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Components of the *Arabidopsis* CBF Cold-Response Pathway Are Conserved in Non-heading Chinese Cabbage

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Abstract Many plants increase in freezing tolerance upon exposure to low non-freezing temperatures, a phenomenon known as cold acclimation. Cold acclimation in *Arabidopsis* involves rapid cold-induced expression of the inducer of C-repeat/dehydration-responsive element-binding factor (CBF) expression (ICE) transcriptional activators followed by expression of the CBF; subsequently, CBF-targeted genes that increase freezing tolerance. Here, we present evidence for a CBF cold-response pathway in non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* L. Makino). We show that non-heading Chinese cabbage encodes *ICE1*-like gene *BrICE1* that bracket an open reading frame of 1,491 bp encoding a protein with a potential bHLH domain, which accumulates rapidly in response to low temperature followed closely by expression of the *BrCBF* gene, an ortholog of the *Arabidopsis CBF3*-like gene, and then *BrCOR14* gene, an ortholog of the *Arabidopsis CBF*-targeted *COR15b* gene. An alignment of the later two genes from *Arabidopsis*, *Brassica napus* revealed the presence of conserved CANNTG core element and AP2 domain in *BrCBF* and a CCG core element in *BrCOR14*. In addition, *BrCBF* and *BrCOR14* showed increased expression induced by low temperature as well as salt and drought, but not by ABA stress which are similar to those of *Arabidopsis*. We conclude that components of the CBF cold-response pathway are highly conserved in non-heading Chinese cabbage.

Keywords Non-heading Chinese cabbage · Cold-responsive genes · *ICE* · *CBF* · *COR*

Abbreviations

ICE	Inducer of CBF expression
CBF	CRT-binding factors
COR	Cold-regulated genes

Introduction

Cold stress adversely affects plant growth and development and thus limits crop productivity. Diverse plant species tolerate cold stress to a varying degree, which depends on reprogramming gene expression to modify their physiology, metabolism, and growth. Cold signal in plants is transmitted to activate C-repeat/drought-responsive element-binding factor (CBF)-dependent and CBF-independent transcriptional pathway, of which CBF-dependent pathway activates CBF regulon. CBF transcription factor genes are induced by the constitutively expressed inducer of CBF expression (ICE)1 by binding to the CBF promoter. ICE1-CBF cold-response pathway is conserved in diverse plant species (Fowler et al. 1996; Goulas et al. 2003; Lee et al. 2005; Chinnusamy et al. 2007; Meng et al. 2008; Wang et al. 2009; Chinnusamy et al. 2010). In this pathway, *ICE*'s core sequence bHLH can recognize DNA with the consensus sequence CANNTG (Chinnusamy et al. 2003; Meshi and Iwabuchi 1995) involving in the CBF or DNA replication-related element-binding (DREB) proteins, while CBF or

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DREB, a family of AP2-domain, are also transcriptional activators binded to the DRE/CRT element (GCC-box pathogenesis-regulated promoter element) and activated transcription (Zhou et al. 2007; Büttner and Singh 1997; Stockinger et al. 1997; Thomashow 1999) of the CBF regulon including genes, specifically *cor15* or *cor47*, thus increasing chilling and freezing tolerance of plants.

Meanwhile, it should be noted that *ICE1* was expressed constitutively, being only slightly up-regulated by cold, but *CBF* expression was induced by cold treatment (Medina et al. 1999; Gao et al. 2002; Thomashow 2001; Chinnusamy et al. 2003). Whereas the overexpression of CBF1 (Jaglo-Ottosen et al. 1998) and DREB1a/CBF3 (Kasuga et al. 1999) in *Arabidopsis* were shown to be able to drive expression of *COR* genes in the absence of low temperature and impart constitutive salt and drought tolerance, while not abscisic acid (ABA) stress, which suggested that it is involved in the expression of cold-, salt-, and drought-regulated genes through an ABA-independent pathway (Kasuga et al. 1999; Yamaguchi-Shinozaki and Shinozaki 1994).

Non-heading Chinese cabbage, like *Arabidopsis*, cold acclimates and is a member of the Cruciferae family. We speculate that non-heading Chinese cabbage may have similar cold acclimation process as Arabidopsis. Recently, several cold-regulated genes have been cloned from *Arabidopsis*, *Capsella bursa-pastoris*, and *Brassica napus* (Jaglo-Ottosen et al. 2001; Wang et al. 2005). Up till now, there has been no report on the cloning of cold-regulated genes from non-heading Chinese cabbage. In this paper, we reported that the molecular cloning of *BrICE1*, *BrCBF*, and *BrCOR14* genes from non-heading Chinese cabbage, bioinformatics analysis revealed that these three genes strongly resembled *ICE*, *CBF*, and *COR* genes from other species.

Materials and Methods

Plant Materials

A non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* L. Makino) cold-resistant inbred line, 043, from non-heading Chinese cabbage project team in Nanjing Agricultural University was used in the present study. Healthy seeds were grown in controlled environmental chambers at 20°C to 22°C under continuous cool-white fluorescent illumination of 100 to 150 $\mu mol m^{-2} s^{-1}$ light intensity as described by Gilmour et al. (1998) Stress treatments were performed with seedlings at three-leaf stage. For cold acclimation, plants were incubated at 4°C under continuous cool-white fluorescent illumination at approximately 50 $\mu mol m^{-2} s^{-1}$ light intensity for varying lengths of time.

Isolation of cDNAs Encoding Cold-Response Proteins

A non-heading Chinese cabbage cDNA fragment encoding an ICE-like polypeptide was isolated by reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers 5′-ATGGTTCTTGACGGAAACA ACGGTG-3′ and 5′-AAAGGGCTTTAGTTCTTCTA ACTCTGCTTC-3′ based on the complete open reading frame (ORF) of *Arabidopsis* cold-responsive gene *ICE1* (Genbank accession number AY195621) and *C. bursa-pastoris ICE53* (Genbank accession number AY506804). The anticipate product size was 1,558 bp. Total RNA was isolated from seedlings incubated at 4°C for 8 h using TaKaRa RNAiso Reagent (Takara, Japan). The first strand cDNA was reversed using TaKaRa RNA PCR Kit (AMV) Ver.2.1 (TaKaRa, Japan). The PCR mixture contained 2.5 μL buffer (10×PCR), 1.5 μL $MgCL_2$ (25 mM), 1.5 μL dNTPs (2.5 mM each), 0.25 μL LA-*Taq* DNA polymerase (5 UmL/L), 10-pmol-specific primers each, 50 ng cDNA, and ddH_2O up to 25 μL. Amplification profile was 94°C for 5 min, 35 cycles of 94°Cfor 30 s, 65°C for 1 min, 72°C for 1 min 30 s, and a final extension of 72°C for 10 min. The products were resolved in 1.0% (*w*/*v*) agarose gel and purified, then cloned into the pGEM-T vector (Tiangen, China) followed by sequencing.

cDNAs encoding full-length CBF-like and COR-like proteins were isolated by RT-PCR and rapid amplification of cDNA ends (RACE) previously (Jiang et al. 2007a, b). The sequences for the entire cDNA insert were determined and deposited.

Bioinformatics Analysis

Associated molecular information was analyzed using software Clustal W, and other databases listed below: NCBI (http://www.ncbi.nlm.nih.gov/), ProtParam (http://us.expasy.org/tools/protparam.html), and TMHMMv2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Alignment scores of the amino acid sequences of the identified cold-responsive genes with other known homologous proteins were processed by PROSITE (http://www.Expasy.org/prosite/), InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) and WU-Blast2 (http://www.ebi.ac.uk/Tools/blast2/index.html). Secondary structure analyses were carried out by SOMPA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopm%20a.html).

Real-Time Fluorescence Quantitative PCR Analysis

For cold acclimation, seedlings were transferred to 4°C for varying lengths of time (0 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 4 days and 7 days), with SaranWrap covered to slow evaporation. For ABA, salt and drought stresses, seedlings

were raised on the water saturated cotton in the growth chamber at 20°C to 22°C. ABA stress was carried out by 200 μM Abscisic acid, Salt stress was induced by 200 mM NaCl and drought stress was imposed by 300 mM mannitol for 0 h, 0.5 h, 2 h, 4 h, 8 h, 24 h and 4 days, respectively. Total RNAs were isolated separately. Gene-specific primers for *BrICE1*, *BrCBF*, and *BrCOR14* were 5′-ACAACAA CGCAACACCCT-3′ and 5′-ACGACGCCAACA CCTCT-3′, 5′-GTGTGTGAAGTGAGGGAACCAAAC-3′ and 5′-CC AAGCCGAGTCCGCATAAT-3′, 5′-TTCTTCTT TCCCCAGCG-3′ and 5′-TTCCATCACCTTTCTCGG-3′ designed based on non-heading Chinese cabbage *BrICE1*, *BrCBF*, and *BrCOR14* genes. Primers for Actins were 5′-ACAACTCCATCATGAAGTGT-3′ and 5′-GAGATC CACATCTGTTGGAA-3′ used as control. Real-time PCR was carried out by One Step SYBR® Primescript™ RT-PCR Kit (Takara, Japan). Mixtures contained 12.5 μl SYBR, 10 pmol adaptor primers each, 50 ng cDNA, ddH_2O up to 25 μl. Amplification profile was 95°C for 120 s, 35 cycles of 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s on Corbett Rotor-Gene 3000A. Each reaction was carried out in a separate PCR system with two replicates and was repeated three times. The data were analyzed using data analysis software that comes with the machine.

Results

Isolation of cDNAs Encoding Cold-Response Proteins

RNA quality was determined by 1% agarose gel electrophoresis. 28S, 18S, and 5S RNA in total extracted RNA had clean bands and proper proportion (Fig. 1). *BrICE1*, *BrCBF*, and *BrCOR14* were obtained by RT-PCR and RACE. Overall information of the putative proteins was shown in Table 1. The BrICE1 protein was estimated to have a half life of about 4.4 h and instability index of 44.13, thus being classified as unstable, while BrCBF and BrCOR14 were classified as stable. Furthermore, alignment scores of the amino acid sequences of the identified cold-responsive genes with other known homologous proteins predicted that BrICE1 was 89% identity to *Arabidopsis* ICE1 and 59% to *Arabidopsis thaliana* ICE2 which illustrated that BrICE1 was more likely an ortholog of ICE1, BrCBF was 73% identical to *A. thaliana* CBF3 and 72% to *A. thaliana* CBF2 and CBF1 which suggest that BrCBF was an ortholog of CBF3, while BrCOR14 was with 76% identity to *A. thaliana* COR15B and 69% identity to *A. thaliana* COR15A which demonstrated that BrCOR14 was more like an orthology of COR15B. Alignment scores of the amino acid sequences of the three genes with other known cold-responsive proteins in Cruciferous family also show high concordance (Table 2).

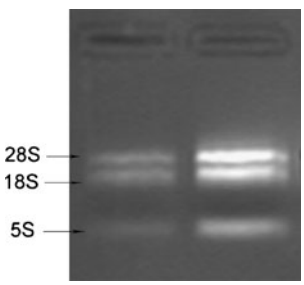

Fig. 1 RNA quality determination

The results also suggested that the BrICE1 was highly conservative to the ICE1 in possessing a bipartite nuclear localization signal (NLS) domain from K_{304} to K_{353} and a bHLH domain from R_{316} to L_{352}, as well as potential recognition sites, for instance basic-leucine zipper (bZIP) that contains a basic region mediating sequence-specific DNA binding followed by a leucine zipper region (required for dimerization) transcription factors, HR1 (shown to bind the small G protein rho or to activate PKN in its GTP-bound form) and Pfam domain (Fig. 2).

Besides *BrCBF* gene contained a consensus sequence, CANNTG (CACCTG). Alignment of the BrCBF proteins indicated that it contained a highly conserved AP2 DNA-binding domain of 60 amino acid residues from I_{52} to A_{109} as well as three potential strikingly conserved elements, YRG (consisted of 23 amino acids containing the conserved YRG amino acid motif and was functionally important for DNA binding), NLS domain (from amino acid R_{42} to R_{59}) and ALA-RICH domain (from amino acid T_{84} to K_{128}). Besides, the prediction also revealed that *BrCBF* contains a dehydration-responsive element (Fig. 3).

In addition, the DNA sequence at the presumed transcription site of *BrCOR14*, CCG, is identified as a common core sequence.

Secondary Structure Analysis

Secondary structure analyses indicated (Fig. 4) that BrICE1 consisted of 158 α-helices, 40 β-turns jointed by 83 extended strands, and 216 random coils which resembled the secondary structure of *Arabidopsis* ICE1 (NP_189309.2) and CbICE53 (*C. bursa-pastoris* AAS79350).

BrCBF consisted of 84 a-helices, nine β-turns jointed by 33 extended strands and 90 random coils which commendably resembled the secondary structure of CBF3 (AF074602) and CbCBF (AY391121.1). It was also note worthy that a-helices occurred predominantly in the structure of BrCBF.

Moreover, the BrCOR14 consisted of 78 α-helices, seven β-turns jointed by 17 extended strands, and 26 random coils, which did not resemble the secondary structure of COR15B (*A. thaliana* NM_129814) and CbCOR15B (AY437888.1) that much. Subsequently, homology modeling analysis was carried out, while no suitable target was found due to the

Table 1 Overall information of *BrICE1*, *BrCBF*, and *BrCOR14* genes

Name	Genbank accession No.	cDNA length (bp)	Open reading frame (bp)	Amino acids	Isoelectric point (pI)	Molecular weight (kDa)	Half life (h)	Instability index
BrICE1	EU374158	1,558	1,491	497	4.98	127.40	4.4 (unstable)	44.13
BrCBF	DQ402470	1,003	648	216	5.32	24.05	30 (stable)	36.73
BrCOR14	DQ192529	549	387	129	5.61	13.75	30 (stable)	23.24

extremely low homology with the proteins deposited in all databases.

Real-Time Fluorescence Quantitative PCR Analysis

Real-time PCR analysis showed that *BrICE1*, *BrCBF* and *BrCOR14* generally displayed a trend of increased first and then decreased with the highest expression at 1, 4, and 24 h, respectively, under 4°C treatment. Comparison of the expression quantity of the three genes at different time points revealed that *BrICE1*'s expression was more than 9.15-fold higher in 1 h after 4°C treatment compared with the basal expression and decreased immediately, followed by accumulation of *BrCBF* at 4 h and which dramatically decreased after that, and *BrCOR14* with the highest expression at 24 h (49.9-fold more than the basal expression) and hold the line till 7 days (Fig. 5).

While for ABA treatment only under 0.5 h did *BrICE1* show 4.3-fold higher expression than the basal. The other two genes showed no significant difference at different time points. For salt (NaCl) treatment, the maximum accumulation of *BrICE1* was 3.48-fold higher. *BrCBF* and *BrCOR14* were 6.13-fold and 117.14-fold higher than the basal, respectively. But maximum accumulation of *BrICE1* was at 24 h, whereas at 8 h *BrCBF* showed the highest expression. In addition, *BrICE1*, *BrCBF*, and *BrCOR14* genes' maximum expression appeared all at 4 h under drought stress carried out by Mannitol treatment with 2.2–6.2–13.7-fold higher than the basal (Fig. 6).

Discussion

Bioinformatics analysis revealed that *BrICE1*, *BrCBF*, and *BrCOR14* genes strongly resemble *Arabidopsis ICE1*, *CBF3*, and *COR15b* separately. Simultaneously sequence analysis showed that BrICE1 contains the highly conserved bHLH domain necessary in *Arabidopsis* ICE families, as well as bZIP and HR1 which strongly suggest that BrICE1 is a DNA-binding protein. Whereas *BrCBF* has CANNTG core element, AP2 domain as well as conserved amino acid sequences, specifically YRG and NLS domain. While AP2 and NLS have been evolutionarily conserved elements necessary for the structure or function of these CBF

Table 2 Comparison of the non-heading Chinese cabbage amino acid sequences of BrICE1, BrCBF, and BrCOR14 with other cold-responsive proteins in the NCBI database

Enzyme source	Number of a. a	Identity (%)	Positives (%)	Genbank accession No.
Brassica campestris ssp. *chinensis* L. Makino (BrICE1)	498	–	–	EU 374158
Arabidopsis thaliana (ICE1)	494	89	92	NP_189309.2
A. thaliana (ICE2)	828	59	67	NP_172746.1
Capsella bursa-pastoris (Cbice53)	492	86	89	AAS79350
B. campestris ssp. chinensis (BrCBF)	216	–	–	DQ402470
A. thaliana (CBF3)	216	73	85	ACI15599.1
A. thaliana (CBF2)	216	72	84	NP_567719
A. thaliana (CBF1)	213	72	82	ABV27062.1
B. juncea (DREB1B)	214	84	91	ABX00639.1
B. napus (CBF)	214	83	91	AAD45623.1
B. campestris ssp. chinensis (BrCOR14)	129	–	–	DQ192529
A. thaliana (COR15B)	141	76	85	NP_181781.1
A. thaliana (COR15A)	139	69	76	NP_181782
B. rapa subsp. *Pekinensis* (COR)	129	97	98	ABF60663.1
B. napus (BN115)	142	78	83	AAA66068.1
Capsella bursa-pastoris (CBCOR15)	139	71	78	AAR99417.1

Fig. 2 The alignment of BrICE1 with ICE1 protein from *Arabidopsis thaliana*. Nuclear localization signal (NLS), bHLH domain are indicated

proteins in *Arabidopsis*, *B. napus*, and *C. bursa-pastoris*, implying they might be indispensable to the function of *BrCBF* in controlling gene expression. Furthermore, *BrCOR14* contains C-repeat DRE sequences common core sequence CGCCGTC, closely resembling the C-repeat DRE sequences in the promoters of the *Arabidopsis* genes *COR15a*, GGCCGAC, and *COR78*/*RD29A*, TACCGAC (Stockinger et al. 1997). An intriguing hypothesis thus raised is that *BrICE1*, the bHLH family, is members of a superfamily of DNA-binding proteins that recognize *BrCBF*, a family of CBF or DREB (DRE binding) proteins having, potentially, CANNTG as a common core sequence, while *BrCBF*, the AP2 domain protein is a superfamily of DNA-binding proteins that recognize *BrCOR14*, a family of cis-acting regulatory elements having, potentially, CCG as a common core sequence (Fig. 7), which strongly suggested that *BrICE1*, *BrCBF* and *BrCOR14* play a critical role in cold-responsive pathway similar to *Arabidopsis ICE1*, *CBF3*, and *COR15b* genes.

Secondary structure prediction is a key element in many different approaches to protein structure analysis, simplifying the 20-state amino acid sequence into typically three states (helix, strand or coil/loop) and is often used to provide constraints for comparative modeling or as a starting point for fold recognition. Indeed, the recent work shows that accurate protein secondary structure information is a useful baseline in fold recognition (Wilson et al. 2002). The secondary structure analysis showed that *BrICE1* and *BrCBF* highly resembled *ICE* and *CBF* genes in *Arabidopsis* and *C. bursa-pastoris*. The N-terminal of the *BrCBF*

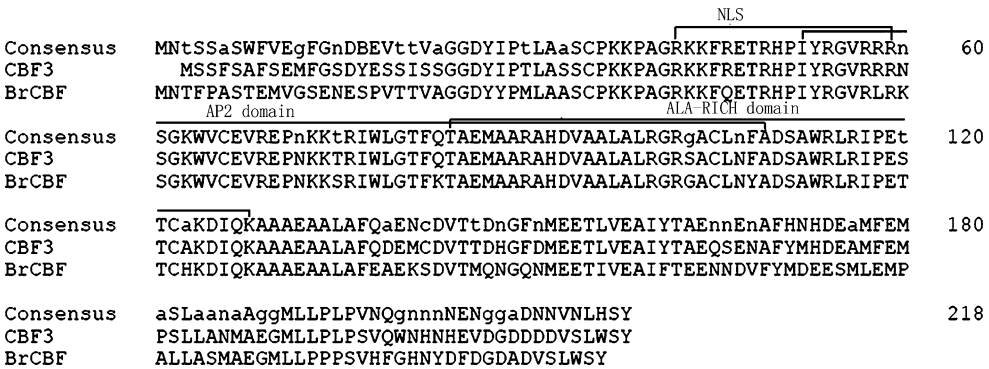

Fig. 3 The alignment of BrCBF with CBF3 protein from *Arabidopsis thaliana*. Nuclear localization signal (*NLS*), AP2domain, and other domains are indicated

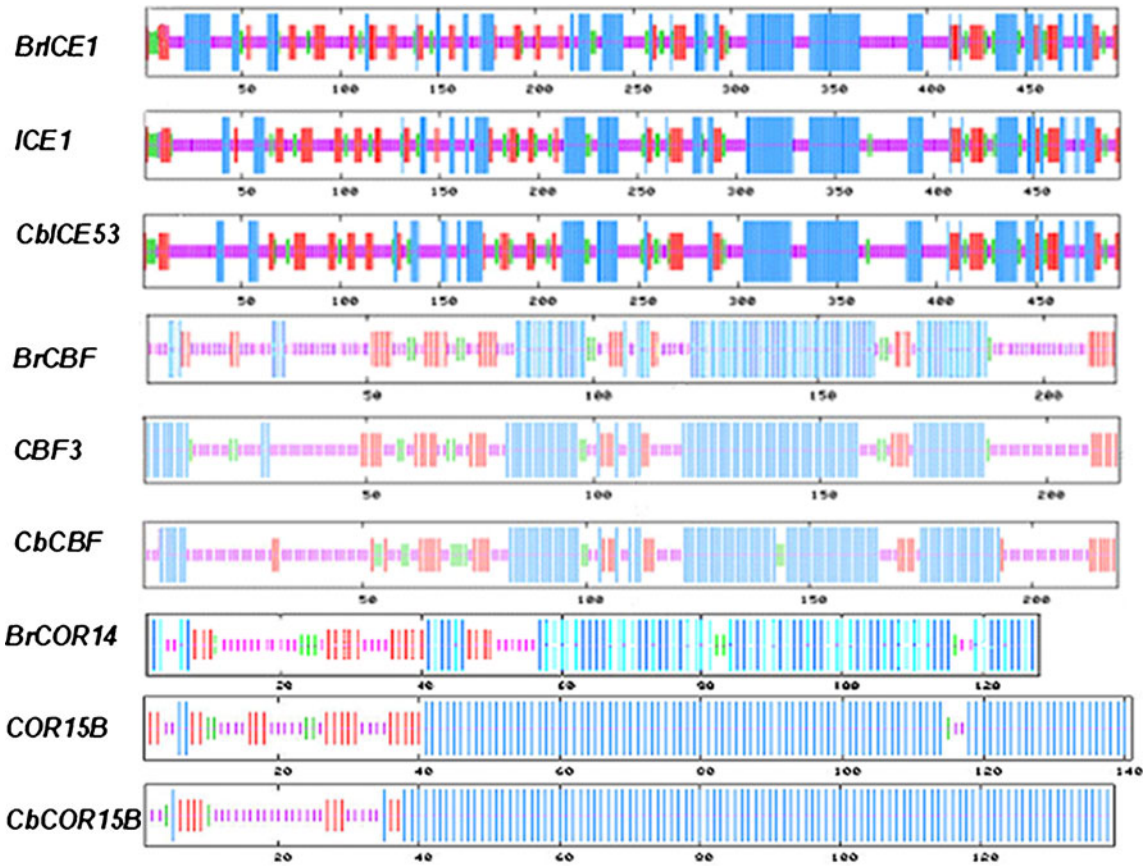

Fig. 4 Comparison of the secondary structure of *Brassica campestris* ssp. *chinensis* L. Makino, *Arabidopsis thaliana* and *Capsella bursa-pastoris* *ICE*, *CBF*, and *COR* genes helix, turn, strand, and coil were indicated in *line* with decreased length in turn, respectively

was composed of some α-helices similar to *CBF3* and *CbCBF* as protection of the protein from being destroyed by some endoenzymes. A hypothesis for the function of these a-helices structures was that they were involved in DNA binding likely through the interaction of their hydrophobic face with the major groove of DNA. Alternatively, these structures might mediate protein–protein interactions which are important for CBF functions. These interactions might involve the ability to form homo- or hetero-dimmers similar to that observed for the MADS box family of plant regulatory proteins (Huang et al. 1996; Riechmann and Meyerowitz 1997). The results also illustrated that the main structure of BrCOR14 was a-helices which suggested that BrCOR14 was not a globulin, and these a-helices might mediate protein–protein interactions which are important for COR functions (Altus et al. 1996).

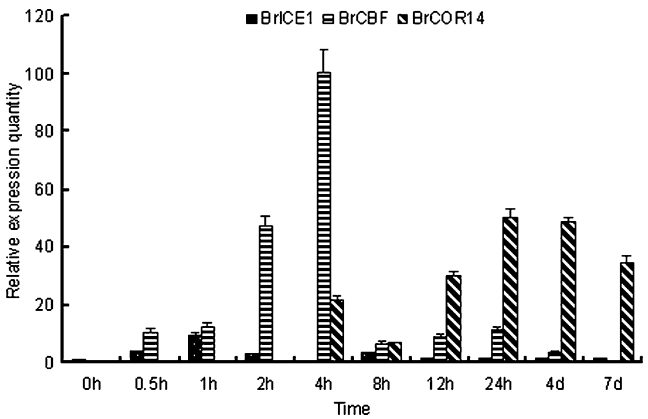

Fig. 5 Real-time fluorescence quantitative PCR analysis of *BrICE1*, *BrCBF*, and *BrCOR14* genes in non-heading Chinese cabbage in response to cold (4°C treatment)

Besides using real-time PCR expression analysis, it was revealed that expression of *BrICE1* increased immediately and showed the highest level of expression at 1 h, followed by dramatic accumulation of *BrCBF* at 4 h and sharply decreased after that. While the highest level of *BrCOR14* expression was observed at 24 h and hold the line till 7 days. These results demonstrated that *BrICE1* was expressed constitutively and might be involved in the cold acclimation process, thus, it might belong to the *ice* gene family. Furthermore, the finding that the expression levels of *BrCBF* increased at early stages of cold exposure and decreased thereafter indicated that *BrCBF*, in the course of cold acclimation, might be regulated by some upstream

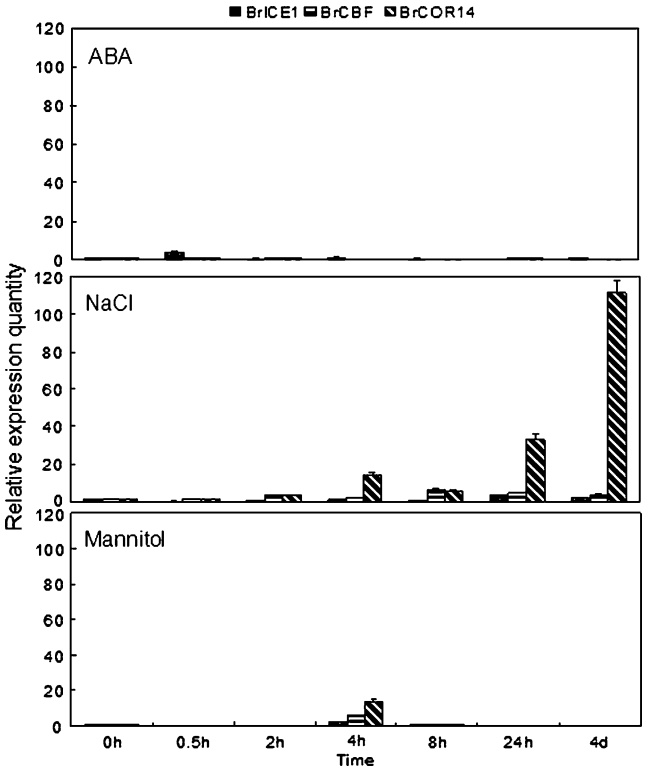

Fig. 6 Real-time fluorescence quantitative PCR analysis of *BrICE1*, *BrCBF*, and *BrCOR14* genes in non-heading Chinese cabbage in response to ABA, salt (NaCl) and drought (Mannitol) stresses

genes or proteins, for instance *BrICE1*, as enhancers and some downstream genes or their products, specifically *BrCOR14*, as suppressors. *BrICE1* increased quickly and then decreased immediately, while *BrCBF* and *BrCOR14* can maintain the higher expression for a relatively long time, which also verified the hypothesis that *BrICE1* protein is unstable whereas *BrCBF* and *BrCOR14* are stable.

In addition, *BrCBF* contains a dehydration-responsive element (Liu et al. 1998) which suggests that *BrCBF* might be responsible not only to cold stress but also to dehydration. While a potential ABA-responsive element (ABRE), CACGTG (Guiltinan et al. 1990; Williams et al. 1992) is not found in *BrCOR14*. ABREs are *cis*-acting

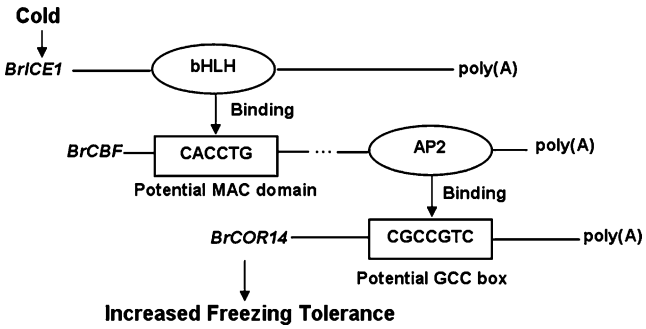

Fig. 7 The anticipated non-heading Chinese cabbage CBF cold-response pathway

elements that had been found to be involved in ABA-regulated gene expression in a number of genes. The other intriguing hypothesis thus raised is that *BrCOR14* was not involved in the ABA-response pathway.

Further studies in ABA, salt and drought stresses also carried out (Yamaguchi-Shinozaki and Shinozaki 1994). Results showed that with salt and drought treatments, the three genes all showed ascending expression, while the highest expression of the three genes all appeared at 4 h under drought treatment, not coming from *ICE*, *CBF*, and *COR* in that order, which might likely indicate that the pathway of salt and drought stresses did not completely resembled that of the cold pathway. Only when treated with ABA within 0.5 h did *BrICE1* show higher expression than the basal while the other two genes showed no significant difference at different time points. These results resembled those of COR genes involved in the expression of cold-, salt-, and drought-regulated genes through an ABA-independent pathway in *Arabidopsis* at the same time verifying the hypothesis that *BrCOR14* is not involved in the ABA-response pathway. While BrICE1 was slightly up-regulated by drought stress which is different from *Arabidopsis* that ICE was not induced by drought stress (Yang et al. 2005). The reason need to be determined further.

Through the above-mentioned bioinformatics analysis and cold acclimation assay, it was found that *BrICE1*, *BrCBF*, and *BrCOR14* have many common characteristics with *Arabidopsis ICE1*, *CBF3*, and *COR15B* genes. There is also a *BrLOS2* gene being cloned from non-heading Chinese cabbage. Therefore, it is highly presumed that non-heading Chinese cabbage may have similar cold acclimation process strongly resembling that of *Arabidopsis* (Jiang et al. 2007c; Yang et al. 2005). Results also suggest that BrCBF and BrCOR14 are involved in the expression of cold-, salt- and drought-regulated genes through an ABA-independent pathway. So these genes might be the potential breeding resources through transformation to improve the cold, salt, as well as drought tolerance. It is important to bear in mind, however, that constitutive high-level overexpression of the *CBF* genes can result in undesirable agronomic traits. In *Arabidopsis*, high-level *CBF* overexpression can cause a "stunted" growth phenotype, a decrease in seed yield, and a delay in flowering (Liu et al. 1998; Gilmour et al. 2000), whether strategies such as using stress-inducible promoters to drive *BrCBF* expression can be developed to attain the potential positive effects of *CBF* regulon engineering without incurring undesirable negative traits remains to be determined (Kasuga et al. 1999). Recently, studies on cold stress signaling and tolerance also revealed that post-transcriptional regulation at pre-mRNA processing and export from nucleus plays a role in cold acclimation. Cold stress-regulated miRNAs have been identified in Arabidopsis and rice

(Chinnusamy et al. 2010). Does non-heading Chinese cabbage have pre-mRNA processing in cold acclimation? Further studies also need to be carried out.

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References

Altus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C, Thomashow MF (1996) Constiutive expression of the cold-regulated *Arabidopsis thaliana* COR15a gene affects both chloroplast and protoplast freezing tolerance. Plant Biol 93:13404–13409

Büttner M, Singh KB (1997) *Arabidopsis thaliana* ethylene responsive element binding protein (AtEBP), an ethylene inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. Proc Natl Acad Sci USA 94:5961–5966

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong XH, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes Dev 17:1043–1054

Chinnusamy V, Zhu JH, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends Plant Sci 12:444–451

Chinnusamy V, Zhu JK, Sunkar R (2010) Gene regulation during cold stress acclimation in plants. Methods Mol Biol 639:39–55

Fowler DB, Limin AE, Wang S, Ward RW (1996) Relationship between low-temperature tolerance and vernalization response in wheat and rye. Can J Plant Sci 76:37–42

Gao MJ, Allard G, Byass L, Flanagan AM, Singh J (2002) Regulation and characterization of four *CBF* transcription factors from *Brassica napus*. Plant Mol Biol 49:459–471

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

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

Goulas E, Dily FL, Ozouf J, Ourry A (2003) Effects of a cold treatment of the root system on white clover (*Trifolium repens* L.) morphogenesis and nitrogen reserve accumulation. J Plant Physiol 160:893–902

Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250:267–271

Huang H, Tudor M, Su T, Zhang Y, Hu Y, Ma H (1996) DNA binding properties of two Arabidopsis MADS domain proteins: binding consensus and dimer formation. Plant Cell 8:81–94

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

Jaglo-Ottosen KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiol 127:910–917

Jiang FL, Hou XL, Shi GJ, Cui XM (2007a) Cloning and characterization of full length cDNA of *BrCBF* gene from *Brassica campestris* ssp. chinensis. J Nanjing Agri Uni 30:18–22 (in *chinensis*)

Jiang FL, Hou XL, Shi GJ, Cui XM (2007b) Cloning and characterization of full length cDNA of *BrCOR14* gene from *Brassica campestris* ssp. *chinensis*. Jiangsu J of Agr Sci 23:34–38 (in Chinese)

Jiang FL, Hou XL, Shi GJ, Cui XM (2007c) Cloning and characterization of full length cDNA of *BrLOS2* gene from *Brassica campestris* ssp. *chinensis*. J Nanjing Agri Uni 30:27–32 (in Chinese)

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, 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

Lee B, Henderson DA, Zhu JK (2005) The arabidopsis cold-responsive transcriptome and its regulation by ICE1. Plant Cell 17:3155–3175

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, Pérez-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 119:463–470

Meng SS, Dane F, Si Y, Ebel R, Zhang CK (2008) Gene expression analysis of cold treated versus cold acclimated *Poncirus trifoliata*. Euphytica 164(1):209–219

Meshi T, Iwabuchi M (1995) Plant transcription factors. Plant Cell Physiol 36:1405–1420

Riechmann JL, Meyerowitz EM (1997) Domain proteins in plant development. Biol Chem 10:1079–1101

Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA 94:1035–1040

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

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

Wang XL, Sun XQ, Liu SX, Liu L, Liu XJ, Sun XF, Tang KX (2005) Molecular cloning and characterization of a novel *ice* gene from *Capsella bursa-pastoris*. Mol Biol 39:18–25

Wang L, Li XW, Zhao Q, Jing SL, Chen SF, Yuan HY (2009) Identification of genes induced in response to low-temperature treatment in tea leaves. Plant Mol Biol Rep 27:257–265

Williams ME, Foster R, Chua NH (1992) Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. Plant Cell 4:485–496

Wilson CL, Hubbard SJ, Doig AJ (2002) A critical assessment of the secondary structure α-helices and their termini in proteins. Protein Eng 15(7):545–554

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

Yang TW, Zhang LJ, Zhang TG, Zhang H, Xu SJ, An LZ (2005) Transcriptional regulation network of cold-responsive genes in higher plants. Plant Sci 169:987–995

Zhou N, Robinson SJ, Huebert T, Bate NJ, Parkin IAP (2007) Comparative genome organization reveals a single copy of CBF in the freezing tolerant crucifer *Thlaspi arvense*. Plant Mol Biol 65:693–705