RESEARCH ARTICLE

Identification of genes from the ICE–CBF–COR pathway under cold stress in *Aegilops–Triticum* composite group and the evolution analysis with those from *Triticeae*

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Abstract Adverse environmental conditions limit various aspects of plant growth, productivity, and ecological distribution. To get more insights into the signaling pathways under low temperature, we identified 10 C-repeat binding factors (CBFs), 9 inducer of CBF expression (ICEs) and 10 cold-responsive (CORs) genes from *Aegilops–Triticum* composite group under cold stress. Conserved amino acids analysis revealed that all CBF, ICE, COR contained specific and typical functional domains. Phylogenetic analysis of CBF proteins from *Triticeae* showed that these CBF homologs were divided into 11 groups. CBFs from *Triticum* were found in every group, which shows that these CBFs generated prior to the divergence of the sub-families of *Triticeae*. The evolutionary relationship among the ICE and COR proteins in *Poaceae* were divided into four groups with high multispecies specificity, respectively. Moreover, expression analysis revealed that mRNA accumulation was altered by cold treatment and the genes of three types involved in the ICE–CBF–COR signaling pathway were induced by cold stress. Together, the results

make CBF, ICE, COR genes family in *Triticeae* more abundant, and provide a starting point for future studies on transcriptional regulatory network for improvement of chilling tolerance in crop.

Keywords *Triticeae* · Cold stress · CBF · ICE · COR

Introduction

Low temperature is a vital environmental factor that restricts growth, productivity, and ecological distribution of plants (Zhuang et al. 2015). Upon exposure to low non-freezing temperatures, many plants have evolved sophisticated mechanisms with the acquisition of freezing tolerance, a process known as cold acclimation (Thomashow 1999; Zhao et al. 2016). During this complex process, extensive changes are involved, including gene expression and regulation, biochemical and metabolic changes (Chinnusamy et al. 2007; Shi et al. 2015). Identifying genes involved in freezing tolerance is crucial for understanding how plants regulate growth at low temperature. Transcription factors and effector genes are precisely regulated in plant cells suffering from cold stress (Ma et al. 2014; Zhou et al. 2011). These regulation cascades or signaling pathways play pivotal roles in adaptation to cold stress (Wingler 2014). C-repeat binding factor/dehydration responsive element binding factor (CBF/DREB) are critical transcription factors that specifically interplay with the C-repeat (CRT)/dehydration-responsive element (DRE) and positively regulated the expression of downstream cold-responsive (COR) genes during cold acclimation (An et al. 2017; Liu et al. 1998; Park et al. 2015; Sakuma et al. 2002; Stockinger et al. 1997; Yamaguchi-Shinozaki and Shinozaki 1994). At the same time, CBF/DREB genes are

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activated by inducers of CBF expression (ICEs) through specific binding to the MYC recognition cis-elements (CANNTG) in the promoter (Chinnusamy et al. 2003). These entities constitute the signal regulatory pathway, ICE–CBF–COR transcriptional cascade, which mediates the cold acclimation process (Chinnusamy et al. 2007; Lissarre et al. 2010; Lu et al. 2017; Ryu et al. 2014; Zhu et al. 2007).

CBF/DREBs which belong to the AP2/ERF (APE-TALA2/Ethylene-Responsive Factor) superfamily have characteristic signature sequence motifs flanking the AP2 domain, PKK/RPAGRxKFxETRHP and DSAWR (An et al. 2017; Gilmour et al. 1998; Jaglo et al. 2001; Jia et al. 2016; Monroe et al. 2016; Park et al. 2015; Skinner et al. 2005). ICE proteins, member of the MYC family transcription factor (Lee et al. 2015; Lu et al. 2017), have a MYC basic helix–loop–helix (bHLH) domain and localize in the nucleus (Peng et al. 2014). CORs generally refer to the proteins encoded by all cold regulated genes (Lee et al. 1999; Uemura et al. 1996), including late embryogenesis abundant protein (LEA) (Tsuda et al. 2000), stress responsive protein (SRP) (Unpublished, from GenBank), cold induced (KIN), low temperature induced (LTI) (Dong and Pei 2014; Nordin et al. 1993; Welin et al. 1995), and so on. In *Arabidopsis*, three CBF genes are cold stress-inducible genes (Liu et al. 1998; Ryu et al. 2014; Stockinger et al. 1997), and the three CBFs are not redundant (Zhou et al. 2011). CBF1 and CBF3 are regulated in a different way from CBF2, and CBF2 is a negative regulator of CBF1 and CBF3 expression during cold acclimation (Novillo et al. 2004, 2007; Qin et al. 2011). Furthermore, the recent research using the CRISPR/Cas9 technology reveals the broad mechanism of CBF genes that CBF1 and CBF3 negatively regulate CBF2 expression (Zhao and Zhu 2016). Coordinately, ICE proteins have distinct functions rather than redundant functions (Rahman et al. 2014). In *Arabidopsis*, ICE1 can activate the expression of CBF3/DREB1A during cold acclimation (Chinnusamy et al. 2003), and ICE2 triggers the expression of CBF1 (Fursova et al. 2009). Finally, many COR genes are induced to resist ambient cold temperature. These elaborate signaling modules constitute a regulatory pathway, which enhances tolerance to cold stress in plants.

Many close wild relatives of common wheat in *Triticeae* carry abundant and valuable stress resistant genes to facilitate genetic improvement, and particularly in species from *Aegilops* with strong resistance to cold stress (Jia et al. 2013). Many CBF genes have been characterized from some *Triticeae* species, including 37 from hexaploid wheat (*Triticum aestivum*) (Badawi et al. 2007), 13 from *Triticum monococcum* (Miller et al. 2006), 10 from durum wheat (*Triticum durum*) (Leonardis et al. 2007), 20 from barley (*Hordeum vulgare*) (Skinner et al. 2005), 11 from

rye (*Secale cereale*) (Siddiqua and Nassuth 2011), 4 from *Brachypodium distachyon* (Li et al. 2012; Ryu et al. 2014), 1 from *Aegilops tauschii* (Badawi et al. 2007) and 9 from *Aegilops biuncialis* (Unpublished, from GenBank). The previous research indicated that CBF14 gene in *A. tauschii* associated with resistance to freezing stress (Masoomi-Aladizgeh et al. 2015), however, the lack of essential functional domains of the CBF14 results in many uncertainties. In addition, the functions of CBF genes from *A. biuncialis* remain largely unknown. Some ICE, COR genes also have been identified from these *Triticeae* species. In *Triticeae*, 2 ICE genes and 5 COR genes were obtained from wheat (*T. aestivum*), including TaICE41, TaICE87, WCS120, Wcor410, Wcor14, WRAB15 and WRAB18 (Badawi et al. 2008; Ganeshan et al. 2008; Soltesz et al. 2013; Talanova et al. 2013), as well as 4 COR genes from barley (*H. vulgare*) including HVA1, DHN5, DHN8 and COR14b (Dal Bosco et al. 2003; Jeknic et al. 2014; Koag et al. 2009; Kosova et al. 2013). However, the deeper researches on ICE, COR genes in *Triticeae* are less. To get better understanding of ICE–CBF–COR pathway and its involvement in the chilling tolerance, more genes related to this regulatory network await identification.

In this study, we identified and characterized 10 CBFs, 9 ICEs and 10 CORs in *Aegilops–Triticum* composite group. The results suggest that all CBF, ICE, COR amino acid sequences contained specific and representative conserved domains. We analyzed phylogenetic relationship of different CBF genes in *Triticeae*, as well as ICE and COR genes in *Poaceae*. Moreover, expression analysis revealed that mRNA accumulation was altered by cold treatment and the genes of three types involved in the ICE–CBF–COR signaling pathway were induced by cold stress. This study concluded that CBF genes may be activated by ICE, and may induce the expression of COR genes to enhance plant tolerance to cold. Consequently, the results provide candidate genes and transcriptional regulatory network for improvement of chilling tolerance in *Triticeae*.

Materials and methods

Plant materials, growth condition and RNA isolation

Four *Aegilops* species together with one winter-hardy wheat cultivar (*T. aestivum*) were selected as research materials in this study, which are presented in Table 1. The winter-hardy wheat cultivar Mironovska 808 (abbreviated as M808) was originally screened for its strong cold tolerance in the Ukraine (Tsvetanov et al. 2000). All seeds were germinated in a petri dish covered with distilled water at 28 °C, then were planted in plastic boxes filled with soil substrates and placed in a controlled growth

Table 1 Research materials in this study

Species	Cultivar	Ploidy	Source	Origin
<i>Triticum aestivum</i>	Mironovska 808	6X	Shenyang Agriculture university, China	Ukraine
<i>Triticum aestivum</i>	China Spring	6X	Shenyang Agriculture university, China	China
<i>Aegilops umbellulata</i>	–	2X	National Clonal Germplasm Repository, USA	Turkey
<i>Aegilops tauschii</i>	–	2X	National Clonal Germplasm Repository, USA	Unknown
<i>Aegilops speltoides</i>	–	2X	National Clonal Germplasm Repository, USA	Turkey
<i>Aegilops neglecta</i>	–	4X	National Clonal Germplasm Repository, USA	France

chamber with the temperature set at 25 °C, a 16 h light/8 h dark photoperiod, and a humidity of 45%. Ten centimeter seedlings were treated with 4 °C cold stress for 6 h before being frozen in liquid nitrogen for total RNA isolation. Total RNA of samples was extracted by TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Japan) according to the manufacturer's instruction. First strand cDNA synthesis was performed using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara), and stored at – 20 °C until use.

Gene isolation and bioinformatic analyses

To isolate the *Aegilops* and *T. aestivum* *CBF*, *ICE* and *COR* genes, homologous sequences from other species in the GenBank were aligned with the Basic Local Alignment Search Tool (BLAST). A set of primers for gene amplification were designed by Primer 5 software (<http://www.Premierbiosoft.com>) (Table 2). The cDNA clone of all *CBF* and *COR* genes were amplified via Polymerase Chain Reaction (PCR) and then were sequenced to test whether they contain identical sequence or not. To obtain the whole sequence of *ICE* genes, 5'-RACE and 3'-RACE experiments were performed using a Full RACE Kit (TaKaRa) after the acquisition of the conserved sequence. To validate the whole coding sequence of *ICE* genes, primers were designed on the basis of the assembled results, and several clones of two independent PCR reactions were sequenced to correct errors introduced during PCR.

Multiple sequence alignments were done using DNAMAN 8 (<http://www.lynnon.com>) and MEGA 5.10 (<http://www.megasoftware.net/mega.php>). Conserved motifs were identified using MEME Suite 4.10.1 (<http://meme-suite.org/>) and WebLogo 3.4 (<http://weblogo.threeplusone.com/>). To compare evolutionary relationship of the isolated *Triticeae* *CBF*, *ICE*, *COR* family members, the phylogenetic trees were constructed by MEGA 5.10 (<http://www.megasoftware.net/mega.php>) based on the neighbor-joining (NJ) method and bootstrap analysis (1000 replicates). Highly similar homologous genes were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>) and presented in Tables 3, 4, and 5.

Expression analysis of *ICE–CBF–COR* genes

To detect the expression of *ICE*, *CBF*, *COR* genes under cold stress, *A. tauschii*, *Aegilops neglecta*, winter-hardy wheat M808 and spring wheat *T. aestivum* L. cultivar China Spring (CS) were placed in growth chambers under long-day conditions (16 h light, 8 h dark) at 25 °C before cold stress. Then, the plants were removed from the growth chambers to a 4 °C cold room, and the leaves were harvested at intervals of 0, 2, 4, 8, 12, 24 h after cold treatment. Total RNA was extracted by TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa). First strand cDNA synthesis was derived using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). Gene-specific primers (Table 2) were designed according to the 3'-untranslated regions (UTR) for qPCR amplification. *18S* (AJ315041) was selected as the reference gene from the GenBank database. According to the instructions of the SYBR R Premix Ex Taq™ (Takara), qRT-PCR was performed using an iQ5 Real-Time PCR System (Bio-Rad, USA). The relative expression level of genes were calculated using iQ5 software and the Microsoft Excel program. Mean values and standard errors of three biological replicates were presented in this analysis.

Results

Identification of *CBF* genes and nomenclature

To enrich the gene family of *Triticeae* cereal *CBF*, a total of 10 distinct *CBF* genes have been isolated from five materials (*Aegilops* species and *T. aestivum*) (Table 3) using PCR amplification with degenerate primers (*CBF1* and *CBF 2*, Table 2). The gene names and GenBank accession numbers are summarized in Table 3. Comparative analysis of the cDNA sequence with genomic sequence confirmed that *CBF* genes are intronless. The identified *CBFs* have amino acid residue numbers ranging from 223 to 269. BLAST searches using the reported *CBF* sequences showed that all *CBFs* displayed strong similarity to the *Triticeae* cereal *CBFs*. Following homology

Table 2 Primers in this study

Primer name	Forward primer sequence (5′–3′)	Rerverse primer sequence (5′–3′)	Purpose	
CBF1	ATGGACGTCGCCGACG	TTAGTCAAACAAATAGCTCCATAGC	Amplification of <i>CBFs</i>	
CBF2	GATGGACGTCGCCGACGC	TTAGTCAACAAGTAGCTC		
ICE-C	GAAGAA(A/G)GG(T/A/G)ATGCC(T/G)GC(T/C)AAGAA	GG(A/G)CACA(A/G)CTC(T/C)TCCTTGA(C/T)(A/G)CG	Amplification of the conserved sequence of <i>ICEs</i>	
ICE-3F	CCAATAGCTTCTCTCCCGTCGA	TCGCCTACATCGCGTTCTGGA	3′ RACE of <i>ICEs</i>	1st round
		CTAGACCGCTGGATGGAACCCC		
ICE-3S	GCCGCATCAAGGAGGAGTTGTG	CTGGAGACCGGCGCAGTGCA		2nd round
		CCTACATCGTGGGATGGAACC		
ICE-5F	AGGGCGTGATGGAGAACTCGG CGATGCTTGTATGACGACAGCT	TGTGCAGGAGCTCCTTGAGGTA	5′ RACE of <i>ICEs</i>	1st round
ICE-5S	GGGGGCGACTGGGGGTACCT	AAGCCCTGTCCATTTTGCTGATC		2nd round
	CGATGCTTCCCGCTCCTCAACC			
ICE-IF1	GCAGAGATAAGTTGCAGTAACTGA	GCGAGCCTCATCCCATCA	Verification of the whole coding sequence of <i>ICEs</i>	1st round
ICE-IS1	GAAGTTGCCAAGAATCCGT	AAAGGAGCGGAACAGCACA		2nd round
ICE-IF2	GCTATGCTGTCGAGTTCAATAG	AAACCATGGGTACTTATTGCAAAT		1st round
ICE-IS2	TTGGGTGCAGGAAGGCGA	TTCCTTTGGTATGCTGTCCTCT		2nd round
COR1	ATGTCTGGTTGGTTTCAAGAGAAG	TCACCCTCCCATCCCCGAG	Amplification of <i>CORs</i>	
COR2	ATGTCGGGTTGGTTCGGGGGTAAC			
18S	TGAGAAACGGCTACCACATC	TCGGCATCGTTTATGGTTGA	qRT-PCR analysis	
CBF4	AGCCCGAGCAACACTCTTT	GCGCCAAGCTGTCGTAGTA		
CBF9	GCAGGGTATGCTCGTGTC	GTCGAACAAGCAGCTCCATA		
ICE41	AGGGGCAGGCGGTGAACA	GGCGAAGCCATCGAAGCAG		
ICE87	AAGGCCTTGGTCTTGACGTG	CGCCTTGATTTCCTCAGCCT		
COR1	TGAGAAAAGCGAGGCTGTCA	TGATCCTTACCGGCTTCCAC		
COR2	GACCAGACGTTCCGGCTTCTT	CGGTCTCCTCGACACACTTT		

comparison and phylogenetic analysis (CBFIa, CBFII, CBFIIIa, CBFIIIb, CBFIIIc, CBFIIId, CBFIVa, CBFIVb, CBFIVc, and CBFIVd) with the established nomenclature of *CBFs* from *H. vulgare*, *S. cereale*, *T. aestivum*, and *T. monococcum* (Badawi et al. 2007; Miller et al. 2006; Skinner et al. 2005), we assigned consistent gene numbers to these identical orthologous genes which belong to the same gene subfamily (Table 3). When the same gene number of several genes arose, a letter designating its order trails *CBF* gene number (e.g. TaCBFIVd-4A).

Phylogenetic analysis of *CBF* genes in *Triticeae*

To establish the evolutionary relationship among the different *CBFs* in *Triticeae*, phylogenetic analysis was

performed using *Triticeae* *CBF* homologs (Table 3). The results revealed that these *CBF* homologs were divided into 11 monophyletic groups (CBFIa, CBFII, CBFIIIa, CBFIIIb, CBFIIIc, CBFIIId, CBFIVa, CBFIVb, CBFIVc, CBFIVd, and CBFV) (Fig. 1). All 10 *CBF* members isolated in this study belonged to Group IVd, with another 8 *CBFs* from *T. aestivum*, 3 *CBFs* from *A. biuncialis* and 2 *CBFs* from *H. vulgare*. In Group IVd, three subgroups were presented (CBFIVd-9, CBFIVd-22 and CBFIVd-4), and 5 genes isolated in this study belonged to Subgroup CBFIVd-9, while another 5 belonged to CBFIVd-4.

In the phylogenetic analysis, only one group contains a single member (TmCBFIIIb-18), which was designated Group IIIb. Group IVb is the only one that contains *CBFs* only from *T. aestivum*. Group IIId is the only one that

Table 3 Description of *CBF* genes used in the study

Proposed gene name	Gene name	Gene accession	Protein accession	Gene fragment	Protein fragment	Species	References
TaCBFIVd-4A	TaCBFIVd-4A	KY931522		672	223	<i>Triticum aestivum</i>	This study
AeuCBFIVd-4	AeuCBFIVd-4	KY931528		672	223	<i>Aegilops umbellulata</i>	This study
AetCBFIVd-4	AetCBFIVd-4	KY931534		672	223	<i>Aegilops tauschii</i>	This study
AesCBFIVd-4	AesCBFIVd-4	KY931540		672	223	<i>Aegilops speltoides</i>	This study
AenCBFIVd-4	AenCBFIVd-4	KY931546		678	225	<i>Aegilops neglecta</i>	This study
TaCBFIVd-9D	TaCBFIVd-9D	KY931521		810	269	<i>Triticum aestivum</i>	This study
AeuCBFIVd-9	AeuCBFIVd-9	KY931527		765	254	<i>Aegilops umbellulata</i>	This study
AetCBFIVd-9	AetCBFIVd-9	KY931533		765	254	<i>Aegilops tauschii</i>	This study
AesCBFIVd-9	AesCBFIVd-9	KY931539		765	254	<i>Aegilops speltoides</i>	This study
AenCBFIVd-9	AenCBFIVd-9	KY931545		765	254	<i>Aegilops neglecta</i>	This study
AebCBFIVd-4	AebCBF7	FR719739	CBX87021	555	184	<i>Aegilops biuncialis</i>	Unpublished, 2010
AebCBFIVd-9	AebCBF9	FR719741	CBX87023	768	255	<i>Aegilops biuncialis</i>	Unpublished, 2010
AebCBFIIIa-6	AebCBF1	FR719733	CBX87015	729	242	<i>Aegilops biuncialis</i>	Unpublished, 2010
AebCBFIIIc-3	AebCBF2	FR719734	CBX87016	741	246	<i>Aegilops biuncialis</i>	Unpublished, 2010
AebCBFIIIc-10	AebCBF3	FR719735	CBX87017	714	237	<i>Aegilops biuncialis</i>	Unpublished, 2010
AebCBFIIId-12	AebCBF4	FR719736	CBX87018	720	239	<i>Aegilops biuncialis</i>	Unpublished, 2010
AebCBFIIId-19	AebCBF5	FR719737	CBX87019	711	236	<i>Aegilops biuncialis</i>	Unpublished, 2010
AebCBFIVa-2	AebCBF6	FR719738	CBX87020	678	225	<i>Aegilops biuncialis</i>	Unpublished, 2010
AebCBFIVd-22	AebCBF8	FR719740	CBX87022	855	284	<i>Aegilops biuncialis</i>	Unpublished, 2010
AtCBFV-1	AtCBF1	U77378	AAC49662	642	213	<i>Arabidopsis thaliana</i> (thale cress)	Stockinger et al. (1997)
AtCBFV-2	AtCBF2	AF074601	AAD15976	651	216	<i>Arabidopsis thaliana</i> (thale cress)	Gilmour et al. (1998)
AtCBFV-3	AtCBF3	AF074602	AAD15977	651	216	<i>Arabidopsis thaliana</i> (thale cress)	Gilmour et al. (1998)
BdCBFIIId-1	BdCBF1	JQ180470	AFD96407	735	244	<i>Brachypodium distachyon</i> (stiff brome)	Li et al. (2012)
BdCBFIIId-2	BdCBF2	JQ180470	AFD96408	777	258	<i>Brachypodium distachyon</i> (stiff brome)	Li et al. (2012)
BdCBFIIId-3	BdCBF3	JQ180470	AFD96409	753	250	<i>Brachypodium distachyon</i> (stiff brome)	Li et al. (2012)
BdCBFIIId-4	BdCBF4	JQ180470	AFD96410	747	248	<i>Brachypodium distachyon</i> (stiff brome)	Li et al. (2012)
HvCBFIa-1	HvCBF1	AF418204	AAL84170	654	217	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Xue (2002)
HvCBFIVa-2	HvCBF2	AF442489	AAM13419	666	221	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Xue (2003)
HvCBFIIIc-3	HvCBF3	AF239616	AAG59618	750	249	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Choi et al. (2002)

Table 3 continued

Proposed gene name	Gene name	Gene accession	Protein accession	Gene fragment	Protein fragment	Species	References
HvCBFIVd-4	HvCBF4B	AY785848	AAX28948	678	225	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2006)
HvCBFII-5	HvCBF5	AY785857	AAX23700	645	214	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2006)
HvCBFIIIa-6	HvCBF6	EU331996	ACA29480	735	244	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	251
HvCBFV-7	HvCBF7	AY785866	AAX23706	660	219	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2006)
HvCBFIVd-9	HvCBF9	AY785880	AAX23709	876	291	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2006)
HvCBFIIIc-10A	HvCBF10A	AY785884	AAX23713	684	227	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2006)
HvCBFIIIc-10B	HvCBF10B	AY785887	AAX23716	723	240	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2006)
HvCBFIa-11	HvCBF11	AY785892	AAX23720	657	218	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2006)
HvCBFIIIId-12	HvCBF12	DQ095157	ABA01491	735	244	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2005)
HvCBFIIIc-13	HvCBF13	DQ095158	ABA01492	759	252	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2005)
HvCBFIVc-14	HvCBF14	DQ095159	ABA01493	645	214	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2005)
ScCBFIa-11	ScCBFIa-11	EU194240	ABY59777	615	204	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFII-5	ScCBFII-5	EU194241	ABY59778	642	213	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFIIIa-6	ScCBFIIIa-6	EU194242	ABY59779	690	229	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFIIIc-10	ScCBFIIIc-10	EU194243	ABY59780	666	221	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFIIIc-3A	ScCBFIIIc-3A	EU194244	ABY59781	687	228	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFIIIc-3B	ScCBFIIIc-3B	EU194245	ABY59782	696	231	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFIIIId-12	ScCBFIIIId-12	EU194246	ABY59783	699	232	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFIIIId-15	ScCBFIIIId-15	EU194247	ABY59784	663	220	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFIIIId-19	ScCBFIIIId-19	EU194248	ABY59785	666	221	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFIVa-2A	ScCBFIVa-2A	EU194249	ABY59786	582	193	<i>Secale cereale</i> (rye)	Campoli et al. (2009)
ScCBFIVa-2B	ScCBFIVa-2B	EU194250	ABY59787	582	193	<i>Secale cereale</i> (rye)	Campoli et al. (2009)

Table 3 continued

Proposed gene name	Gene name	Gene accession	Protein accession	Gene fragment	Protein fragment	Species	References
TaCBFIa-11	TaCBFIa-A11	EF028751	ABK55354	657	218	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFII-5A	TaCBFII-5.1	EF028752	ABK55355	648	215	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFII-5B	TaCBFII-5.2	EF028753	ABK55356	660	219	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFII-5C	TaCBFII-5.3	EF028754	ABK55357	657	218	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIIa-6B	TaCBFIIIa-6.1	EF028755	ABK55358	711	236	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIIa-6C	TaCBFIIIa-6.2	EF028756	ABK55359	729	242	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIIa-6A	TaCBFIIIa-D6	EF028757	ABK55360	717	238	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIIC-3B	TaCBFIIIC-3.1	EF028758	ABK55361	708	235	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIIC-3C	TaCBFIIIC-3.2	EF028759	ABK55362	741	246	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIIC-10	TaCBFIIIC-B10	EF028761	ABK55364	723	240	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIIC-3A	TaCBFIIIC-D3	EF028760	ABK55363	738	245	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIID-12B	TaCBFIIID-12.1	EF028762	ABK55365	738	245	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIID-15B	TaCBFIIID-15.2	EF028765	ABK55368	726	241	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIID-15A	TaCBFIIID-A15	EF028764	ABK55367	720	239	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIID-19B	TaCBFIIID-A19	EF028766	ABK55369	705	234	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIID-12A	TaCBFIIID-B12	EF028763	ABK55366	738	245	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIID-19C	TaCBFIIID-B19	EF028767	ABK55370	705	234	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIIID-19A	TaCBFIIID-D19	EF028768	ABK55371	705	234	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVa-2C	TaCBFIVa-2.2	EF028770	ABK55373	693	230	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVa-2A	TaCBFIVa-2.3	EF028771	ABK55374	579	192	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVa-2B	TaCBFIVa-A2	EF028769	ABK55372	678	225	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVb-21A	TaCBFIVb-21.1	EF028775	ABK55378	609	202	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVb-20A	TaCBFIVb-A20	EF028772	ABK55375	654	217	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVb-20B	TaCBFIVb-B20	EF028773	ABK55376	639	212	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVb-20C	TaCBFIVb-D20	EF028774	ABK55377	639	212	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVb-21B	TaCBFIVb-D21	EF028776	ABK55379	609	202	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVc-14C	TaCBFIVc-14.1	EF028777	ABK55380	639	212	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)

Table 3 continued

Proposed gene name	Gene name	Gene accession	Protein accession	Gene fragment	Protein fragment	Species	References
TaCBFIVc-14A	TaCBFIVc-14.3	EF028779	ABK55382	645	214	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVc-14 B	TaCBFIVc-B14	EF028778	ABK55381	645	214	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVd-4C	TaCBFIVd-4.1	EF028780	ABK55383	669	222	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVd-9B	TaCBFIVd-9.1	EF028782	ABK55385	810	269	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVd-22 A	TaCBFIVd-A22	EF028785	ABK55388	828	275	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVd-22 B	TaCBFIVd-B22	EF028786	ABK55389	873	290	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVd-4B	TaCBFIVd-B4	EF028781	ABK55384	669	222	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVd-9A	TaCBFIVd-B9	EF028783	ABK55386	810	269	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVd-22 C	TaCBFIVd-D22	EF028787	ABK55390	828	275	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TaCBFIVd-9C	TaCBFIVd-D9	EF028784	ABK55387	810	269	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2007)
TmCBFIIIc-3	TmCBF3	AY951949	AAY32553	741	246	<i>Triticum monococcum</i>	Miller et al. (2006)
TmCBFII-5	TmCBF5	AY951947	AAY32551	633	210	<i>Triticum monococcum</i>	Miller et al. (2006)
TmCBFV-7	TmCBF7	AY785904	AAX28965	828	275	<i>Triticum monococcum</i>	Skinner et al. (2005)
TmCBFIIIc-10	TmCBF10	AY951950	AAY32554	720	239	<i>Triticum monococcum</i>	Miller et al. (2006)
TmCBFIId-12	TmCBF12	EU076381	ABW87011	744	247	<i>Triticum monococcum</i>	Knox et al. (2008)
TmCBFIIIc-13	TmCBF13	AY951951	AAY32555	720	239	<i>Triticum monococcum</i>	Miller et al. (2006)
TmCBFIVc-14A	TmCBF14	EU076382	ABW87012	639	212	<i>Triticum monococcum</i>	Knox et al. (2008)
TmCBFIVc-14B	TmCBF14	AY951948	AAY32552	639	212	<i>Triticum monococcum</i>	Miller et al. (2006)
TmCBFIId-15	TmCBF15	EU076383	ABW87013	726	241	<i>Triticum monococcum</i>	Knox et al. (2008)
TmCBFIId-16	TmCBF16	EU076384	ABW87014	864	287	<i>Triticum monococcum</i>	Knox et al. (2008)
TmCBFIIIb-18	TmCBF18	AY951946	AAY32550	738	245	<i>Triticum monococcum</i>	Miller et al. (2006)

contains CBFs from the whole five *Triticeae* subfamilies used in this study. Groups IIIa, IIIc and IVa are the ones that contain CBFs from four *Triticeae* subfamilies (*Aegilops*, *Hordeum*, *Triticum*, *Secale*), suggesting that the ancestral *CBFIIIa*, *CBFIIIc* and *CBFIVa* genes were already present before divergence of these subfamilies. Similarly, Groups Ia and II are the ones that contain CBFs from three *Triticeae* subfamilies (*Hordeum*, *Triticum*, *Secale*), manifesting that the ancestral *CBFIa* and *CBFII* genes were already present before divergence of these subfamilies. In addition, 5 CBF homologues belonging to Group V are somewhat distant from those belonging to the rest of the groups, and it may reveal that sequence discrepancies of amino acids play a critical role in the classification reflecting their evolutionary relationships. CBFs from *Triticum* were found in every group, showing that

these CBFs generated prior to the divergence of *Triticum* and other members of *Triticeae*. At present, all four genes from *B. distachyon* were found in the *CBFIId* group, indicating that *BdCBFs* had appeared inferior to the radiation of these subfamilies.

Sequence structure analysis of CBF proteins

To identify some of the structural differences associated with the *CBF* genes, the genes involved in the subgroup *CBFIVd-9* and *CBFIVd-4* were selected to perform multiple sequences alignments on the basis of the evolutionary relationship of *Triticeae CBFs*. Conserved amino acids analysis was performed to highlight conservation of domains by MEME and WebLogo. It was showed that all CBF amino acid sequences contained an AP2 DNA binding

Table 4 Description of *ICE* genes used in the study

Proposed gene name	Gene name	Gene accession	Protein accession	Gene fragment	Protein fragment	Species	References
AeuICE1	AeuICE1	KY931524		1149	382	<i>Aegilops umbellulata</i>	This study
AetICE1	AetICE1	KY931530		1151	383	<i>Aegilops tauschii</i>	This study
AesICE1	AesICE1	KY931536		1149	382	<i>Aegilops speltoides</i>	This study
AenICE1	AenICE1	KY931542		1151	382	<i>Aegilops neglecta</i>	This study
TaICE2A	TaICE2A	KY931518		1329	442	<i>Triticum aestivum</i>	This study
AeuICE2	AeuICE2	KY931523		1331	443	<i>Aegilops umbellulata</i>	This study
AetICE2A	AetICE2A	KY931529		1331	443	<i>Aegilops tauschii</i>	This study
AesICE2	AesICE2	KY931535		1331	443	<i>Aegilops speltoides</i>	This study
AenICE2	AenICE2	KY931541		1331	443	<i>Aegilops neglecta</i>	This study
AetICE2B	AetICE1-like	XM_020312906	XP_020168495	1503	500	<i>Aegilops tauschii</i>	RefSeq, 2017
AtICE1	AtICE1	AY195621	AAP14668	1485	494	<i>Arabidopsis thaliana</i>	Chinnusamy et al. (2003)
AtICE2	AtICE2	KM210288	AIU34717	1335	444	<i>Arabidopsis thaliana</i>	Unpublished, 2014
BdICE1	BdICE1	XM_003567379	XP_003567427	1116	371	<i>Brachypodium distachyon</i> (stiff brome)	RefSeq, 2015
HvICE2	HvICE2	DQ151537	ABA25897	579	192	<i>Hordeum vulgare</i> subsp. <i>vulgare</i> (domesticated barley)	Skinner et al. (2006)
OsICE1	OsICE1	XM_015795517	XP_015651003	1239	412	<i>Oryza sativa Japonica Group</i> (Japanese rice)	RefSeq, 2016
SbICE1	SbICE1-like	XM_002458977	XP_002459022	1131	376	<i>Sorghum bicolor</i> (sorghum)	Paterson et al. (2009)
SiICE1	SiICE1-like	XM_004971094	XP_004971151	1131	376	<i>Setaria italica</i> (foxtail millet)	RefSeq, 2015
TaICE1	TaICE41	EU562183	ACB69501	1146	381	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2008)
TaICE2B	TaICE87	EU562184	ACB69502	1332	443	<i>Triticum aestivum</i> (bread wheat)	Badawi et al. (2008)
ZmICE2	ZmICE2	EU974475	ACG46593	1131	376	<i>Zea mays</i>	Alexandrov et al. (2009)

domain in the N-terminal and an acidic C-terminal domain (Fig. 2a). The AP2 DNA binding domain directly flanked by two special signature motifs, which were used to distinguish the CBF subfamily from other members of the AP2/ERF superfamily (Fig. 2b). The former is a leader sequence of 16 amino acids (PKRPAGRTKFXExRHP) and the latter is the DSAWR motif, with the exception of AenCBFIVd-4 possessing the first amino acid position of an alanine (A) in place of the more common aspartic acid (D). Analysis of all placed CBFs revealed that three distinct group *CBF* genes were present with the C-terminal sequence discrepancies (Fig. 2a, c). As shown in the Fig. 2a, c, the Cluster I includes AenCBFIVd-9, AesCBFIVd-9, AetCBFIVd-9 and AeuCBFIVd-9 with the presence of fragment 2, 3, but the absence of fragment 4. The rest of members from the subgroup CBFIVd-9 belonged to the Cluster II with fragment 1, 2, 3 but no fragment 4. All CBF amino acid sequences from the subgroup CBFIVd-4 fall into the Cluster III with the unique fragment 4. Another

conserved domain is typically present in the C-terminal, the LWSC/Y motif (Fig. 2c). Members of the subgroup CBFIVd-9 (Cluster I and Cluster II) have a preference for cysteine (C) at the end of this motif, compared to the tyrosine (Y) of the subgroup CBFIVd-4 (Cluster III). The phylogenetic relationship, structural conservation and consistent function of these CBF proteins would reflect the close genetic relationship among *Triticeae*.

Identification of *ICE* genes and phylogeny of *ICE* genes in *Poaceae*

As master regulators of *CBF* expression, ICEs bind to the promoter of *CBF* genes, and positively induce the expression of *CBF* genes. We therefore isolated *ICEs* from the above five contextual materials. Firstly, a partial conserved cDNA fragment of 230 bp was produced. Then 5' end (650 bp) and 3' end (360 bp) of *ICE* were obtained. The assembled whole coding sequences of *ICE* genes were verified by several clones and

Table 5 Description of *COR* genes used in the study

Proposed gene name	Gene name	Gene accession	Protein accession	Gene fragment	Protein fragment	Species	References
TaCOR1	TaCOR1	KY931520		515	163	<i>Triticum aestivum</i>	This study
AeuCOR1	AeuCOR1	KY931526		518	164	<i>Aegilops umbellulata</i>	This study
AetCOR1	AetCOR1	KY931532		518	164	<i>Aegilops tauschii</i>	This study
AesCOR1	AesCOR1	KY931538		518	164	<i>Aegilops speltoides</i>	This study
AenCOR1	AenCOR1	KY931544		518	164	<i>Aegilops neglecta</i>	This study
TaCOR2	TaCOR2	KY931519		528	166	<i>Triticum aestivum</i>	This study
AeuCOR2	AeuCOR2	KY931525		525	165	<i>Aegilops umbellulata</i>	This study
AetCOR2	AetCOR2	KY931531		528	166	<i>Aegilops tauschii</i>	This study
AesCOR2	AesCOR2	KY931537		528	166	<i>Aegilops speltoides</i>	This study
AenCOR2	AenCOR2	KY931543		528	166	<i>Aegilops neglecta</i>	This study
AcLEA	AcLEA	JN007028	AEJ88291	630	209	<i>Agropyron cristatum</i>	Unpublished, 2011
AetPHV A1-like	AetPHV A1-like	XM_020294635	XP_020150224	501	166	<i>Aegilops tauschii</i>	RefSeq, 2017
AtCOR15a	AtCOR15a	U01377	AAB87705	420	139	<i>Arabidopsis thaliana</i>	Baker et al. (1994)
AtCOR6.6	AtCOR6.6	X55053	CAA38894	201	66	<i>Arabidopsis thaliana</i>	Gilmour et al. (1992)
EtLEA3	EtLEA3	KJ123698	AIZ11400	600	199	<i>Eremopyrum triticeum</i>	Sun et al. (2014)
HvES2A	HvES2A	X79466	CAA55976	534	177	<i>Hordeum vulgare</i>	Speulman and Salamini (1995)
OsLEA-like	OsLEA-like	NM_001062730	NP_001056195	603	200	<i>Oryza sativa Japonica Group</i> (Japanese rice)	Rice Annotation et al. (2008)
PpLEA3	PpLEA3	GU947647	ADF36679	552	183	<i>Pogonatherum paniceum</i>	Wang et al. (2012)
TaLEA	TaLEA	HQ718763	ADW41578	570	189	<i>Triticum aestivum</i> (bread wheat)	Min et al. (2012)
TaSRP3	TaSRP3	JQ923472	AFN10738	570	189	<i>Triticum aestivum</i> (bread wheat)	Unpublished, 2012
WCOR615	WCOR615	U73217	AAB18208	528	175	<i>Triticum aestivum</i> (bread wheat)	Unpublished, 1996
Wrab17	Wrab17	AF255053	AAF68628	501	166	<i>Triticum aestivum</i> (bread wheat)	Tsuda et al. (2000)
Wrab19	Wrab19	AF255052	AAF68627	540	179	<i>Triticum aestivum</i> (bread wheat)	Tsuda et al. (2000)

sequencing of two independent PCR reactions. We identified 9 potential ICE homologues, and the gene names and GenBank accession numbers were summarized in Table 4. Nomenclature of *ICEs* was similar to the previous description, but group information of phylogenetic analysis was not displayed in the name. These *ICEs* encode proteins ranging from 382 to 443. ICE1s shared higher homology with the published TaICE1 (ACB69501), but ICE2s shared lower homology with the TaICE1. Similarly, ICE2s shared higher homology with the published TaICE2B (ACB69502), but ICE1s shared lower homology with the TaICE87. By further analysis, we found that ICE1s and ICE2s had low homology with the full amino acid sequences for less conservative N-terminal sequences. However, these two groups of proteins are characterized by the presence of the identically C-terminal conserved regions. To get rid of the influence of less conservative N-terminal

sequences, we retained the conserved C-terminal domain to perform the phylogenetic analysis.

Various ICEs from *Poaceae* and two ICEs in *Arabidopsis* were selected to establish the relationship, and these candidate proteins were listed in Table 4. The evolutionary relationship among the ICE proteins showed that these ICEs were divided into 4 groups (Group I, Group II, Group III, Group V) (Fig. 3). Four ICE1s isolated in this study belong to Group I with the published TaICE1 and HvICE2, but five ICE2s isolated in this study belong to Group V with the published TuICE1, AetICE2B and TaICE2B. ICEs from *B. distachyon*, *Oryza sativa*, *Setaria italica*, *Sorghum bicolor* and *Zea mays* were classified into the same cluster, Group II. Two ICEs in *Arabidopsis* fell into Group III. Group I and Group V are the ones that contain ICEs from two *Triticeae* subfamilies (*Aegilops* and *Triticum*), suggesting that the ancestral *Group I* and *Group*

Fig. 1 Phylogenetic analysis of CBF proteins in *Triticeae*. The tree was constructed using the neighbor-joining (NJ) method with a Poisson correction model and 1000 bootstrap replicates. The tree was

V genes were already present before divergence of these subfamilies. Interestingly, ICE1s were distantly relative to ICE2s, and the results indicated that ICE1s may have large sequences differences in the C-terminal regions with ICE2s.

Further analysis of the conserved domains was performed. As other ICE-like proteins, these proteins contain a highly conserved basic helix-loop-helix (bHLH) signature domain, which contains the ICE-specific sequence KMDRASILGDAID/EYLKELL (Fig. S1). These structurally different ICE proteins all possess a predicted SUMO

divided into 11 groups, and the solid points before the sequence names indicate the gene isolated in this study

binding domain, but members of the Group I have a preference for IKEE, compared to VKEE of the other groups.

Identification of *COR* genes and phylogeny of *COR* genes in *Poaceae*

As CBF target genes or effector molecules, *COR* genes have a decisive role in enhancing crop cold tolerance. To dissect potential impacts on the downstream cold responsive genes of CBFs, we isolated 10 *COR*s from the above five contextual materials (Table 5). The details of the gene

(fragment 1, 2, 3, 4), and all CBF proteins contained another conserved domain (LWSC/Y). The length of schemes does not reflect protein size. **b** Details of the N-terminal structure of CBF proteins. All characteristics were marked in the figure. **c** Details of the C-terminal structure of CBF proteins. All characteristics were marked in the figure

Various kinds of CORs from *Poaceae* and two CORs in *Arabidopsis* were chosen to construct the relationship, and 23 total candidate proteins were listed in Table 5. The results indicated that these CORs were assigned into four groups (Group I, Group II, Group III, and Group V) (Fig. 4). Five COR1s deriving from this study belong to Group I with the published Wrab17, TaSRP3, TaLEA, AetPHV A1-like and HvES2A. However, five COR2s

Fig. 3 Phylogenetic analysis of ICE proteins in *Poaceae*. The tree was constructed using the NJ method with a Poisson correction model and 1000 bootstrap replicates. The tree was divided into 4 groups

deriving from this study and the only WCOR615 fell into the same cluster, Group II. CORs from *Pogonatherum paniceum*, *O. sativa*, *Agropyron cristatum*, *Eremopyrum triticeum* and Wrab19 from *T. aestivum* were classified into Group III. The rest of CORs from *Arabidopsis* belong to Group V. Generally, the evolutionary relationship of CORs showed high multispecies specificity. Exceptionally, unlike other members of COR from *T. aestivum* which either belong to Group I or Group II, Wrab19 belongs to Group III. The appearance of being distantly related to the core

group genes stated that the wheat COR genes continued to evolve following the divergence of species.

Sequences variations on proteins from Group I and Group II were performed. Further analysis of amino acid composition showed that these fragments had high hydrophilic amino acid contents, including alanine (A), aspartic acid (D), glutamic acid (E), glycine (G), lysine (K) and threonine (T) (Fig. S2). The maximum of hydrophilic amino acid contents are as high as 70% with the absence of proline (P), cysteine (C) and tryptophan (W).

Fig. 4 Phylogenetic analysis of COR proteins in *Poaceae*. The tree was constructed using the NJ method with a Poisson correction model and 1000 bootstrap replicates. The tree was divided into 4 groups with high multispecies specificity

Expression analysis of *ICE*, *CBF*, *COR* genes

To comprehensively understand the precise mechanism and further details of the ICE–CBF–COR transcriptional cascade in *Triticeae*, expression analysis of the related genes was assessed. Overall, mRNA accumulation was altered by cold treatment (Fig. 5). The changing pattern of three kinds of genes was similar. Without cold treatment, the basal expression of *CBF*, *ICE*, *COR* genes were flat in all four materials. At the preliminary stage of cold treatment (2 h), these genes were not induced by low temperature. With the continuous cold stress, transcript levels of six genes are slightly higher than that without cold induction. After 4 additional hours of cold treatment, the expression of *CBF* and *ICE* genes were significantly elevated in *A. tauschii*, *A. neglecta* and winter wheat M808, compared with the spring wheat China Spring (CS). The expression of *COR* genes after cold treatment for 4 h had no obvious changes in comparison to that for 2 h. Then, the expression of six related genes reached the peak level after 4 °C for 12 h. At this peak point, transcript levels of genes in *A. tauschii* and *A. neglecta* are moderately higher than that in winter wheat. Whereas, we noticed that transcript

levels of six genes were slightly reduced after longer exposure to cold stress (4 °C for 24 h). In *Triticeae*, transcript levels of all genes in spring wheat were the lowest, followed by winter wheat and *A. neglecta*, and transcript levels are the highest in *A. tauschii*. Together, these results indicate that all genes (*ICE*, *CBF*, *COR*) were cold-induced.

Discussion

Wheat is an interesting model species for studying cold tolerance, however as a typical allohexaploid plant, complex genome has limited the further research on its tolerance. *Aegilops*, a progenitor of the allohexaploid wheat, is a close wild relative of common wheat with cold hardiness. *Aegilops tauschii* is D genome donor of common wheat with abundant genetic diversity and many important and excellent genes related to the stress resistance, which has been sequenced with a smaller genome (Jia et al. 2013). Consequently, the *Aegilops–Triticum* composite group is useful for the study and dissection of molecular, transcriptional, and evolutionary components of cold tolerance

Fig. 5 Expression analysis of *ICE*, *CBF*, *COR* genes under cold stress. The Y-axis indicates the normalized fold change in expression of each *ICE*, *CBF*, *COR* genes. The X-axis indicates the cold (4 °C) treatments for 0, 2, 4, 8, 12, 24 h. Error bars represented the standard error of the mean, and the asterisks represented the significance

in cereals. Low temperature induces the expression of many genes, and identifying and comparing the functional domains of key genes involved in the transcriptional cascade are necessary.

To better understand the exact functions and the evolutionary relationships of gene families involved in ICE–CBF–COR pathway during cold stress in the *Poaceae*, we initiated a study to identify and characterize *CBF*, *ICE*, *COR* genes from the *Aegilops–Triticum* composite group and obtained 10 *CBFs*, 9 *ICEs* and 10 *CORs*. Conserved amino acids analysis revealed that all amino acid sequences had typical functional domains. Generally, all CBF amino acid sequences contained an AP2 DNA binding domain and an acidic C-terminal domain (Fig. 2a). The above two features were also generally observed in CBFs from other species, which indicated the possible functional significance. The structural conservation and phylogenetic relationship of the CBF proteins would reflect the close genetic relationship among members of *Triticeae*. However, there existed differences on one of special signature motifs among different protein members, with ASAWR directly flanked of AP2 DNA binding domain of AenCBFIVd-4 (Fig. 2b). Similarly, sequence discrepancies on the CBFs C-terminal with various combinations of four fragments were obvious (Fig. 2a, c). At the end of C-terminal, members of the subgroup CBFIVd-9 (Cluster I and Cluster II) had a preference for LWSC rather than LWSY of subgroup CBFIVd-4 (Cluster III) (Fig. 2c). As transactivation domain, the structural differences described on C-terminal of CBFs in this study may have significant impacts on transcription activities. With less conservative N-terminal sequences of *ICEs*, we cloned *ICE* genes through cloning three fragments respectively (a partial conserved fragment, 5' end and 3' end), and obtained 9 assembled *ICE* sequences. These *ICE* proteins contain a highly conserved bHLH domain, which includes a DNA binding motif and a dimerization motif. The former contributes to binding to the MYC consensus sequence and activates the expression of target genes (Peng et al. 2014), and the latter functions as the formation of homodimers or heterodimers (Mullen et al. 1994; Xu et al. 2014). The *ICE*-specific sequence of 19 amino acids is highly conserved, but has only an amino acid difference between orthologous genes (D/E). Sumoylation and ubiquitination motifs were essential elements and present in most of *ICE* proteins (Miura et al. 2007). Structural differentiation of the predicted SUMO binding domain (IKEE/VKEE) may have impacts on the activity and stability of *ICE* proteins. As downstream effectors, *COR* genes in this study putatively encoded hydrophilic proteins, which are different from the hydrophobic protein Wrab19 ($pI = 10.3$) (Tsuda et al. 2000). It has been revealed that the ICE–CBF–COR transcriptional cascade in cold regulation have differences

among various organs in *Arabidopsis* including pollen, leaves and roots (Chinnusamy et al. 2007; Liao et al. 2014), and we also discovered that structure of the members of the transcriptional networks differ among *Triticeae* subspecies, as well as notable structural differences are displayed by these proteins may have influences on the functions.

On the basis of previous studies on CBFs in *Poaceae*, we further established the relationship among the different CBFs in *Triticeae*, and our results revealed that these CBFs were subdivided into 11 groups with the extra CBFV (Fig. 1). A highly conserved AP2 DNA binding domain were observed among CBFs, which demonstrated that members of CBF families in *Triticeae* should also bind the CRT motif, then promote the expression of target genes. Meanwhile, CBFs belonging to Group V are somewhat distant from other groups with larger sequence discrepancies including AtCBFVa-1, AtCBFVa-2, AtCBFVa-3, HvCBFVa-7, and TmCBFVa-7. CBFs in *Arabidopsis* belong to *Brassicaceae*, which is evolutionarily distant from *Triticeae* and serves as members of outgroup. HvCBFVa-7 and TmCBFVa-7 are highly conserved in AP2 DNA binding domain, but the C-terminal and N-terminal of which are not conserved in comparison to other CBFs. Different *ICEs* from *Poaceae* and two *ICEs* in *Arabidopsis* were divided into 4 groups (Fig. 3). The analysis on multiple conserved domains of *ICE* proteins indicated that these *ICEs* can act on initiating the expression of CBFs through binding the MYC motif. In *Arabidopsis*, different *ICE* proteins are not redundant but have distinct functions (Rahman et al. 2014). As it turns out, *ICE1s* in this study were distantly relative to *ICE2s*, which could imply that the functions of *ICE1s* and *ICE2s* are diverse. Similarly, *CORs* in *Poaceae* and two *CORs* in *Arabidopsis* were assigned into four groups (Fig. 4). Compared with *ICE* and *CBF* proteins, as functional proteins rather than transcription factors, *CORs* displayed greater sequences diversity, which may shed light on important evolutionary trends coupling the functions. Unlike other *CORs* with high multispecies specificity, Wrab19 presented a long genetic distance away from the core group members, showing that the wheat *COR* genes continued to evolve following the divergence of species. Recently, functions of every members involved in this pathway in *Arabidopsis* were explicit, but size and complexity of these genes families in *Triticeae* are enormous without further research on concrete function of every member so far, as well as functional redundancy of genes families.

The diversity or complexity of different plants depends not on genes but on various regulatory networks and the research on regulation of gene expression under low temperature have recently received more attention. Cold tolerance of plants, as a complex quantitative trait, is

regulated by multi-gene, and improvement of single-gene is difficult to completely enhance cold resistance of plants. As a result, this study implies that the ICE–CBF–COR transcriptional networks are likely present in all higher plants and could be a primary and essential component of cereal cold tolerance. After expose to low temperature, all genes in this study involved in the ICE–CBF–COR pathway were cold induced. Under cold conditions, *ICE* genes were induced and activated downstream *CBF* genes through binding the MYC recognition motifs in the promoter, subsequently, the target *COR* genes were accumulated to a higher level of contents. Previous studies had already noted that expression of the upstream regulatory factors occurred before that of the downstream genes in signaling pathways (Lee et al. 2005). In this study, the expression of *COR* genes after cold treatment for 4 h had no obvious changes in comparison to that for 2 h, but the expression of *ICE* and *CBF* genes were slightly elevated during this period, which is consistent with the aforementioned study.

In conclusion, recent studies focus on urgently seeking to improve plant chilling tolerance, and the ICE–CBF–COR transcriptional cascade play a pivotal role. The results showed that the cold stress signaling pathway is conserved in *Triticeae*. To be specific, *ICE* genes were activated under cold stress, then *CBF* genes are induced, and *COR* genes are ultimately induced to enhance plants chilling tolerance after the two-step cascade of transcriptional activators (Thomashow et al. 2001). In *Arabidopsis*, cold-activated OST1 kinase can phosphorylate AtICE1 and modulate chilling tolerance by enhancing ICE1 stability (Ding et al. 2015). PHYA (Benedict et al. 2006) and AtSIZ1 protein (Rahman et al. 2014) also can increase the stability of AtICE1, but JAZ1/4 suppresses transcriptional activity of ICE1 (Hu et al. 2013). Moreover, ICE1/SCRM and ICE2/SCRM2 in *Arabidopsis* are related to stomatal development (Kanaoka et al. 2008). Thus, as a central component in cold signaling pathway or a convergence point integrating cold and other signaling pathways, how ICE1 elaborately modulated covering couple pathways still remains unclear. Cold tolerance is a complex multigenic trait, we should consider it from many aspects of pathways with an aim of extending our understanding on temperature responses in plant, rather than view it as a single entity. In addition, it is notable that how plants response to cold stimulation is unknown. On the whole, this study enriches members of *CBF*, *ICE*, *COR* genes family in *Triticeae*, and provide a starting point for future studies on transcriptional regulatory network for improvement of chilling tolerance in crop.

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