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Structure and Functional Analysis of Wheat *ICE* (Inducer of CBF Expression) Genes

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Two different inducers of CBF expression (*ICE1*-like genes), *TaICE41* and *TaICE87*, were isolated from a cDNA library prepared from cold-treated wheat aerial tissues. *TaICE41* encodes a protein of 381 aa with a predicted MW of 39.5 kDa while *TaICE87* encodes a protein of 443 aa with a predicted MW of 46.5 kDa. TaICE41 and TaICE87 share 46% identity while they share 50 and 47% identity with *Arabidopsis* AtICE1 respectively. Expression analysis revealed that mRNA accumulation was not altered by cold treatment suggesting that both genes are expressed constitutively. Gel mobility shift analysis showed that TaICE41 and TaICE87 bind to different MYC elements in the wheat *TaCBFIVd-B9* promoter. Transient expression assays in *Nicotiana benthamiana*, showed that both TaICE proteins can activate *TaCBFIVd-B9* transcription. The different affinities of TaICE41 and TaICE87 for MYC variants suggest that ICE binding specificity may be involved in the differential expression of wheat *CBF* genes. Furthermore, analysis of MYC elements demonstrates that a specific variant is present in the wheat *CBF* group IV that is associated with freezing tolerance. Overexpression of either *TaICE41* or *TaICE87* genes in *Arabidopsis* enhanced freezing tolerance only upon cold acclimation suggesting that other factors induced by low temperature are required for their activity. The increased freezing tolerance in transgenic *Arabidopsis* is associated with a higher expression of the cold responsive activators *AtCBF2*, *AtCBF3*, and of several cold-regulated genes.

Keywords: Arabidopsis — DNA binding — Freezing tolerance — Promoter — Transcription factor — Transactivation.

Abbreviations: AP2, Apetala2; bHLH, basic helix-loop-helix; CBF, C-repeat binding factor; CRT, C-repeat; CA, cold-acclimated; COR, cold-regulated; DRE, dehydration responsive element; DREB, drought responsive element binding; EMSA, electrophoretic mobility shift assay; EREBP, ethylene responsive element binding protein; ESTs, expressed sequenced tags; FT, freezing tolerance; GFP, green fluorescent protein; HOS1, high expression of osmotically responsive genes 1; ICE, inducer of CBF expression; LTREs, low-temperature-responsive elements; MYB15, Myb domain protein 15; NA, non-acclimated; WT, wild type.

The nucleotide sequences reported in this paper have been submitted to Genbank under accession numbers: *TaICE87* cDNA (EU562184); *TaICE41* cDNA (EU562183); *TaCBFIIIa-6.1* promoter (EU562186); *TaCBFIIIc-3.2* promoter (EU562187); *TaCBFIIIc-B10* promoter (EU562188); *TaCBFIIId-D19* promoter (EU562189); *TaCBFIVd-B9* promoter (EU562190); *TaCBFIVb-D20* promoter (EU562191); *TaCBFIVd-4.1* promoter (EU562185); *TaCBFIVd-D22* promoter (EU562192).

Introduction

Plants have developed strong mechanisms to adapt their cellular metabolism to survive under different stress conditions by undergoing several physiological, biochemical and molecular changes. During cold acclimation, temperate plants increase freezing tolerance (FT) by prior exposure to low, non-freezing temperatures (Guy 1990, Thomashow 1999). During this period of low temperature exposure, the accumulation of several cold-regulated (COR) genes is correlated with the development of FT (Knight et al. 1999, Thomashow 1999). The promoters of several cold and dehydration-responsive genes in *Arabidopsis* have been shown to contain the C-repeat (CRT)/dehydration responsive element (DRE) (Baker et al. 1994, Yamaguchi-Shinozaki and Shinozaki 1994). The DRE/CRT-related motifs have also been reported as low-temperature-responsive elements (LTREs) in the promoters of the *Brassica napus BN115* (Jiang et al. 1996) and the wheat *WCS120* (Ouellet et al. 1998) genes. C-repeat/dehydration-responsive element Binding Factor (CBF)/Drought Responsive Element Binding (DREB) proteins

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are the most characterized transcription factors that bind to the CRT/DRE element and activate cold- and/or drought-responsive gene expression (Stockinger et al. 1997, Gilmour et al. 1998, 2000, 2004, Liu et al. 1998, Shinwari et al. 1998, Thomashow 2001). These transcripts encode transcriptional activators that are members of the Apetala2 (AP2)/ethylene responsive element binding protein (EREBP) family of DNA-binding proteins (Riechmann and Meyerowitz 1998). In *Arabidopsis*, three CBF transcripts designated *CBF1*, *CBF2* and *CBF3* (Jaglo-Ottosen et al. 1998, Medina et al. 1999), or *DREB1b*, *DREB1c* and *DREB1a* respectively (Liu et al. 1998) start to accumulate within 15 min of plant exposure to low temperature. Constitutive overexpression of the *CBF1*/*DREB1b*, *CBF2*/*DREB1c* or *CBF3*/*DREB1a* (Gilmour et al. 2004, Jaglo-Ottosen et al. 1998, Kasuga et al. 1999, Liu et al. 1998) genes in transgenic *Arabidopsis* plants induces the expression of multiple cold-responsive CRT/DRE-containing genes without a low temperature stimulus and enhances FT. However, the mechanism of *CBF*/*DREB1* gene induction in response to low temperature stimuli is not completely understood.

Several reports identified direct regulators of *CBF*/*DREB1* expression, including MYB15, ICE1, and HOS1 (Agarwal et al. 2006, Dong et al. 2006, Chinnusamy et al. 2003, 2006). MYB15 binds to *CBF*/*DREB1* promoter regions to repress its expression and negatively regulate FT (Agarwal et al. 2006). ICE1 (for inducer of CBF/DREB1 expression) physically interacts with the Myb domain protein 15 (MYB15) and may attenuate directly (through binding to *MYB15* promoter) or indirectly (through its downstream genes) *MYB15* expression in response to cold (Agarwal et al. 2006). ICE1 is a MYC-like basic helix-loop-helix transcription factor that activates *CBF*/*DREB1* expression in response to low temperature (Chinnusamy et al. 2003). ICE1 binds to canonical MYC *cis*-elements (CANNTG) in the *CBF3*/*DREB1A* promoter to induce its expression, which then leads to the induction of the *CBF*/*DREB1* regulon (Chinnusamy et al. 2003, Lee et al. 2005). Gilmour et al. (1998) hypothesized that ICE is present in an inactive state at normal growth temperature and becomes active upon exposure to low temperature to stimulate transcription of the *CBF*/*DREB1* genes. The activity of ICE is under the negative control of the HOS1 ubiquitin E3 ligase that targets ICE for proteasome degradation (Ishitani et al. 1998, Lee et al. 2001, Dong et al. 2006). During cold treatment, ICE degradation is prevented by the SIZ1 SUMO E3 ligase which sumoylates AtICE1 at position K393 (Miura et al. 2007). These results indicate that the ubiquitin E3 ligase HOS1 and MYB15 act as negative regulators while the MYC transcription factor ICE1 and SIZ1 act as a positive regulator to modulate expression of *CBF3*/*DREB1A*. *LOS-4* encodes a DEAD-box RNA helicase that appears to have a positive effect on CBF expression since *los-4* mutant plants are chilling sensitive while ectopic expression of CBF3 in these mutants restores the normal phenotype (Gong et al. 2002, 2005). On the other hand, the mutation of ICE1, ice1 results in complete elimination of *CBF3* transcript accumulation (Chinnusamy et al. 2003). Different MYC elements potentially involved in the cold response were also identified in the *CBF2* promoter (Zarka et al. 2003). The consensus sequence CACATG was suggested as a potential binding site for the ICE1 protein. However, the ice1 mutation has little effect on *CBF2* transcript accumulation under low temperature indicating that cold regulation of the different *CBF*/*DREB1* gene family members is independent.

In wheat, Badawi et al. (2007) have identified several *CBF* genes that are subdivided into 10 different groups. Several CBF groups are amplified only in Pooideae and most of these amplified groups (five out of six) are expressed at a higher level in the freezing-tolerant cultivar. These observations raise the question whether wheat possesses specific *ICE*-like genes having the potential to differentially regulate *CBF* genes. A combined bioinformatics, phylogenetic, expression and genetic analysis of wheat *bHLH* genes revealed that two *ICE* genes, named *TaICE41* and *TaICE87*, encode potential activators of the cold-responsive CBF transcription factors in hexaploid wheat. DNA/protein interactions studies in vitro and in vivo demonstrated that the two wheat *ICE* genes bind to different MYC elements and activate the transcription of *CBF* genes. Overexpression of the wheat *ICE* genes enhanced FT in the heterologous *Arabidopsis* system.

Results

Identification and characterization of wheat ICE genes

Data mining of public databases resulted in the identification of several *Arabidopsis* ICE1 homologs from different species including two genes from rice (AK109915 and NM_001074519). Since rice is a monocot relative of wheat, the rice *ICE1*-like genes were used as query to identify wheat expressed sequenced tags (ESTs). Four wheat ESTs (CD900164, BE422944, CA714228, and BJ260527) were used to design forward and reverse primers to clone wheat *ICE1*-like genes from a cDNA library prepared from wheat aerial tissues exposed to short periods of cold-acclimation (Houde et al. 2006). Two different *ICE1*-like genes were amplified and designated *TaICE41* and *TaICE87*. The *TaICE41* cDNA encodes a protein of 381 amino acids with a predicted molecular mass of 39.5 kDa while *TaICE87* encodes a protein of 443 amino acids with a predicted molecular mass of 46.5 kDa. The complete TaICE41 and TaICE87 proteins share 46% identity and have 50% and 47% identity with AtICE1, respectively.

Alignment of TaICE41 and TaICE87 with ICE1 proteins from *Arabidopsis* and the closest homologs from rice and poplar shows that ICE1-like proteins share highly conserved regions in the bHLH domain and in their C-terminal region (Fig. 1). A short region of 11 amino acids (Box I in Fig. 1) is conserved between TaICE87, AtICE1, CbICE53 and Os11g0523700 suggesting that these amino acids may be part of an important domain for ICE activity. The *ice1* mutant of *Arabidopsis* causing a loss of ICE function was found to have a substitution of R236 with H236 in this region (Chinnusamy et al. 2003). The box I is absent from the wheat TaICE47 protein and from a rice homolog (Os01g0928000 in Fig. 1) suggesting that this may represent an *ICE* gene with distinct properties. Although the N-termini of ICE1-like proteins were distinct among plant species, a weakly conserved domain was found preceding the bHLH domain (between Box I and Box II, Fig. 1).

A total of 28 ICE1 homolog sequences were collected from 17 plant species (Tables S1 and S2). The highly conserved region from the bHLH domain to the C-terminal region was subjected to phylogenetic analysis using the closest non-ICE bHLH proteins from *Arabidopsis*, rice and poplar as *outgroup* genes. The different ICE proteins are separated into three major clades (Fig. 2). ICE-like proteins in dicots form one clade while the ICE-like proteins from five monocots species (barley, maize, rice, sugarcane and wheat) form two distinct clades. Although 133 genes were identified as *bHLH* genes in *Arabidopsis* (Heim et al. 2003), only AtICE1 and AtbHLH033 are included in the dicot ICE1 clade (Fig. 2). AtbHLH061 is the closest homolog to ICE1 among the remaining *Arabidopsis* bHLH proteins, but it is clearly separated from ICE1 proteins.

So far, at least two *ICE1*-like genes are localized on distinct chromosomes in *Arabidopsis*, barley, poplar, and rice. The ICE1-like pair of proteins in dicots (i.e. AtICE1/AtbHLH033 in *Arabidopsis* and PtrLGXII1027/PtrLGXV000793 in poplar) show high homology especially in the C-terminal region (89% and 97%, respectively). TaICE87 forms a separate clade with several monocot genes and is classified as ICE1 because it is closer to the dicot ICE1 clade. TaICE41 forms a distinct clade with other monocot genes and is named ICE2. HvICE2 is among this ICE2 clade and was identified as a monocot homolog to AtICE1 that is closely related but distinct from HvICE1 (Tondelli et al. 2006, Skinner et al. 2006). Two other wheat ESTs besides TaICE87 are included in the ICE1-like clade and represent distinct ICE1-like proteins since their sequences possess 74–90% identity between each other and contain one or two gaps in their alignment (data not shown).

Real-time PCR quantification of mRNA expression levels of *TaICE87* and *TaICE41* during cold acclimation was performed using gene-specific primers and TaqMan probes spanning the exon–intron junction. The pattern of mRNA expression was not significantly altered by low temperature treatment in both winter and spring wheat, demonstrating that *ICE1* mRNA expression is constitutive. The relative expression level of *TaICE87* was 1 ± 0.4 at 22°C ($n = 3$) and 1.4 ± 0.3 after 24 h at 4°C in the winter cultivar. A similar result was obtained for the spring cultivar. For *TaICE41*, the relative expression level of both cultivars varied from 1 ± 0.3 at 22°C to 0.8 ± 0.3 after 24 h of cold exposure.

CBF promoter analysis and binding specificity

Analysis of the *TaCBF* genes showed the presence of MYC recognition sequences in their promoters (Fig. 3A). The motif search revealed that the MYC binding sites in wheat and *Arabidopsis CBF* promoters contain all the 16 different possible MYC permutation sequences of the CANNTG core (Table S3). We have subdivided the different MYC elements into four groups according to the third base of the CANNTG core sequence (MYC1 through MYC4). Each group is then subdivided into subgroups according to the fourth base (namely for MYC1: MYC1a, MYC1c, MYC1g, and MYC1t). It should be noted that these sequences are palindromic and that on a double-stranded structure, eight of the MYC variants will be complementary to the other eight sequences. This classification needs to be revised when we better understand the role of the bases adjacent to the MYC element in the binding specificity. Based on this classification, differences in the composition of MYC variants between *CBF* genes in *Arabidopsis* and wheat were observed. The *AtDDF1*, *AtDDF2* and *AtCBF4* are members of the *DREB-A1* subfamily of transcription factors in *Arabidopsis* but are not responsive to low temperature (Haake et al. 2002, Magome et al. 2004). The MYC2 elements are found in two or three copies in the promoter of cold-responsive CBFs (highlighted in blue in Table S3) but are generally absent from the genes that do not respond to low temperature (except for *AtCBF4* which contains one copy of a MYC2 variant). ICE1 from *Arabidopsis* was shown to bind to the MYC2a sequence in the *AtCBF3* promoter and to activate its transcription in transgenic plants (Chinnusamy et al. 2003). Analysis of 19 wheat *CBF* promoter regions revealed that the MYC2a element is present in several promoters, suggesting that it is conserved and plays a role in both wheat and *Arabidopsis*. MYC3 and MYC4g elements are generally absent in *Arabidopsis* (except MYC3a in *AtDDF1*) but found in several wheat promoters. Most interestingly, the MYC4g element is present in most of group IV *CBF* promoters suggesting that this element may have evolved specifically in wheat (Table S3). MYC3 elements are found in all wheat CBF groups and thus are not likely to confer specificity between wheat CBFs.

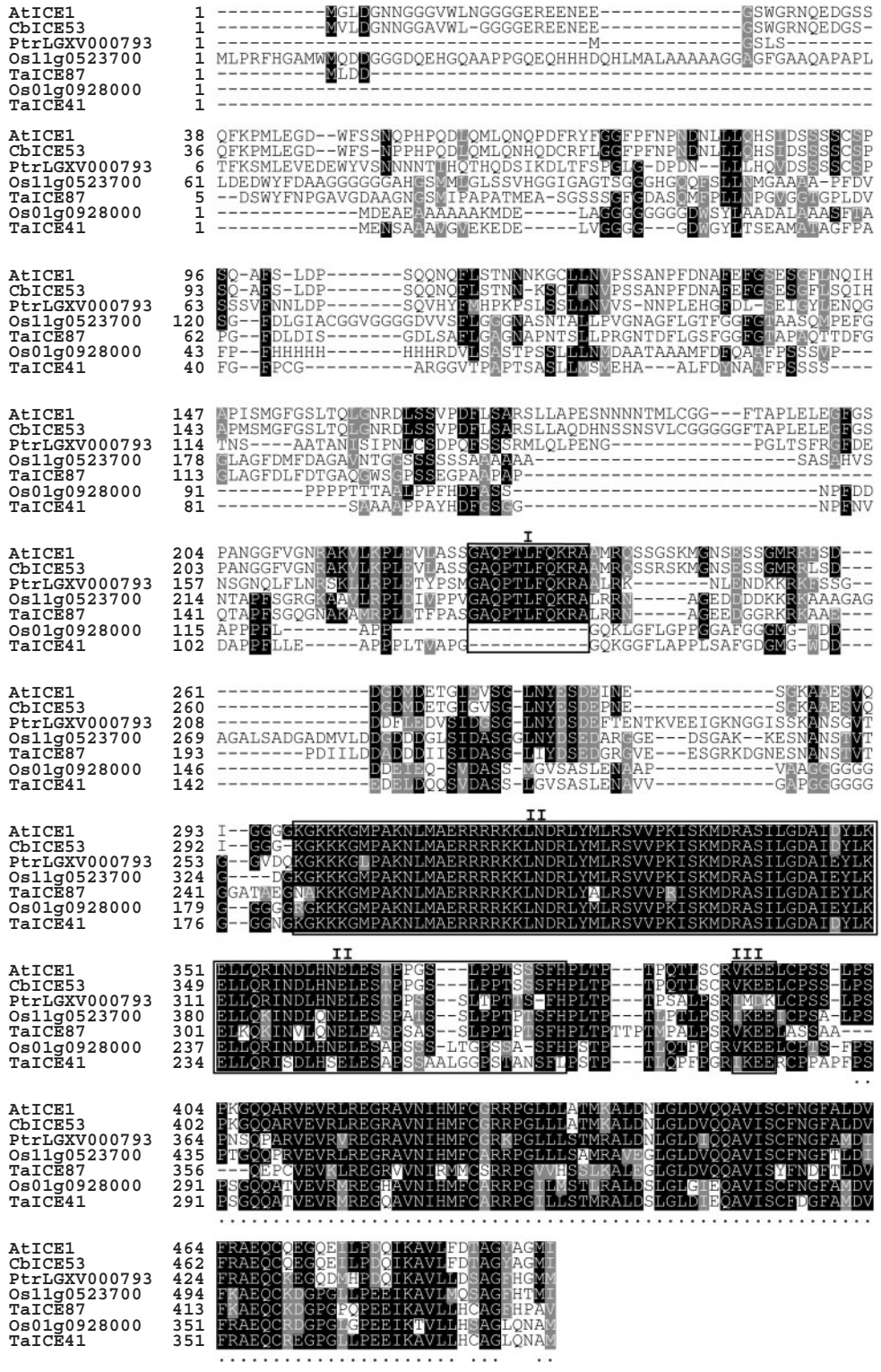

Fig. 1 Sequence alignment and phylogenetic analysis of ICE-like proteins from different species. ICE1 sequences from *Arabidopsis*, *Capsella bursa-pastoris*, rice (one close homolog of TaICE87 and one close homolog of TaICE41), poplar and wheat ICE1-like proteins were aligned using ClustalW. Identical and similar residues are highlighted in black and gray, respectively. A stretch of 11 amino acids (GAQPTLFQKRA) that is totally conserved between TaICE87, AtICE1 and CbICE53 and specifically found in ICE1-like proteins is boxed (box I). The 56 aa bHLH domain and ZIP region are indicated by Box II; and the C-terminal conserved region is indicated by dots. The SUMO conjugation motif is indicated (Box III).

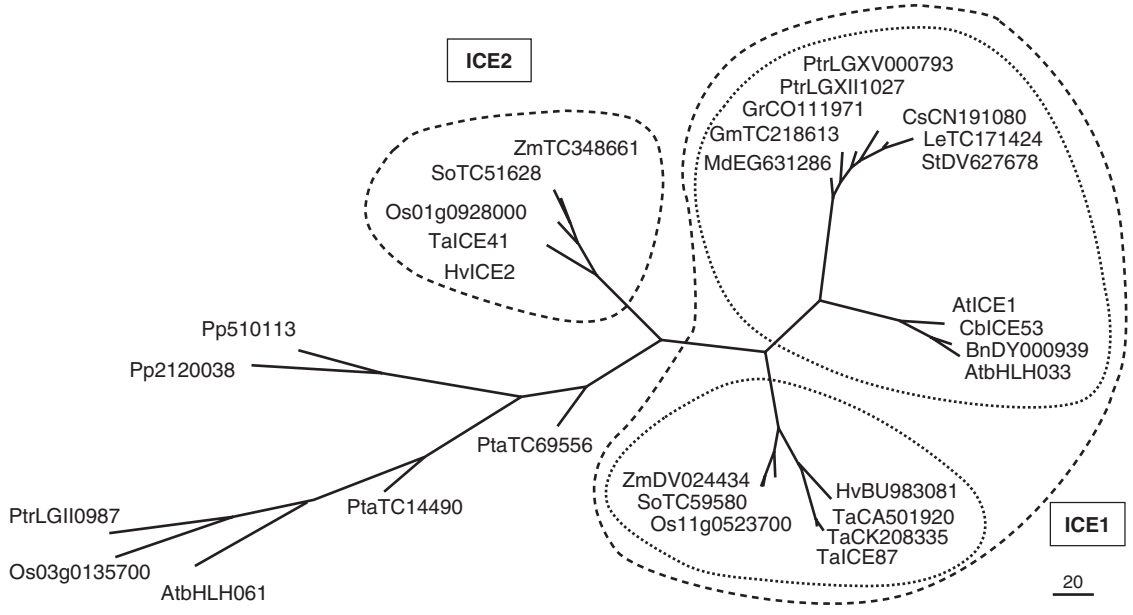

Fig. 2 Phylogenetic tree of ICE1-like proteins from plants. The maximum parsimony tree is constructed using the deduced amino acid sequences of bHLH domain and the conserved C-terminal region. The scale indicates the number of amino acid substitutions between taxons. All the ICE1-like proteins from dicots form a single clade. TaICE87 and TaICE41 are separated into distinct clades with other ICE proteins from monocots. The clade involving TaICE87 is clustered close to the ICE1-like clade from dicots, suggesting that proteins in this clade are ICE1-like monocot proteins. TaICE41 was included in another clade with monocot proteins that diverged from the ICE1-like clade and was named ICE2-like. At: *Arabidopsis thaliana*, Bn: *Brassica napus*, Cb: *Capsella bursa-pastoris*, Cs: *Citrus sinensis*, Gm: *Glycine max*, Gr: *Gossypium raimondii*, Hv: *Hordeum vulgare*, Le: *Lycopersicon esculentum*, Md: *Malus domestica*, Os: *Oryza sativa*, Pp: *Physcomitrella patens*, Pta: *Pinus taeda*, Ptr: *Populus tremula*, So: *Saccharum officinarum*, St: *Solanum tuberosum*, Ta: *Triticum aestivum*, Zm: Zea mays.

Among the isolated wheat promoters, the *TaCBFIVd-B9* promoter is the only one that contains both a MYC2a element and a MYC4g element. Sequence analysis of the 1544 bp *TaCBFIVd-B9* promoter revealed a number of conserved motifs found in several eukaryotic promoters (Fig. 3B). A typical TATA box was recognized at position −144 bp (TATATA) upstream of the ATG translation initiation codon. Potential regulatory elements associated with hormone- and stress-related responses were located within the *TaCBFIVd-B9* promoter region: five MYB binding sites, 14 MYC binding sites, two LTREs, one ABRE-like, one CArG-box binding site for MADS transcription factors and one MeJA-like element.

Since MYC2a was the preferred MYC element bound by AtICE in *Arabidopsis* and since it is present in the promoter of several wheat *CBF* genes, this element was used for electrophoretic mobility shift assay (EMSA). Furthermore, the MYC4g element was selected because it is specifically found in the wheat CBF Group IV genes which are expressed at higher levels in a freezing tolerant cultivar (Badawi et al. 2007). Also, the six bases of MYC4g are complementary to MYC2a (palindrome) indicating that the double-stranded sequence will contain MYC2a in the opposite direction. Among 14 MYC-recognition core elements that were found within the 1544 bp of *TaCBFIVd-B9* promoter (Fig. 3B and Table S3), one MYC2a, two MYC4g and 11 other MYC elements were identified. The MYC2a and the two different MYC4g sequences including the adjacent bases (MYC4g1 and MYC4g2, in Fig. 3B) were used to design DNA probes for EMSA studies (Fig. 4). Purified TaICE87 did not bind to MYC2a (Fig. 4B, panel I) while it bound to MYC4g1 and MYC4g2 elements (Fig. 4B, panels II and III). Competition with unlabeled MYC4g (1 or 2) completely inhibited the binding of TaICE87 to its target (Fig. 4B, panels II and III). In contrast, TaICE41 bound specifically to MYC2a (Fig. 4C, panel I) but not to MYC4g1 or MYC4g2 (Fig. 4C, panels II and III). Taken together, TaICE87 and TaICE41 have preferences in binding to MYC elements. Since MYC4g is complementary to MYC2a, the third and fourth bases of the MYC element are not critical for binding specificity. It also suggests that bases outside the MYC elements are important for binding specificity.

TaICE87 *and* TaICE41 *transactivate the wheat TaCBFIVd-B9 promoter*

The ability of the two wheat ICE proteins to activate the wheat *TaCBFIVd-B9* promoter was determined using the transient expression system of Agroinfiltration of

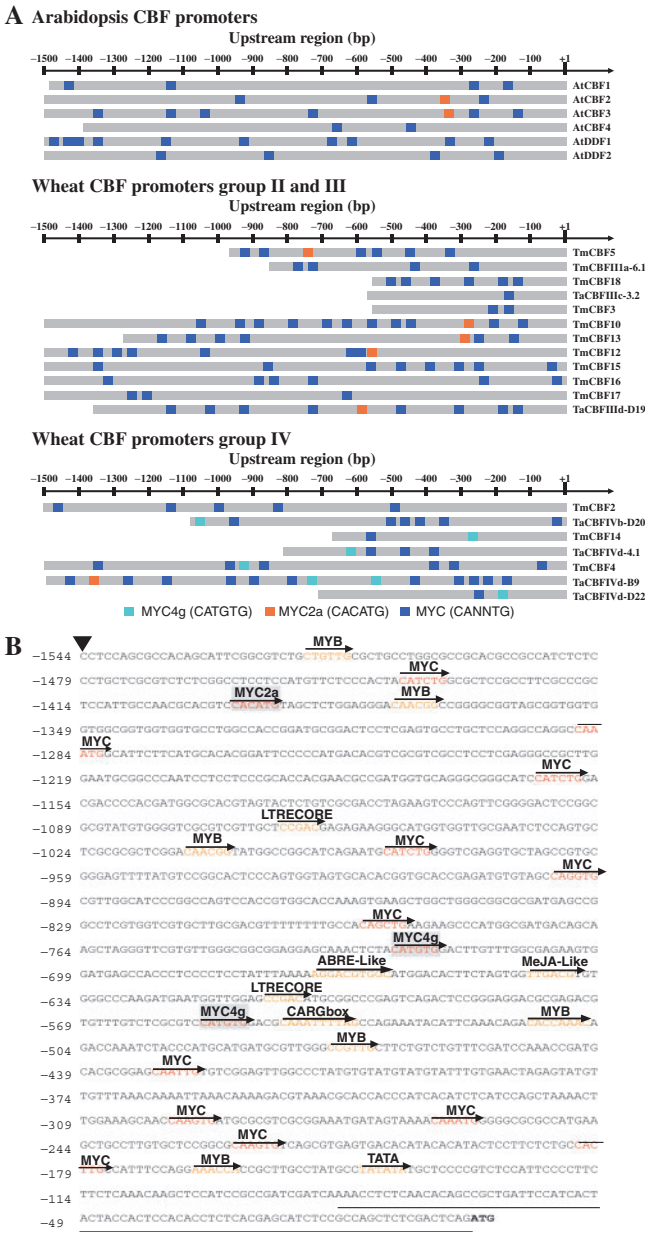

Fig. 3 Distribution of MYC elements in different CBF promoters and structure of the *TaCBFIVd-B9* promoter in wheat. (**A**) Mapping of MYC elements (CANNTG) in representative CBF promoters from *Arabidopsis* or from wheat Group-II, Group III and Group IV genes showing the relative position and distribution of two previously identified MYC elements (CACATG: MYC2a; and CATGTG: MYC4g). (**B**) Nucleotide sequence of 5′-flanking promoter region and putative *cis*-acting elements of the *TaCBFIVd-B9* gene. The 5′ untranslated region is underlined. The plant CARE and PLACE programs were used for promoter analysis.

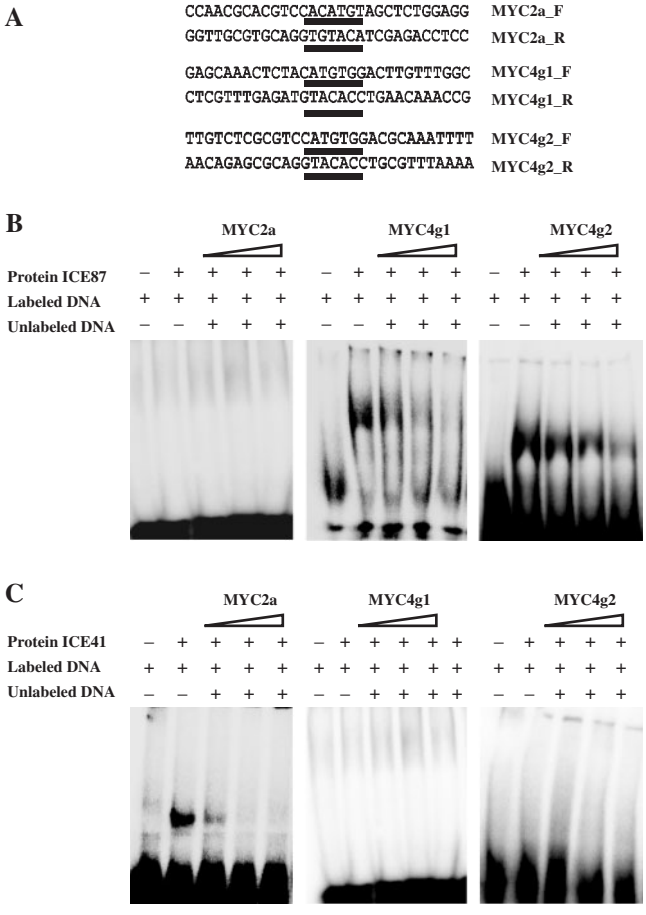

Fig. 4 DNA-binding affinities of the recombinant TaICE87 and TaICE41 proteins to different MYC elements of the *TaCBFIVd-B9* promoter. (**A**) Double-stranded oligomers of MYC2a, MYC4g1 and MYC4g2 used as probe in the DNA-binding assays. (**B**) and (**C**) Electrophoretic mobility shift assays. Gel mobility shift assay showing a different binding affinity between the recombinant TaICE87 (B) and TaICE41 (C) proteins and the MYC2a, MYC4g1 and MYC4g2 sequences. HIS-tagged recombinant proteins were incubated with or without competitors on ice for 20 min. The ^{32}P-labeled probe was then added and the mixture incubated at 25°C for 30 min. The labeled MYC fragment used in each experiment is indicated at the top of each panel. Triangles indicate increasing amounts of unlabeled competitor which corresponds to 50-, 100- and 500-fold excess of each probe.

Nicotiana benthamiana plants. The plasmid pGreenII0029 containing the *CaMV35S* promoter (PGII) was used to overexpress TaICE87 or TaICE41, while a reporter plasmid was constructed to express the green fluorescent protein (GFP) under the control of the *TaCBFIVd-B9* promoter (CBFp) (Fig. 5A). Control experiments with non-infected plants or plants infected with the empty vector (PGII vector) showed no green fluorescence using a UV hand lamp (Fig. 5B, panel 1) or using confocal microscopy (Fig. 5B, panels 2 and 3). Similarly, plants infected with CBFp and treated at 24°C did not show any GFP fluorescence in whole plants (Fig. 5B, panel 4) or under the microscope (Fig. 5B, panel 5). However, plants infected with CBFp and treated at 4°C showed no visible fluorescence under the UV hand lamp (not shown) but

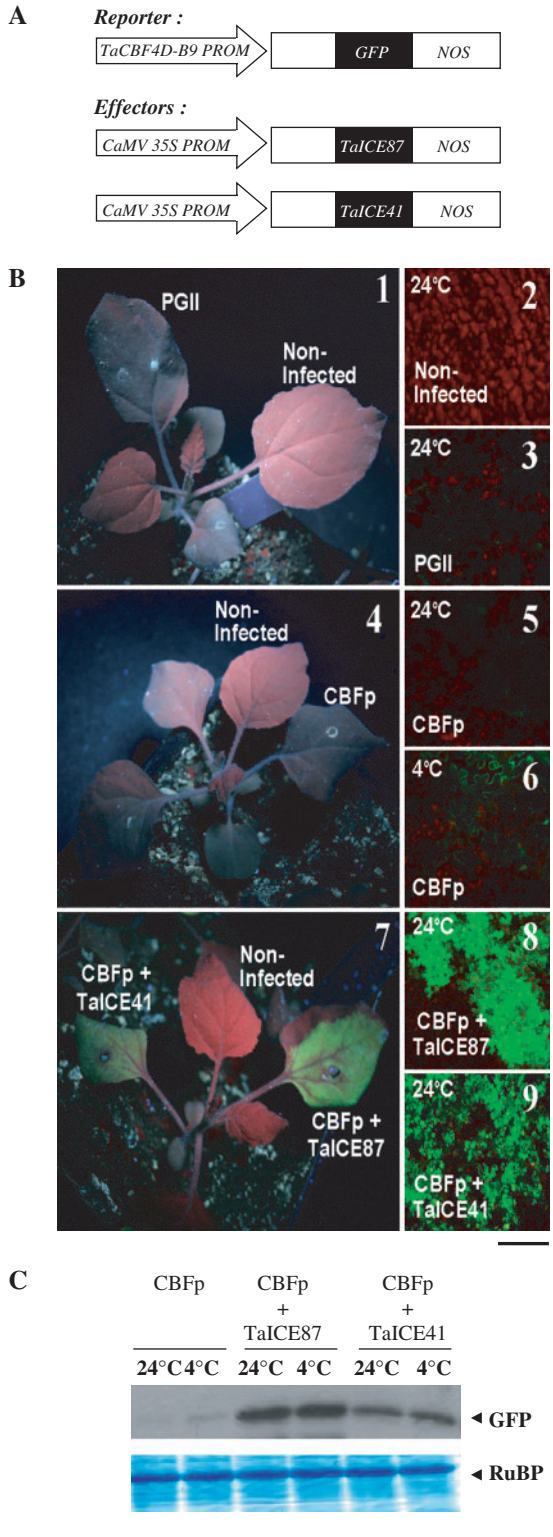

Fig. 5 Transactivation of the *TaCBFIVd-B9* promoter-GFP fusion gene by the TaICE87 or TaICE41 proteins. (**A**) Reporter and effector constructs used in the transient assays. CBFp: GFP reporter gene under the control of the wheat *TaCBF4D-B9* promoter; *TaICE87* or *TaICE41*: *TaICE87* or *TaICE41* cDNA under the control of the *CaMV35S* promoter; *GFP*: green fluorescent protein; *NOS*: nopaline synthase terminator. (**B**) Transactivation experiments in *Nicotiana benthamiana*. Intact leaves were infiltrated with *Agrobacterium* strains carrying the CBFp construct with or without ICE effectors. Panels 1, 4 and 7: whole plants exposed to a UV hand lamp. Panels 2, 3, 5, 6, 8 and 9: GFP fluorescence detection by laser scanning confocal microscopy in leaf epidermal cells 7 d post-infection. Non-infected: normal leaf without *Agrobacterium* infection; PGII: leaf infiltrated with the pGreenII0029 vector containing the *CaMV35S* promoter only; CBFp: reporter constructs (GFP under the control of the wheat CBF promoter); GFP + TaICE87: co-infection with the reporter and *TaICE87* effector constructs; GFP + TaICE41: co-infection with the reporter and *TaICE41* effector constructs. Bar: 60 µm. The data shown are representative of at least three independent experiments ($n = 16$ plants). (**C**) Immunoblot analysis of GFP protein accumulation in the Agroinfiltrated *Nicotiana* leaves. Soluble proteins were separated by SDS-PAGE, transferred to PVDF and probed with the anti-GFP antibody. The different constructs are described in A. A Coomassie brilliant blue stained gel is shown as loading control. The same results were obtained with proteins extracted from three independent experiments.

a weak GFP-fluorescence was observed under the microscope indicating that tobacco can weakly respond to low temperature and activate the wheat CBF promoter (Fig. 5B, panel 6). When plants were co-infected with CBFp and either of the effector genes (*TaICE87* or *TaICE41*), a strong GFP expression was clearly seen in the whole leaf using a UV hand lamp (Fig. 5B, panel 7) and a strong fluorescence is seen in epidermal cells under confocal microscopy at 24°C (Fig. 5B, panels 8 and 9) or at 4°C (data not shown). These results demonstrate that both wheat *ICE* genes strongly transactivate the *TaCBFIVd-B9* promoter in *Nicotiana benthamiana*. Immunoblot analysis of GFP expression showed that TaICE87 was more efficient in transactivating the *CBF* promoter compared to TaICE41 (Fig. 5C). Taken together, these results showed that both TaICE87 and TaICE41 proteins function as transcription activators of the *TaCBFIVd-B9* promoter.

TaICE41 and TaICE87 overexpression enhances freezing tolerance in Arabidopsis

The effect of overexpressing *TaICE87* and *TaICE41* on FT were evaluated in the non-acclimated (NA) and cold-acclimated (CA) transgenic and control plants exposed to a freezing temperature of –6.5°C or –10.5°C, respectively. Plant survival was scored after 2 weeks of recovery under normal growth conditions, and representative results are shown (Fig. 6A and 6C) for *TaICE87* and *TaICE41*, respectively. No difference in FT was observed between the wild-type (WT) and PBI-transformed plants (control), or with the transgenic lines overexpressing *TaICE87* or *TaICE41* gene under NA conditions. However, after cold acclimation, the overexpression of *TaICE87* or *TaICE41* gene resulted in increased FT compared to the WT or PBI (Fig. 6B and 6D).

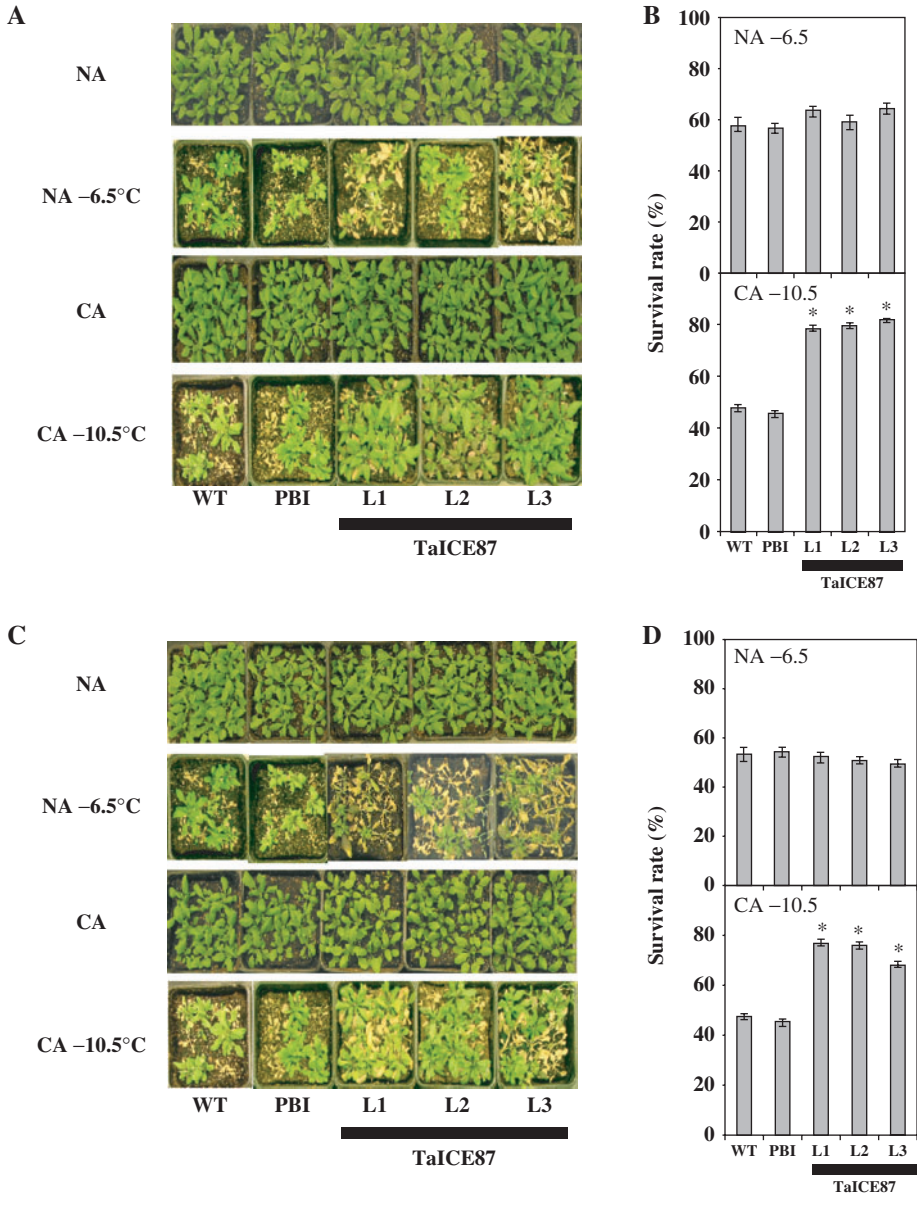

Fig. 6 *TaICE87* and *TaICE41* enhance tolerance of *Arabidopsis* to freezing stress. (**A** and **C**) Plants were grown for 3 weeks at 22°C (NA) or grown at 22°C then transferred at 4°C for 7 d (CA). Wild-type plants (WT), *pBI121* transformed plants (PBI, control) and three lines overexpressing *TaICE87* (A) or *TaICE41* (C) were used in this experiment. Pictures were captured before the freezing test (NA, CA) and the same plants were subjected to freezing (NA frozen to –6.5°C and CA frozen to –10.5°C) and allowed to recover for a period of 2 weeks. (**B** and **D**) Survival rate after freezing stress is expressed as a percent of surviving plants (from A and C respectively). Statistical analysis was performed by one-way ANOVA, and the asterisks (*) indicate differences that are significant at the $P<0.001$ level.

The overexpression of *TaICE87* and *TaICE41* resulted in the greater accumulation of the cold responsive genes, *AtCBF2*, *AtCBF3*, *COR15a* and *COR47* compared to WT or the PBI vector (control) (Fig. 7A and 7B). The *AtCBF1* expression was more variable and showed no clear difference in expression between the controls (WT and PBI) and the *TaICE* (*TaICE87* or *TaICE41*) overexpressing lines. The *AtCOR6.6* gene was more upregulated after 48 h in the transgenic lines overexpressing *TaICE87* but not *TaICE41*. The most striking differential induction between the controls and the *TaICE* overexpressing lines was observed with *AtCOR47*. These data demonstrate that *TaICE87* and *TaICE41* are functional homologs of *AtICE* genes that can function in the heterologous *Arabidopsis* system even though they have different binding specificities.

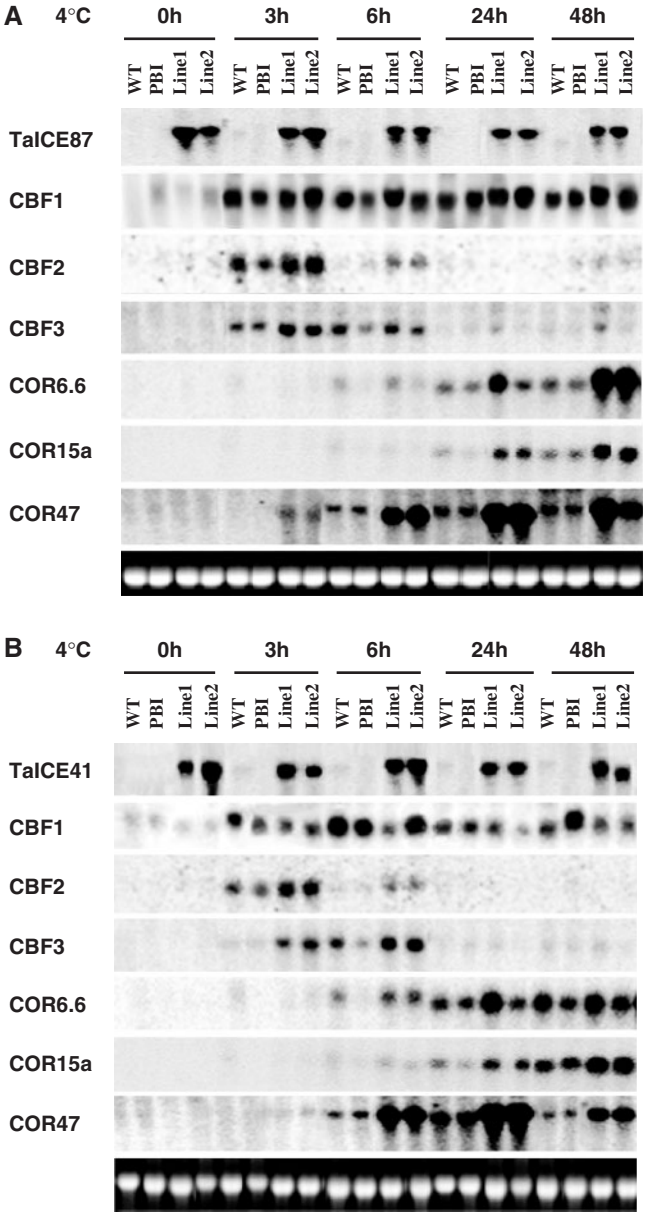

Fig. 7 Effect of *TaICE87* and *TaICE41* overexpression on the accumulation of cold-regulated transcripts. Total RNA was extracted from leaves of 15-day-old transgenic *Arabidopsis* plants grown under LD conditions, and exposed at 4°C for 3–48 h. Transcript levels were measured by Northern blot. (**A**) *TaICE87* overexpressing lines. (**B**) *TaICE41* overexpressing lines. Two independent lines overexpressing *TaICE87* or *TaICE41* were used in this experiment. Each experiment was repeated three times using RNA prepared from two biological samples with similar results. WT, wild-type Columbia *Arabidopsis*; PBI: *pBI121* transformed plants as control.

Discussion

We isolated and characterized two functional wheat *ICE*-like *bHLH* genes that are candidate regulators of *CBF* gene expression and FT in wheat. Sequence comparison showed that TaICE87 and TaICE41 shared high amino acid identity with *Arabidopsis* ICE1 transcription factors, particularly in the bHLH domain and the C-terminal region. Phylogenetic analysis of 28 ICE1 homologs from monocot and dicot species revealed the presence of three major clades. One ICE1 clade was found in eudicots while two ICE clades were identified in monocots suggesting that *ICE*-like genes have diverged from an ancestral *ICE* gene. The presence of two distinct clades in monocots suggests that these ICE proteins may have different properties. The TaICE87 protein is classified in the ICE1 clade because it is phylogenetically closer to the eudicot ICE1 clade while the TaICE41 protein is in a distinct clade which was named ICE2. One of the major differences between the two ICE clades is the presence of additional amino acids towards the end of Box II (near aa 371 of AtICE1) that modifies the conserved LPPT sequence, and the absence of Box I in the *ICE2* genes (shown only for TaICE41 and OS01g092800 in Fig. 1).

The overexpression of either *TaICE87* or *TaICE41* genes in *Arabidopsis* enhanced FT only after cold acclimation suggesting that other factors induced by low temperature exposure are needed. This is consistent with the result obtained by Chinnusamy et al. (2003) who showed that cold-induced modification of the AtICE1 protein or of a transcriptional cofactor may be necessary for AtICE1 to activate the expression of CBFs. Furthermore, Miura et al. (2007) showed that sumoylation of AtICE1 by SIZ1, a SUMO E3 ligase, plays a role in its activation and/or stability. A potential sumoylation site was found in both TaICE41 and TaICE87 proteins using SUMOplot (Minty et al. 2000) (Fig. 1). Since the sumoylation site is conserved between AtICE1, TaICE87 and TaICE41, it suggests that TaICE41 and TaICE87 activity could be regulated by sumoylation via the SUMO E3 ligase.

AtICE1 binds to MYC recognition sites in the *AtCBF3* promoter to induce its expression but has a minimal effect on the expression of *AtCBF1* and *2* (Chinnusamy et al. 2003). We have found that TaICE87 and TaICE41 activate both *AtCBF2* and *AtCBF3* suggesting that the wheat ICE proteins may have a broader spectrum of affinities than AtICE1 to bind MYC elements in the *Arabidopsis* promoters. The presence of several MYC variants in these cold-responsive *CBF* promoters may provide appropriate binding sites for the wheat ICE proteins. Since AtICE1 was shown to regulate only *AtCBF3*, other AtICE-like proteins may be involved in the regulation of the other COR CBF in *Arabidopsis*. The *AtbHLH033* gene which is found within the eudicot ICE1 clade contains a Box I that is well conserved (except for one amino acid) but has a short deletion of five amino acids that covers the LPPT sequence which is well conserved between AtICE1 and TaICE87 towards the end of Box II. This deletion may modify the

binding specificity of this ICE-like protein. However, further studies will be required to characterize the role of the different protein regions in relation to the binding specificity of ICE proteins. The presence of a MYC2a element in the promoter of *AtCBF2* (Table S3), which is not activated by AtICE1 (Chinnusamy et al. 2003), suggests that upstream and downstream sequences surrounding MYC2a might have a significant effect on the binding affinity of AtICE1. Furthermore, analyses of the MYC element composition of the *DREB-1A* promoters show that the MYC2c variant is present only in the promoter of *CBF3* (Table S3) suggesting that this variant may be important to increase the binding strength of AtICE1 or other ICE-like proteins for cold activation. The MYC2c variant has not been tested in previous studies and quantitative analysis of the binding affinity (Xue 2005) will be needed to compare the different MYC elements. Similarly, there are three different MYC4 elements in the *AtCBF3* promoter while there is only one in *AtCBF1* and *AtCBF2*. The presence of these elements (and surrounding DNA sequence) may provide more specific binding to AtICE1. In the same manner, the greater specificity of TaICE87 to bind wheat MYC4g rather than wheat MYC2a and its ability to activate *AtCBF3* and *AtCBF2* may indicate that MYC4 or other MYC variants may be able to recruit the wheat TaICE87 protein to activate the transcription of *AtCBF3* and *AtCBF2*. The ability of TaICE41 to bind to wheat MYC2a and its ability to activate both *AtCBF2 and AtCBF3* promoters suggest that TaICE41 may bind to different MYC elements than AtICE1. Detailed quantitative characterization of the binding affinities for the different MYC variants will be required to understand the difference in binding specificities between wheat and *Arabidopsis* ICE proteins. Overall, overexpression of *TaICE87* or *TaICE41* in the heterologous *Arabidopsis* system showed that the two wheat genes are functional homologs of AtICE1 with different properties.

Analysis of the wheat *CBF* promoters indicated that the *TmCBF* and *TaCBF* genes also contain several MYC recognition sequences. Transient expression assays in tobacco showed that TaICE87 or TaICE41 activate the *TaCBFIVd-B9* promoter. *In vitro* binding assays showed that TaICE87 can bind to two different oligomers containing MYC4g motifs in the *TaCBFIVd-B9* promoter while it could not bind the MYC2a element. In contrast, TaI CE41 binds to MYC2a rather than MYC4g elements. This demonstrates that TaICE87 and TaICE41 can act as direct activators of *TaCBFIVd-B9* expression in distinct ways. These results suggest that at least two different ICEs can activate *CBF* genes in wheat. Without the upstream gene sequence for all the *TaCBF* genes and extensive EMSA studies to test all the potential MYC binding sites, it would be premature to speculate as to the preference of TaICE87 or TaICE41 proteins for the different *TaCBF* genes. However, our survey of potential MYC recognition sites in wheat *CBF* promoters revealed that these promoters are enriched in specific MYC element subtypes supporting the potential for differential regulation of *CBF* genes by the two *TaICE* genes. In particular, the MYC2 elements are rarely found in the *TaCBF* group IV promoters while the MYC4g is found exclusively within this group. Furthermore, the MYC3 element is underrepresented in COR *Arabidopsis CBF*s while they are found in most wheat *CBF* promoters suggesting that these elements may be important for wheat *CBF* transcription. Although our previous work has shown that specific *CBF* genes are much more expressed in the cold-hardy winter wheat compared to less hardy spring wheat (Badawi et al. 2007), it is difficult to make any correlations between the level of expression of the different *CBF* genes in winter/spring cultivars regarding the role of particular MYC sequences. Phylogenetic analysis showed that wheat Group II and Group III *CBF* genes are closer to *AtCBF*, compared to the Group IV which were amplified more recently in Pooideae (Badawi et al. 2007). This amplification of *CBF* genes in wheat appears to be associated with a specialization of TaICE function. The impact of this specialization increases the complexity of the regulatory network but may also be a redundant mechanism that increases the robustness of the cold-acclimation process in the hardy winter wheat. The production of transgenic wheat overexpressing the different *TaICE* genes will be required to compare their ability to activate different wheat *CBF* genes. Alternatively, the isolation of additional wheat *CBF* promoters from the different groups and the quantitative analysis of promoter efficiency using the identified MYC elements will help to evaluate the ability of the different ICE proteins to specifically bind to particular *CBF* promoters. Transactivation experiments will also be useful to document the specificity of *CBF* gene regulation. The cloning of *CBF* promoter regions from winter and spring wheat within the *CBF* groups that are more expressed in winter wheat may reveal subtle differences in MYC element composition that contribute to the differences observed in their expression (Badawi et al. 2007).

In conclusion, we describe here the cDNA cloning and functional characterization of two ICE-like transcription factor genes, *TaICE87* and *TaICE41*. Both proteins possess a conserved bHLH domain and are constitutively expressed. The DNA-binding domain of TaICE87 and TaICE41 interacts with MYC2a and MYC4g respectively in the *TaCBFIVd-B9* promoter *in vitro* and activate its transcription. Their overexpression in *Arabidopsis* activates CBF transcription and enhanced FT. This is consistent with their putative role in wheat.

Materials and methods

Plant material and growth conditions

Two varieties of hexaploid wheat (*Triticum aestivum* L.), a spring cultivar (Manitou) and a winter hardy cultivar (Norstar), were grown in environmentally controlled growth chambers as previously described (Danyluk et al. 2003).

Cloning of cDNAs and promoters isolation

cDNAs were isolated from winter wheat Norstar as described previously (Danyluk et al. 2003, Kane et al. 2005). For promoter isolation, several genomic *T. aestivum* libraries were constructed using the Genome Walker Kit (CLONTECH, Palo Alto, CA) according to the manufacturer's instructions. The wheat promoter regions were PCR-amplified from genomic DNA using a *CBF* gene-specific primer (first primer described in Table S4) with the AP1 primer followed by a second PCR (nested PCR primer described in Table S4) with the AP2 primer. Promoter sequences were then analyzed using Plant-CARE (Lescot et al. 2002), Genomatix Promoter Database (http://www.genomatix.de/products/GPD/index.html), and PLACE (Higo et al. 1999).

Search for ICE1 sequences and phylogenetic analysis

The protein sequence of TaICE87, TaICE41 and *Arabidopsis* ICE1 were used as query sequences for TBLASTN analyses to collect *ICE1*-like genes in plants. The survey was conducted against the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/), the TIGR (http://compbio.dfci.harvard.edu/tgi/plant.html), the Rice Annotation Project DataBase (http://rapdb.lab.nig.ac.jp/index.html), the JGI *Physcomitrella patens subsp patens* v1.1 (http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html), and the JGI *Populus trichocarpa* v1.1 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) databases. Genes and accession numbers are listed in Table S1. The sequences were translated and protein sequences were aligned with ClustalW. Character-based parsimony analysis was used for phylogenetic analyses in PAUP 4.0 (Swofford 2003). Since *ICE1* in *Arabidopsis* belongs to the bHLH domain family, three non-*ICE bHLH* genes from *Arabidopsis*, poplar and rice were used as outgroup (Table S1).

Electrophoresis mobility shift assays (EMSA)

The full length *TaICE87* and *TaICE41* coding regions were cloned in the pENTR4 vector then transferred to the pDEST15 vector by recombination using the Gateway technology (Invitrogen; for primers, see Table S4). The resulting plasmids pDEST15-*TaICE87* and pDEST15-*TaICE41* were independently transformed into *Escherichia coli* BL21-A1 to express GST-fusion proteins. The proteins were purified on GST-Bind affinity resin (Novagen) and used in EMSA to determine their binding affinity towards the *TaCBF* promoter MYC elements. Sense and complementary oligonucleotides (Fig. 4A) corresponding to MYC elements were annealed and radiolabeled with [γ-^{32}P]ATP (Amersham) to generate double-stranded probes. DNA-binding reactions were performed in a total volume of 20 µl of buffer (10 mM Tris–HCl pH 7.5, 4% glycerol, 20 mM KCl, 20 mM dithiothreitol) containing 1 µM of poly(dI-dC), 0.2% (v/v) Triton X-100, 2 ng ($\sim 5 \times 10^4$ CPM) of probe, and 100 ng of the recombinant GST-tagged proteins. The binding specificity was assessed by competition with a 50-, 100- and 500-fold excess of unlabelled double-stranded oligonucleotides. Binding reaction mixtures were incubated for 15 min at room temperature and then resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel, prepared in 0.5 × TBE, at 100 V for 90 min. The gels were dried, exposed to K screens and the signal was detected with a Personal Molecular Imager FX System (Bio-Rad).

Transient expression by Agroinfiltration of Nicotiana benthamiana

Three binary constructs based on pBin19 or pGreenII0029 were generated for transient expression assays. The primers used for cloning are indicated in Table S4. The CBFp:GFP reporter plasmid contained the *m-GFP5-er* gene under the control of the *TaCBFIVd-B9* promoter while the effector constructs contained the *TaICE41* or *TaICE87* genes under the control of the *CaMV35S* promoter (*35S:TaICE87* and *35S:TaICE41*). The plasmids were independently transformed into *Agrobacterium tumefaciens* strain EHA105. The transformed Agrobacteria were used individually or in combination to infiltrate intact leaves of *N. benthamiana* as described by Kane et al. (2007). After infiltration, plants were kept at 24°C for a 3-d recovery period. Plants were then maintained at 24°C or exposed at 4°C for an additional 2 d (cold treatment). GFP expression was evaluated using a 100 W hand-held long-wave ultraviolet lamp (UV products, Upland, CA, Black Ray model B 100AP) or using a confocal system (Bio-Rad MRC1024) with a Nikon Eclipse TE300 inverted microscope, and analyzed using the LaserSharp software (Bio-Rad). The accumulation of GFP was confirmed using an anti-GFP antibody (Clontech).

Overexpression of TaICE87 and TaICE41 in Arabidopsis

The *35S:TaICE87* and *35S:TaICE41* constructs (Fig. 5A and Table S4) were used for the transformation of *Arabidopsis thaliana* ecotype Columbia by floral-dipping (Clough and Bent, 1998). Transformants were selected on medium containing MS salts and vitamins supplemented with 50 mg liter^{-1} kanamycin, and resistant T2 seedlings

were transferred to soil and grown to seed under LD conditions (16h photoperiod) at 24/20°C (day/night). Wild-type Columbia and plants transformed with *pBI121* (PBI: *35S*:GUS) were used as controls.

Molecular analyses

Genomic DNA and total RNA were isolated from aerial parts (wheat), or leaves (*Arabidopsis*) using DNAzol or TRIzol reagents following the manufacturer's instructions (Invitrogen). For northern blot analysis, 10 μg of total RNA were separated on formaldehyde–agarose gels, transfered to nylon membranes and hybridized with ^{32}P-labeled probes. Probes for the different *CBF*s and *COR* genes were amplified using specific primers (Table S4).

Determination of freezing tolerance

A Caltec Scientific Ltd. Model 8-792 Large Capacity Temperature Stress Chamber was used to perform the FT tests. This instrument consists of four major component systems: a Sanyo Model MDF-792 24.75 ft^3 capacity ultra-low temperature chest freezer, a custom designed stainless steel plenum box with its integral blower and heater (provides air circulation and heating) and an Omega Engineering Inc. Model CN3002 programmable profile controller (monitors the test-chamber air temperature). The controlled action of the heater combines with the constant cooling of the freezer to achieve the desired temperature at any given time.

NA and CA soil-grown plants (3 weeks old) were subjected to the following freezing treatment. The temperature was lowered gradually (2°C h^{-1}) to –6.5°C for NA or –10.5°C for CA plants and maintained at this temperature for 6 h. The temperature was then gradually increased (2°C h^{-1}) to 4°C. To determine temperature variability in the freezer, temperatures were monitored by four independent thermocoupled T probes distributed in the freezer and connected to an Agilent 3497-0A data acquisition/switch unit. Freezing regimes that showed more than 0.5°C discrepancies between the different probes were rejected. To minimize light stress effects after the freezing treatment, plants were thawed at 4°C for 24 h in the dark and away from direct light in the growth chamber (20°C) for an additional 24 h before returning to normal light conditions. Representative pictures were taken 2 weeks after the freezing treatment. Eighteen plants were frozen per line per assay, and the experiment was repeated three times.

Supplementary data

Supplementary data mentioned in the article is available at *Plant and Cell Physiology* online.

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