functional requirement. Zhang et al. (2004b) also opined that the regulon size or the number and kind of target genes vary among freezing sensitive and tolerant plants.

In order to elucidate the potential function of *LICBF* in response to different stimuli, the expression pattern of the *LICBF* gene was analysed under various abiotic stress conditions. Expression of *CBF* genes was extensively investigated in various crops in compliance to different abiotic stresses (Agarwal et al. 2006b). Expression analysis of *CBF* genes has been primarily focused on the leaves. In limited reports, the expression levels of *CBFs* were higher in roots than in leaves (Wang et al. 2010a). So, in the present study, the transcription level of *LICBF* was studied in leaves only. The *LICBF* expression was analyzed by semi-quantitative RT-PCR, which revealed very low level of expression in unstressed control leaves and therefore suggests that *LICBF* could have constitutive expression albeit at low levels. Expressions of *PeDREB1* from *Phyllostachys edulis* and *PtCBFb* from *Poncirus trifoliata* were also observed under normal condition (Liu et al. 2012; Wang et al. 2009). Expression of *LICBF* gene was up-regulated by low temperature, salt and dehydration. The *Arabidopsis* *CBF1*, *CBF2* and *CBF3* expresses specifically under cold. In practical, more than one environmental stresses are often encountered simultaneously. It is probable that during the summer, plant may experience both drought and high temperature stress simultaneously, while during the winter, because of the impaired water absorption and transport system, low-temperature might also bring with it osmotic stress (Xiong et al. 1999). The different abiotic stress signalling pathways are assumed to interact and share some common elements that act as potential 'node' for cross-talk, such as Ca^{2+} , Calcium-regulated proteins, transcription factors and so on (Qin et al. 2007). The *LICBF* gene was observed to induce strongly within 30 min of exposure to drought, salinity and low temperature stress and therefore said to be early responsive to multiple stresses. Medina et al. (1999) also reported that *CBF* genes showed high expression in response to low temperature stress treatment and the transcript was detectable as early as 30 min of exposure to 4 °C, and reached to maximum expression level at 1 h after the exposure. Our result also corroborate to those described earlier in *LpCBF3* from rye grass, *Ptcbfb* from *Poncirus*

Fig. 5 RT-PCR analysis of expression pattern of LICBF transcript after treatment of cold stress (A), salt stress (B) and drought stress (C). *Lepidium* 26SrRNA gene act as internal control (D) to ensure equal amount of templates were added to each RT-PCR reaction. 0 h time point serves as control

trifoliata, *OsAP211* from rice, *VviDREB1* from *Vaccinium vitis-idaea*, *MbDREB1* from apple, but confront with those observed in *DREB1* from aloe, *BrCBF* from chinese cabbage, *OsDREB1B* under cold stress (Wang and He 2007; Wang et al. 2009; Qin et al. 2007; Gao et al. 2009; Wang et al. 2010b; Yang et al. 2011; Jiang et al. 2011). The difference in timing and strength of expression of CBFs across the different plant species may be due to variation in the organization of various cis-elements between the orthologs

(Chen et al. 2006). Adequate amount of *LICBF* transcript was still observed even after 24 h and 48 h of exposure. Similarly, *OsDREB1A/CBF3* and *ZmDREB1A* were also found to induce by cold within a period of 40 min and 60 min, respectively, and remain detectable even at 24 h after exposure to cold stress (Dubouzet et al. 2003; Qin et al. 2004).

In summary, our results indicate cloning of a gene, *LICBF*, from *L. latifolium* L that established high homology with members of DREB1/CBF TF. The *LICBF* encode a protein

having a conserved AP2 domain and other typical features of DREB1/CBF TFs like nuclear localization signal (NLS) and acidic domain. The expression patterns indicate that *LICBF* is early responsive gene to drought, salt and cold stresses.

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Conflicts of interest The authors declare that they have no conflict of interest.

References

- Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X, Zhu JK (2006a) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *J Biol Chem* 281:37636–37645
- Agarwal PK, Agarwal P, Reddy MK, Sopory SK (2006b) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Rep* 25:1263–1274
- Allen MD, Yamasaki K, Ohme-Takagi M, Tateno M, Suzuki M (1998) A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. *EMBO J* 17:5484–5496
- Aslam M, Sinha VB, Singh RK, Anandhan S, Pandey V, Ahmed Z (2009) Isolation of cold stress-responsive genes from *Lepidium latifolium* by suppressive subtraction hybridization. *Acta Physiol Plant* 32:205–210
- Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F, Houde M (2008) Structure and functional analysis of wheat ICE (inducer of CBF expression) genes. *Plant Cell Physiol* 49:1237–1249
- Benedict C, Skinner JS, Meng R, Chang Y, Bhalerao R, Huner NPA, Finn CE, Chen TH, Hurry V (2006) The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus spp.* *Plant Cell Environ* 29:1259–1272
- Cao ZF, Li J, Chen F, Li YQ, Zhou HM, Liu Q (2001) Effect of two conserved amino-acid residues on DREB1A function. *Biochem* 66:623–627
- Chang SM, Lu YQ, Rausher MD (2005) Neutral evolution of the nonbinding region of the anthocyanin regulatory gene *Ipmyb1* in *Ipomoea*. *Genetics* 170:1967–1978
- Chaurasia OP, Ahmed Z, Ballabh B (2007) Ethnobotany and plants of Trans Himalayas. In: Edible wild plants: *Lepidium latifolium* L., 1st edn. Satish serial publishing house, Delhi, p 197
- Chen Y, Chen P, de los Reyes BG (2006) Differential responses of the cultivated and wild species of soybean to dehydration stress. *Crop Sci* 46:2041–2046
- Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev* 17:1043–1054
- Chinnusamy V, Schumaker K, Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *J Exp Bot* 55:225–236
- Chinnusamy V, Zhu JK, Sunkar R (2010) Gene regulation during cold stress acclimation in plants. *Meth Mol Biol* 639:39–55
- Doherty CJ, Buskirk HAV, Myers SJ, Thomashow MF (2009) Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *Plant Cell* 21:972–984
- Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encoded transcription activators that function in drought-, high salt- and cold- responsive gene expression. *Plant J* 33:751–763
- Gao F, Chen JM, Xiong AS, Peng RH, Liu JG, Cai B, Yao QH (2009) Isolation and characterization of a novel AP2/EREBP-type transcription factor *OsAP211* in *Oryza sativa*. *Biol Plant* 53(4):643–649
- Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol* 124:1854–1865
- Gong Z, Lee H, Xiong L, Jagendorf A, Stevenson B, Zhu JK (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *Proc Natl Acad Sci USA* 99:11507–11512
- Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annu Rev Plant Physiol Plant Mol Biol* 41:187–223
- Hong JP, Kim WT (2005) Isolation and functional characterization of the *Ca-DREBLP1* gene encoding a dehydration-responsive element binding-factor-like protein 1 in hot pepper (*Capsicum annuum* L. cv. Pukang). *Planta* 220:875–888
- Hsieh TH, Lee JT, Yang PT, Chiu LH, Charng YY, Wang YC, Chan MT (2002) Heterology expression of the *Arabidopsis C-repeat/dehydration response element binding factor 1* gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. *Plant Physiol* 129:1086–1094
- Jaglo-Ottesen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. *Science* 280:104–106
- Jiang F, Wang F, Wu Z, Li Y, Shi G, Hu J, Hou X (2011) Components of the *Arabidopsis* CBF cold-response pathway are conserved in non-heading chinese cabbage. *Plant Mol Biol Rep* 29:525–532
- Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat Biotechnol* 17:287–291
- Kirch HH, Schlingensiepen S, Kotchoni S, Sunkar R, Bartels D (2005) Detailed expression analysis of selected genes of the aldehyde dehydrogenase (ALDH) gene superfamily in *Arabidopsis thaliana*. *Plant Mol Biol* 57:315–332
- Knight H, Veale EL, Warren GJ, Knight MR (1999) The *sfir6* mutation in *Arabidopsis* suppresses low-temperature induction of genes dependent on the CRT/DRE sequence motif. *Plant Cell* 11:875–886
- Lee SC, Kim SH, An SH, Yi SY, Hwang BK (2006) Identification and functional expression of the pepper pathogen-induced gene, CAIP2, involved in disease resistance and drought and salt stress tolerance. *Plant Mol Biol* 62:151–164
- Li XP, Tian AG, Luo GZ, Gong ZZ, Zhang JS, Chen SY (2005) Soybean DRE-binding transcription factors that are responsive to abiotic stresses. *Theor Appl Genet* 110:1355–1362
- Liu L, Cao XL, Bai R, Yao N, Li LB, He CF (2012) Isolation and characterization of the cold-induced *Phyllostachys edulis* AP2/ERF family transcription factor, *peDREB1*. *Plant Mol Biol Rep* 30:679–689
- Liu L, Duan L, Zhang J, Zhang Z, Mi G, Ren H (2010) Cucumber (*Cucumis sativus* L.) over-expressing cold-induced transcriptome regulator ICE1 exhibits changed morphological characters and enhances chilling tolerance. *Sci Hortic* 124:29–33
- 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
- Medina J, Bargues M, Terol J, P'erez-Alonso M, Salinas J (1999) The *Arabidopsis* CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. *Plant Physiol* 119:463–469
- Mohapatra SS, Wolfrum L, Poole RJ, Dhindsa RS (1989) Molecular cloning and relationship to freezing tolerance of cold-acclimation-specific genes of Alfalfa. *Plant Physiol* 89:375–380
- Nakashima K, Yamaguchi-Shinozaki K (2006) Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants. *Plant Physiol* 126:62–71
- Qin F, Sakuma Y, Li J, Liu Q, Li YQ, Shinozaki K, Yamaguchi-Shinozaki K (2004) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L. *Plant Cell Physiol* 45:1042–1052
- Qin Q, Liu J, Zhang Z, Peng R, Xiong A, Yao Q, Chen J (2007) Isolation, optimization, and functional analysis of the cDNA encoding transcription factor *OsDREB1B* in *Oryza Sativa* L. *Mol Breed* 19:329–340
- Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem Biophys Res Commun* 290:998–1009
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring expression profile of 7000 *Arabidopsis* genes under drought, cold-and high-salinity stresses using a full-length cDNA microarray. *Plant J* 31:279–292
- Shen YG, Zhang WK, He SJ, Zhang JS, Liu Q, Chen SY (2003) An EREBP/AP2 type protein in *Triticum aestivum* was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. *Theor Appl Genet* 106:923–930
- Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr Opin Plant Biol* 6:410–417
- Tahtiharju S, Palva T (2001) Antisense inhibition of protein phosphatase 2C accelerates cold acclimation in *Arabidopsis thaliana*. *Plant J* 26:461–470
- Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* 50:571–599
- Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. *Curr Opin Biotechnol* 17:113–122
- Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Curr Opin Biotechnol* 16:123–132
- Wang HL, Tao JJ, He LG, Zhao YJ, Xu M, Liu DC, Sun ZH (2009) cDNA cloning and expression analysis of a *Poncirus trifoliata* CBF gene. *Physiol Plant* 53(4):625–630
- Wang X, Dong J, Liu Y, Gao H (2010a) A Novel dehydration-responsive element-binding protein from *Caragana korshinskii* is involved in the response to multiple abiotic stresses and enhances stress tolerance in transgenic tobacco. *Plant Mol Bio Rep* 28:664–675
- Wang QJ, Xu KY, Tong ZG, Wang SH, Gao ZH, Zhang JY, Zong CW, Qiao YS, Zhang Z (2010b) Characterization of a new dehydration responsive element binding factor in central arctic cowberry. *Plant Cell Tissue Organ Cult* 101:211–219
- Wang YM, He CF (2007) Isolation and characterization of cold-induced DREB gene from *Aloe vera* L. *Plant Mol Biol Rep* 25:121–132
- Xiang DJ, Hu XY, Zhang Y, Yin KD (2008) Over-expression of ICE1 Gene in transgenic rice improves cold tolerance. *Rice Sci* 15:173–178
- Xiong LM, Ishitani M, Zhu JK (1999) Interaction of osmotic stress, temperature, and abscisic acid in the regulation of gene expression in *Arabidopsis*. *Plant Physiol* 119:205–212
- Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu Rev Plant Biol* 57:781–803
- Yang W, Liu XD, Chi XJ, Wu CA, Li YZ, Song LL, Liu XM, Wang YF, Wang FW, Zhang C, Liu Y, Zong JM, Li HY (2011) Dwarf apple *MbDREB1* enhances plant tolerance to low temperature, drought, and salt stress via both ABA-dependent and ABA-independent pathways. *Planta* 233:219–229
- Zhang JZ, Creelman RA, Zhu JK (2004a) From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiol* 135:615–621
- Zhang X, Fowler SG, Cheng H, Lou Y, Rhee SY, Stockinger EJ, Thomashow MF (2004b) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differ from that of freezing-tolerant *Arabidopsis*. *Plant J* 39:905–919
- Zhao TJ, Sun S, Liu Y, Liu JM, Liu Q, Yan YB, Zhou HM (2006) Regulating the drought-responsive element (DRE)-mediated signaling pathway by synergic functions of trans-active and trans-inactive DRE binding factors in *Brassica napus*. *J Biol Chem* 281:10752–10759
- Zhu JK (2002) Salt and drought stress signal transduction in plants. *Annu Rev Plant Physiol Plant Mol Biol* 53:247–273
- Zubay G, Doty PJ (1959) The isolation and properties of deoxyribonucleoprotein particles containing single nucleic acid molecules. *J Mol Biol* 7:1–20