Differential and coordinated expression of Cbf and Cor/Lea genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance

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The cold acclimation process in plants is primarily regulated through the signal transduction pathways that lead to the induction and enhancement of expression of different sets of Cor/Lea genes. Winter wheat ‘Mironovskaya 808’ (M808) exhibited a much higher level of freezing tolerance than spring wheat ‘Chinese Spring’ (CS), and the difference became clearer after the long-term cold acclimation. To understand the molecular basis of this cultivar difference, we isolated two CBF/DREB1 homologs, Wcbf2, which are the candidate gene for a transcription factor of the Cor/Lea genes. Expression of the Wcbf2 gene was induced rapidly by low temperature (LT) and drought but not by abscisic acid (ABA). The gene expression was temporal and at least twice up-regulated by LT. The first up-regulation occurred within 1–4 h, which might correspond to the rapid response to LT, while the second up-regulation occurred during 2–3 weeks of cold acclimation. After the second up-regulation, the amount of Wcbf2 transcript greatly decreased in CS, while it increased again in M808 after 4 weeks until 9 weeks (end of the test period). The maintenance of this high level of the Wcbf2 transcript might represent the long-term effect of cold acclimation. The activation of Cor/Lea genes followed the accumulation of Wcbf2 transcript suggested direct involvement of the Wcbf2 gene in the induction and enhancement of the Cor/Lea gene expression. The cultivar difference in freezing tolerance developed during different stages of cold acclimation can be at least partly explained by the differential and coordinated regulation of the predicted Cor/Lea gene signal transduction pathway that is mediated by the CBF/DREB1 transcription factors in common wheat.

Key words: Cold acclimation, CRT/DRE element, CBF/DREB1 transcription factor, Freezing tolerance, Triticum aestivum L.

INTRODUCTION

Cold/freezing temperatures represent a significant abiotic stress limiting geographical distribution of plants and reducing crop quality and productivity. Plants in temperate regions have evolved varying degrees of ability to survive cold/freezing stress. One prominent adaptive mechanism to this stress is known as cold hardening or acclimation (Levitt, 1980), which is triggered by induction of a battery of Cor (cold-responsive) genes after exposure of plants to low but non-freezing temperatures (LT) for certain periods of time (Guy, 1990). Ample volume of information is now available from a model dicotyledonous plant Arabidopsis thaliana and it has been established that this adaptive process is tightly associated with the development of cold/freezing tolerance (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). Compared to Arabidopsis system, however, available information on cold acclimation and freezing tolerance in monocotyledonous crops such as wheat, barley and rice is still limited.

In Arabidopsis, a functional cis-acting element of the Cor genes, i.e. the CCGAC core motif known as a CRT (C repeat)/DRE (dehydration responsive element) sequence, was proven to play a critical role in the promoter function...
of COR15A/RD29A genes (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker et al., 1994). Both ABA-dependent and -independent pathways regulate the expression of Arabidopsis Cor genes (Shinozaki and Yamaguchi-Shinozaki, 2000). A family of transcription factors called CBF-binding factors (CBFs) or DRE-binding proteins (DREBs) is involved in the ABA-independent pathway and regulates the Cor gene expression through binding to the CRT/DRE cis elements. These transcription factors contain a DNA binding domain found in the ethylene-responsive element binding protein/APETALA2 (EREBP/AP2) family (Stockinger et al., 1997; Liu et al., 1998). Three intensively studied genes that are collectively called CBF/DREB1 genes (including CBF1/DREB1B, CBF2/DREB1C and CBF3/DREB1A) are transiently induced and their transcripts start accumulating within 15 min of exposure to LT (Gilmour et al., 1998). The CBF/DREB1 transcript levels reach a maximum at about 3 h LT treatment and then decline significantly, and the transcripts remain at a low level over the course of the 3-week LT treatment (Zarka et al., 2003). Recent characterization of the cbf2 mutant of Arabidopsis has indicated that the CBF2/DREB1C protein negatively regulates the CBF1/DREB1B and CBF3/DREB1A genes, thus causing the transient expression of these transcription factor genes (Novillo et al., 2004). Moreover, it has recently been suggested that the CBF/DREB1 gene expression can partly be activated by ABA (Knight et al., 2004). The CBF-mediated cold-response mechanism in Arabidopsis appears to be conserved in both dicotyledonous and monocotyledonous plants including rape, tomato, wheat and rye (Jaglo et al., 2001; Hsieh et al., 2002).

Wheat and its relatives grow under widely different climatic conditions and thus exhibit a large genetic variability in cold/freezing tolerance. A number of Cor and related Lea (late embryogenesis abundant) genes have been analyzed in cereals including wheat (Cattivelli and Bartels, 1990; Houde et al., 1992; Sarhan et al., 1997). The levels of accumulation of Cor/Lea transcripts positively correlate with the levels of freezing tolerance in seedlings of common wheat cultivars (Ohno et al., 2001; Kobayashi et al., 2004). The LT response of the wheat Cor/Lea genes is also regulated through either ABA-dependent or -independent pathway. At least four of the wheat Cor/Lea genes, Wrab17, Wrab18, Wrab19 and Wcor825, are responsive to exogenous ABA (Tsuda et al., 2000; Kobayashi et al., 2004). An ABA-independent wheat Cor gene Wcor15 encodes a chloroplast-targeted COR protein analogous to the Arabidopsis protein COR15a (Lin and Thomashow, 1992; Thomashow, 1999; Takumi et al., 2003). Expression of the other wheat Cor/Lea genes including Wcor14, Wcs19, Wlt10 and Wcs120 is also ABA-independent (Houde et al., 1992; Tsvetanov et al., 2000; Ohno et al., 2001; NDong et al., 2002). The conserved CRT/DRE-like sequence motifs were found in the promoter regions of the Wcor15 and Wcs120 genes, and their promoter sequences were proven to be LT-inducible in both monocotyledonous and dicotyledonous transgenic plants (Quellet et al., 1998; Takumi et al., 2003). These results strongly suggest that the functional Cor/Lea gene system involving the CBF/DREB1 trans-acting factors and the CRT/DRE cis element is conserved in wheat.

To understand the molecular basis of cold acclimation and freezing tolerance and cultivar differences in these two important phenotypes in wheat, we monitored the developmental time course of freezing tolerance and the expression profiles of newly isolated wheat CBF/DREB1 homologs and their putative downstream Cor/Lea genes. The study was performed during the cold acclimation period extending for up to 63 days and using two wheat cultivars with distinct levels of freezing tolerance. Based on the results obtained, we discuss about conservation and divergence of the CBF/DREB1-mediated LT signaling pathway(s) between wheat and Arabidopsis.

**MATERIALS AND METHODS**

**Plant materials and bioassay conditions for freezing tolerance.** Two cultivars of common wheat (*Triticum aestivum* L.), a winter-type ‘Mironovskaya 808’ (M808) and a spring-type ‘Chinese Spring’ (CS), were used. Bioassay conditions for freezing tolerance were according to Ohno et al. (2001). Seeds were imbibed under tap water for 5 h and kept overnight at 4°C to stimulate synchronized germination. Twenty-five imbibed seeds from each of the two cultivars were planted as separate groups in the same pots (25 cm × 12 cm in width and 12 cm in depth) with soil, and incubated in a growth chamber under the following standard temperature and light conditions; 25°C with a 16 h photoperiod at a light intensity of 110–120 µm photons m⁻²s⁻¹ provided by cool white fluorescent lamps. Seedlings were watered every other day with 0.1% Hyponex solution (N-P-K=5-10-5, Hyponex, Osaka, Japan). Seven-day-old seedlings were cold-acclimated at 4°C for 2 to 9 weeks, and then frozen at −15°C for 9 or 24 h in the darkness. Frozen seedlings were thawed overnight at 4°C and transferred back to the standard temperature condition. At the 5th day of the transfer, numbers of surviving seedlings were recorded and the data were statistically analyzed.

**Isolation and sequencing of wheat CBF homologs.** Partial cDNA fragments corresponding to the wheat CBF-like cDNA clone TaCBF (accession no. AF376136) (Jaglo et al., 2001) were amplified using total RNA template extracted from the cold-acclimated seedlings of M808 in reverse transcriptase PCR (RT-PCR). Primer sequences designed for RT-PCR were 5’-TGA-CGCTGCAACTGATGGAC-3’ and 5’-AGTTCCAAAGCG-
CGTGTAG-3'. The amplified fragments were cloned into pGEM-T vector (Promega, USA) and their sequences were determined by the automated fluorescent dye deoxy terminator cycle sequencing system using ABI PRISM™ 310 Genetic Analyser (PE Applied Biosystems, USA). Based on the sequences of these partial cDNAs, a following primer set was designed for determining the 5' and 3' ends of the CBF-homologous cDNA; 5'-GGTGCCGGC-TCTCCCTGAACCTTGCCG-3' and 5'-GGCAGCTTGTG-GACGAGCAGCTTGGTGG-3'. Rapid amplified cDNA end (RACE)-PCR was conducted using a Marathon cDNA Amplification Kit (CLONTECH). Single-stranded cDNA for the RACE-PCR was produced using the total RNA extracted from cold-acclimated seedling leaves. To isolate the complete open reading frames (ORFs) of the wheat CBF homologs, RT-PCR was conducted with the following two gene-specific primer sets: 5'-CTCAACCCCTGTGCAAC-3' and 5'-AAGCGTTTTTGACATTACATTA-3' for Ubi-1 and 5'-CTCAACCCCTGTGCAAC-3' and 5'-TACAAATTCAGTCCTTCCCT-3' for Wcbf2/2-2 (see Results). The amplified cDNA fragments were cloned into pGEM-T vector and sequenced. Nucleotide sequences of the cDNAs and their deduced amino acid sequences were analyzed by DNASIS (Hitachi, Tokyo, Japan). Amino acid sequence homology was searched with the BLAST algorithm (Karlin and Altschul, 1993) and a multiple alignment was calculated according to Waterman (1986).

**Southern blot analysis and gene expression studies.** For genomic Southern blot analysis, a single accession each of the wild and cultivated A genome diploid wheat, *T. boeoticum* and *T. monococcum*, respectively, was used. Total DNA was single-digested with HindIII, BamHI and EcoRI. The digested DNA was fractionated by electrophoresis through 0.8% agarose gel and transferred to Hybond N+ nylon membranes (Amersham Biosciences). Signal detection using the polyclonal antibodies against WCOR/LEA proteins was performed according to Ohno et al. (2003).

For studies of gene expression, 7-day-old seedlings of M808 and CS grown under the standard temperature condition were transferred to the cold-acclimation condition at 4°C as already described. Seven-day-old seedlings were also treated by desiccation for 4 h on dry filter papers in petri dishes. Exogenous ABA was sprayed according to Kobayashi et al. (2004). Total RNA was extracted by guanidine thiocyanate from the aboveground tissues and also from the seedling leaves. RNA (15 µg) was fractionated by electrophoresis through 1.2% formaldehyde/agarose gel and transferred to the Hybond N+ nylon membranes. RNA blots were hybridized with the 32P-labeled whole sequences of the *Wdhn13, Wcor14* and *Wcor15* cDNA clones as probes. Probe labelling, hybridization, washing and autoradiography were performed in the same way as for Southern blot analysis. Because of low levels of gene expression of the wheat CBF homologs, the amount of their transcripts was determined by semi-quantitative RT-PCR analysis using a first strand cDNA synthesis kit (TOYOBO, Japan). The total template RNA samples for the cDNA synthesis were treated with DNaseI to remove contaminated DNA. RT-PCR with the gene-specific primer sets resulted in amplification of the single 790-bp fragment for *Wcbf2-1*. The amplification at fewer cycles was in the exponential range of amplification. As an internal control, a fragment from the wheat ubiquitin gene (*Ubi-1*) was amplified. Primer information for *Ubi-1* and the PCR condition were according to Murai et al. (2003). Intensity of the RT-PCR fragments was assessed by scanning the electrophoregrams with NIH IMAGE 1.61 software, and the relative values were calculated after normalized by the *Ubi-1* transcripts. For studying light/dark response of *Wcbf2*, 7-day-old seedlings were grown for 0–8 days at 4°C under different light/dark regimes (see Results), and total RNA was extracted at different time points for RT-PCR analysis.

For immunoblot analysis, soluble proteins were extracted from seedling leaves according to Kobayashi et al. (2004). Protein concentration was determined using the Bio-Rad Protein Assay Dye Reagent Concentrate with IgG as a standard. Soluble proteins (10 µg) were resolved through SDS-PAGE with 15% (w/v) polyacrylamide gel and electro-blotted onto nitrocellulose membranes, Hybond-C Extra (Amersham Biosciences). Signal detection using the polyclonal antibodies against WCOR/LEA proteins was performed according to Ohno et al. (2003).

**Interaction of WCBF2 with wheat Cor/Lea gene promoters.** The 1.1-kb 5' upstream region of *Wdhn13* was amplified with the following promoter-specific primer set in addition to the *HindIII* or *XbaI* linker; 5'-CCAA-GCTTCGATCGGGAGAGAGTTAT-3' and 5'-CCTCTAC-GACTGACACTGTTCTGTGCT-3'. The PCR-amplified fragment was digested with *HindIII* and *XbaI* and replaced by the CaMV35S promoter of pBI121 (Clontech) to produce *Wdhn13::GUS* constructs. The *Wcor15::GUS* construct containing 1.7-kb 5' upstream region was after Takumi et al. (2003). The *Wcbf2* cDNA sequence was amplified with the following primer set in addition to the *XbaI* or *SacI* linker; 5'-CCCTCTTAGATTACGGTGC-ACTGATTGA-3' and 5'-CCCCAGCTCGAGATTATGTTCAAAAGC-3'. The PCR fragment was digested with *XbaI* and *SacI* and replaced with the GUS gene of the pBI121. The chimeric *Wcbf2* construct was named 35S::*Wcbf2*. The constructs were purified by the MaxiV500 Ultrapure Plasmid Extraction System (VIOGENE, CA, USA) and introduced into a wheat callus line 'HY-1'
by particle bombardment according to Takumi et al. (1999). The GUS activity was quantified according to Jefferson (1987) and estimated as a relative value to the GUS activity of pBI121. The means with standard error were calculated based on 4–6 independent experiments.

RESULTS

Developmental time course of cold/freezing tolerance during the long-term cold acclimation. Our previous studies of the short-term cold acclimation up to 7 days showed that the LT treatment significantly increased the levels of freezing tolerance in both cultivars, and that a much higher level of freezing tolerance was developed in M808 than in CS after 3 days of cold acclimation (Ohno et al., 2001; Kobayashi et al., 2004). To examine the effect of long-term acclimation on freezing tolerance, 7-day-old seedlings were placed under the LT condition and kept for 2 to 9 weeks. As observed in the previous studies, the aboveground tissues of all examined seedlings became wilted and withered within a day of transfer back to the standard temperature condition after freezing treatment, but freezing-tolerant plants showed a rapid recovery and developed new shoots from the surviving shoot apical meristems. The bioassay showed that the cold acclimation for 2–3 weeks gave high levels of freezing tolerance in both M808 and CS and that the long-term LT treatment dramatically increased the level of freezing tolerance only in M808 (Fig. 1). This winter cultivar developed perfect freezing tolerance against −15°C for 9 h after 5–7 weeks cold acclimation. By contrast, in the spring cultivar CS only about 20% seedlings could survive the freezing treatment after 5-week acclimation and all seedlings were killed after more than 6-week cold acclimation. The long-term cold acclimation thus demonstrated the clear difference in the ability of freezing tolerance between the two wheat cultivars.

Isolation of WCBF2 cDNAs. One wheat CBF-like gene designated TaCBF was isolated by screening a cold-acclimated wheat cDNA library using three CBF gene fragments of rye (AF370728, AF370729, and AF370730) as probes (Jaglo et al., 2001). The deduced polypeptides of the wheat TaCBF and rye ScCBF cDNA clones showed high degrees of identity to Arabidopsis CBF1/DREB1B within the AP2/EREBP DNA-binding domains. To isolate additional wheat CBF homologs, we designed a primer set for 5′- and 3′-RACE-PCR based on the TaCBF cDNA sequence and obtained two cDNA clones using RNA template extracted from cold-acclimated seedling leaves of M808. These two cDNA sequences with 790-bp and 941-bp in length contained complete ORFs and showed high homology to the original TaCBF fragment.

ORFs of the two CBF homologs putatively encoded polypeptides of 212 amino acid residues (MW=23.3 kDa) (Fig. 2A). They contained a conserved AP2/EREBP domain and a nuclear localization signal (NLS) and shared the amino acid identity of 94.3%. WCBF2-2 differed from TaCBF by one amino acid and thus it likely represented a cultivar difference of the same gene. The amino acid sequences of the three wheat CBF homologs also showed high similarity to other plant CBFs. A phylogenetic tree of the CBF proteins was constructed by UPGMA method (Fig. 2B). The tree was divided into two clusters containing monocot-derived proteins and one cluster containing dicot-derived proteins. Our cloned CBF homologs showed the highest levels of identity with the wheat and rye CBFs, followed by barley HvCBF2 (Xue, 2003), rice OsDREB1B (Dubouzet et al., 2003) and another barley HvCBF1 (Xue, 2002a, b). Because of the high homology with the barley HvCBF2 gene, the wheat CBF homologs were designated as Wcbf2-1 and Wcbf2-2. A cluster containing barley HvCBF3 (Choi et al., 2002) and rice OsDREB1A (Dubouzet et al., 2003) was distantly related to the CBF members in the main monocot cluster. Transgenic study of the rice OsDREB1A gene, however, indicated its functional similarity to the Arabidopsis CBF3/DREB1A gene (Dubouzet et al., 2003). The multiple alignment of the AP2/EREBP domains of wheat and other plant CBF homologs is shown in Fig. 2C. The amino acid sequence of the WCBF2-1 AP2 domain showed perfect identity with those of the WCBF2-2, TaCBF, and ScCBF proteins. Therefore, the Wcbf2-1 gene seemed to be a paralog of the Wcbf2-2 and TaCBF genes in wheat.

To study copy number of the Wcbf2 genes in the wheat genome, Southern blot analysis was conducted using total DNA isolated from the two A genome diploid species of

Fig. 1. Cultivar difference in freezing tolerance after cold-acclimation. Bioassay was performed under the standard assay conditions: 4°C for the indicated period for cold acclimation and −15°C for 9 h ( ); 24 h ( ) for freezing. Results represent means ± standard deviations (n=3–6 per time point) in the two wheat cultivars (gray bar: CS, shaded bar: M808). Survival rate after 7-w acclimation was 100% in M808, and that after 7- and 9-w acclimation in CS was 0%.
Expression profiles of wheat CBF and Cor/Lea genes

Because of the high homology between the two Wcbf2 ORFs, only the 3’-RACE PCR product of Wcbf2-1 was used as a probe. Southern blotting generated more than five HindIII bands (Fig. 3), suggesting that Wcbf2 belonged to a small multigene family in the A genome of wheat. Many bands were generated in Southern blots using DNA extracted from common wheat, suggesting the multigene nature also of the B and D genome Wcbf2 homologs (data not shown).

Expression profiles of Wcbf2 and Cor/Lea genes during cold acclimation. Arabidopsis CBF/DREB1
transcripts begin accumulating within 15 min after exposure of plants to LT (Jaglo et al., 2001). Expression of the monocot CBF genes (barley HvCBF1 and HvCBF3 and rice OsDREB1A and OsDREB1B) is also induced by LT within at least 2 h (Choi et al., 2002; Xue, 2003; Dubouzet et al., 2003). HvCBF2, on the other hand, is constitutively expressed in barley leaves and the transcript level is rapidly enhanced by LT (Xue, 2003). To examine whether such rapid induction or enhancement of gene expression occurs in the Wcbf2 gene, RT-PCR analysis was conducted using the primer sets designed in the untranslated regions of the Wcbf2 genes. The Wcbf2-1 transcript was detected at a low level under non-stress condition, and it level rapidly increased within 15 min after exposure of wheat seedlings to the LT condition (Fig. 4A). The transcript level reached a high plateau by 1–2 h, and the time-dependent increase occurred slightly earlier in M808 (1 h) than in CS (2 h). The amount of Wcbf2-1 transcript then showed a gradual decrease and became undetectable within 24 h under the LT condition. The values of the Wcbf2 transcript levels were calculated as fragment intensity relative to the highest value (Fig. 4B), and the expression patterns clearly showed the rapid response to LT in M808. RT-PCR using the Wcbf2-1- and Wcbf2-2-specific primer sets gave the same result, and therefore only the Wcbf2-1-specific primer set was used in the subsequent Wcbf2 expression analyses. In our previous study, expression of the cold inducible Wcor15 gene was detected within 4 h in M808, while it was delayed until 6 h in CS (Takumi et al., 2003). We therefore compared the expression profiles of two other wheat Cor/Lea genes, Wcor14 and Wdhn13, and found that they were also induced earlier and at higher levels in M808 than in CS (Fig. 4C). The transcript level of Wcor14 gene was much higher than that of the Wdhn13 gene in both cultivars. It was notable that induction of the Wcbf2 gene occurred prior to that of the putative downstream Cor/Lea genes during this early stage of LT response. Putative CRT/DRE elements were found in the 5' upstream regions of Wcor15 (Takumi et al., 2003) and Wdhn13 (our unpublished data), suggesting that Wcor14 also possesses this cis element and that these Cor/Lea genes are transcriptionally regulated by the CBF/DREB1A homologs in wheat.

After the temporal disappearance within 12–24 h, the Wcbf2 transcript reappeared on and after 1-day under the LT condition in the seedling leaves. The transcript level increased until day 10 in M808, while it remained constant in CS (Fig. 4D). No significant changes were observed in the transcript level after 1-day de-acclimation of 3-day acclimated seedlings, suggesting that the Wcbf2 transcript was stable for at least one day under the normal temperature condition. The level of the Wcbf2 transcript was influenced by the LT condition with different light/dark regimes in both CS and M808 (Fig. 5). The increase in the Wcbf2 transcript level during 3- and 5-day LT treatment was observed only under the standard light condition. The result suggested that the Wcbf2 transcription was either enhanced by light or suppressed by darkness under the LT condition, similar to the expression patterns of a majority of Cor/Lea genes (Kobayashi et al., 2004).

Since the two wheat cultivars showed a clear-cut difference in their levels of freezing tolerance after the long-term exposure to LT (Fig. 1), we compared the long-term cold-responses of the Wcbf2 expression in the two cultivars. The Wcbf2 transcript accumulated to reach maximum levels at day 14–21 in both cultivars under the LT condition (Fig. 6A). In CS, the level of Wcbf2 transcript declined after day 21 and became undetectable at day 63, whereas in M808 the transcript level showed a temporal decrease after day 28 but it re-increased until day 42 and remained at a high level towards day 63 (Fig. 6B). The observed difference in the Wcbf2 transcript level between the two cultivars appeared to be correlated with the difference in their freezing tolerance (Fig. 1). Expression profiles of the three Cor/Lea genes were also studied under the long-term LT condition. The level of Wdhn13 transcript decreased towards the later stages in CS, while it remained at a fairly constant in M808 (Fig. 6C). Not much difference was observed in the levels of transcript accumulation of Wcor14 and Wcor15 between the two cultivars. The Wdhn13 gene showed a much
Expression profiles of wheat CBF and Cor/Lea genes during the short-term cold acclimation in the two wheat cultivars. Transcript accumulation in response to LT was monitored by semi-quantitative RT-PCR analysis using gene-specific primers (A, B, D) and by Northern blot analysis using 32P-labeled probes (C). The RT-PCR products of Wcbf2-1 and Wcbf2-2 transcripts were quantified as means of relative values to the highest level in (B), and the data were from two independent experiments. A3D1 in (D) represents deacclimation under the standard conditions for 1 d after 3-d LT treatment. The ubiquitin and rRNA genes were used as control.
lower level of expression than Wcor14 and Wcor15 in both cultivars, but a quite different expression pattern was observed between the two cultivars. The Wdhn13 transcript appeared at day 21 and decreased thereafter in CS, while in M808 the transcript level showed two maximums at day 21 and day 42. The expression profile of Wdhn13 agreed well with that of Wcbf2. Accumulation of the COR/LEA proteins was also studied under the long-term acclimation condition. Immunoblot analysis showed the continuous and high accumulation of these proteins throughout the tested period in M808 (Fig. 6D). In CS, the amount of WCOR15 protein showed a similar increase, while the level of WCOR14 protein decreased after day 42. The amount of WDHN13 protein in CS was much lower than that in M808 throughout the tested period, agreeing well with its transcript profile. Some additive bands were observed in immunoblots of WCOR14 and WCOR15. The barley COR14b immunologically cross-reacts with a related, chloroplast-imported protein COR14a (Crosatti et al., 1999). The polyclonal antibodies against WCOR14 and WCOR15 appeared to cross-react with homologous proteins.

**Wcbf2 response to drought stress and ABA treatment.** All Arabidopsis CBF/DREB1 genes except for CBF4 are activated by LT but not by salt and drought (Haake et al., 2002). Only a recent study reported possibility of the Arabidopsis CBF1-3 activation by exogenous ABA (Knight et al., 2004). To study the effects of abiotic stresses other than LT on the Wcbf2 expression, wheat seedlings were treated with dehydration and also with 20 µM ABA under the standard temperature condition. The level of Wcbf2 transcript increased quite rapidly within 15–30 min after exposure to drought in both cultivars (Fig. 7). M808 accumulated the Wcbf2 transcript earlier (a maximum level at 30 min) than CS (1 h). Expression of the Wcbf2 gene under the drought condition was transient and after reaching maximums the amount of Wcbf2 transcript decreased gradually. The result showed that the Wcbf2 gene was responsive not only to LT but also to drought. However, the expression level of Wcbf2 did not change after the ABA treatment.

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**Fig. 5. Light/dark modulation of the Wcbf2 expression.** (A) Semi-quantitative RT-PCR analysis in the seedling leaves of M808. The ubiquitin gene was used as a control. (B) Quantification of the Wcbf2 transcripts in CS and M808. The values represent the means of the Wcbf2 transcript levels relative to the value in 3 days acclimation under light/dark regime. NA, non-acclimated control; 3L and 5L, 3 and 5 days accumulation under standard light/dark regime; 3D and 5D, 3 and 5 days acclimation under continuous dark; 5L3D and 5D3L, 5L followed by 3D and 5D followed by 3L, respectively.
Fig. 6. Expression profiles of the Wcbf2 and Cor/Lea genes during the long-term cold acclimation in the two wheat cultivars. (A) Transcript accumulation of the Wcbf2 gene in response to LT was monitored by semi-quantitative RT-PCR analysis using gene-specific primers. The ubiquitin gene was used as a control. (B) Quantification of the Wcbf2 transcripts. The values represent the means of the Wcbf2 transcript levels relative to the value in 21 days acclimation. The data were from two independent experiments. (C) Northern blot analysis of the Cor/Lea transcripts using 32P-labeled probes. The rRNA gene was used as a control. (D) Immunoblot analysis of the COR/LEA proteins using polyclonal antibodies. The bottom lane shows CBB-stained gel.
The result indicates that the Wcbf2 gene is involved in an ABA-independent cold signal pathway in wheat.

**Interaction between the Cor/Lea gene promoters and WCBF2 protein.** We investigated the trans-activation of downstream target genes by the WCBF2 protein. The 5' upstream regions of the Wcor15 and Wdhn13 genes were combined with the GUS gene and the Nos terminator. The two chimeric constructs, designated as Wcor15::GUS and Wdhn13::GUS, were introduced via particle bombardment into the cultured cell line HY-1, which was derived from scutellum of common wheat cultivar ‘Haruyutaka’. A chimeric construct of the Wcbf2 cDNA under control of the CaMV35S promoter designated as 35S::Wcbf2 was co-bombarded with the Cor/Lea promoter::GUS constructs. After bombardment, the GUS activities were estimated as relative values to that of pBI121. In both Wcor15::GUS and Wdhn13::GUS constructs, bombardment with the 35S::Wcbf2 construct gave higher GUS activities than that without the 35S::Wcbf2 (Fig. 8), although the differences were not statistically significant.

**DISCUSSION**

CBF/DREB1 transcription factors are known to be involved as key regulators of the cold signal transduction in various plant species (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). We isolated cDNA clones encoding wheat CBF/DREB1 homologs from cold-acclimated seedling leaves of a freezing-tolerant winter cultivar M808, and designated them as Wcbf2 based on the highest homology to the barley HvCBF2 gene (Fig. 2). Expression analyses of Wcbf2-1 were conducted by the semi-quantitative RT-PCR method, and the Wcbf2-1 transcript levels were compared in the exponential range of amplification. This method is not applicable for precise assessment of the Wcbf2-1 mRNA quantification, but able to use for comparison of the expression patterns between M808 and CS. We monitored the expression profiles of the Wcbf2 gene and its putative downstream Cor/Lea genes up to 9-week exposure to LT in M808 and a spring cultivar CS. After the cold acclimation for 2–3 weeks, both cultivars showed similarly high levels of freezing tolerance and Wcbf2 transcript (Figs. 1 and 6). The long-term cold acclimation (5–9 weeks) resulted in more dramatic cultivar difference in freezing tolerance (Fig. 1) than the short-term cold acclimation (1–7 day) in our previous studies (Ohno et al., 2001; Kobayashi et al., 2004). M808 showed higher levels of Wcbf2 transcript than CS at least during the long-term cold acclimation (Fig. 6). The overall expression profiles of the Wcbf2 gene showed a good correlation with development of freezing tolerance. This coordinated relationship strongly indicates that Wcbf2 plays an important role in the cold signal transduction and the development of freezing tolerance in wheat. We noted that the Wcbf2 gene expression was up-regulated at least twice during the cold
acclimation period in both cultivars. The first up-regulation occurred within 1–4 h exposure to LT, and the activation of the Cor/Lea genes followed this first up-regulation. This rapid and transient induction and enhancement of the Wcbf2 gene expression was consistent with the reported expression patterns of the CBF/DREB1 gene family in other plant species. The amount of Wcbf2 transcript reached a maximum level earlier in M808 than in CS, and more abundant Cor/Lea transcripts were accumulated in M808 than in CS. The more rapid enhancement of the gene expression in M808 likely explained why M808 has superior acclimation ability against a quick temperature drop (Kobayashi et al., 2004). The second up-regulation was observed during 2–3 weeks of cold acclimation, and after this period the amount of Wcbf2 transcript continuously decreased in CS, while in M808 it remained at a high level until 9 weeks (end of the experiment) after a transient decrease. The result suggests that the maintenance of the Wcbf2 expression in M808 well represents its high cold-acclimation ability. In Arabidopsis, the transcript levels of the CBF/DREB1 gene family are kept low over the course of 3-week LT treatment (Zarka et al., 2003). Therefore, the second up-regulation and subsequent maintenance of the Wcbf2 transcript levels might be a wheat-specific phenomenon. The mechanisms of these regulatory processes should be solved in future studies.

The down-regulation of the Wcbf2 expression after 2–3 weeks was correlated with decrease of the Wdhn13 transcript level in both cultivars (Fig. 6). Because the northern blots of Wcor14 and Wcor15 reached high plateau levels, the down-regulation was not clearly observed. Some wheat Cor/Lea genes including Wcor14 and Wcor15 show no clear difference of transcript accumulation patterns between CS and M808 in the short-term cold acclimation, while the other Cor/Lea genes such as Wdhn13 and Wcs120 clearly show the difference explaining the cultivar-dependent levels of freezing tolerance (Kobayashi et al., 2004). During cold acclimation, a battery of the Cor/Lea genes is induced (Guy, 1990; Thomasaw, 1999). Not necessarily apparent correlation between the freezing tolerance levels and the Cor/Lea transcript levels (Grossi et al., 1998; Kobayashi et al., 2004) suggests that only a part of the Cor/Lea genes might be able to explain the observed cultivar difference. To evaluate quantitative contribution of Wcbf2 to total increase of the expression levels of all LT-induced Cor/Lea genes in wheat, further transcriptome studies such as microarray should be required. Nevertheless, our results suggest that the cultivar difference in the Wcbf2 transcript level reflects those of at least some members of the Cor/Lea genes, supporting the implication that Wcbf2 plays an important role in cold acclimation as well as in the rapid response to the LT treatment. Genomic Southern blot analysis showed that the Wcbf2 gene and its homologs consisted of a small multigene family, similar to other members of the CBF/DREB1 family (Fig. 3). In this study, we analyzed expression profiles of Wcbf2-1 as a representative of the Wcbf2 family. To understand the regulatory mechanisms of the Cor/Lea expression via these transcription factors during cold acclimation, cloning of the Wcbf2 homoeologs/paralogs and comparative studies of their expression profiles should be required.

The differential and coordinated expression of the Ch2 and Cor/Lea genes in response to LT suggests that the cold-regulated CBF/DREB1 transcription factors can directly induce and enhance the expression of the downstream Cor/Lea genes. ABA treatment did not change the level of Wcbf2 gene transcript, similar to all other CBF/DREB1 genes except for Arabidopsis CBF4 (Haake et al., 2002). The Wcbf2 expression was also regulated by light (Fig. 5), which agrees with the light/dark response of most of the Cor/Lea genes (Kobayashi et al., 2004). In Arabidopsis, LT-specific Cor expression is greatly enhanced by light signaling (Kim et al., 2002). Surprisingly, however, a rapid enhancement of the Wcbf2 gene expression was observed under drought stress in wheat (Fig. 7), unlike CBF/DREB1 genes of Arabidopsis and other plants. The 5’ upstream region of the LT-specific Wcor15 gene contains putative CRT/DRE core sequences and induced a reporter GUS gene expression in response to LT and light in transgenic tobacco plants (Takumi et al., 2003). The Wdhn13 gene, which is responsive to both LT and drought, also contains the CRT/DRE motifs in the promoter region (our unpublished data). Increase of the GUS activities under control of these 5’ upstream sequences via WCBF2 protein was not significantly observed in cultured cells of common wheat (Fig. 8), which might be due to using suspension cell culture and more than 1-kb sequences of the Cor/Lea promoter regions. Further studies are required to prove the direct physical interaction of the CRT/DRE elements in the promoter regions of Wcor15 and Wdhn13 genes with the WCBF2 protein, and to understand the role of the transient expression of the Wcbf2 gene in response to drought stress.

The apparent cultivar difference observed in the levels of the Wcbf2 transcript strongly predicts the presence of yet unidentified players that regulate the Wcbf2 gene and its homoeologs/paralogs thus determine the observed cultivar difference. Group 5 chromosomes of Triticeae including wheat and barley contain major quantitative trait loci (QTLs) for winter hardiness, and the major frost resistance genes (Fr-1) were also identified on the same chromosomal regions containing QTLs (Galiba et al., 1995; Cattivelli et al., 2002). These chromosome regions affect the Cor/Lea gene expression (Vágújfalvi et al., 2000). The reported map position of the barley HcCBF3, however, was not closely linked with the major QTL (Fr-H1) on the chromosome 5H, and thus the gene was sug-
gested as a minor QTL in some genetic backgrounds (Choi et al., 2002). The wheat sequence homologous to the HvCBF3 gene showed a tight linkage with the Fr-A2 QTL for frost tolerance on the T. monococcum map (Vágújfalvi et al., 2003). The Fr-A2 is mapped on a distal region of the long arm of chromosome 5A. Chromosomal location of wheat CBF/DREB1 homologs remains unknown, but the high homology of the Wcbf2 genes to the barley HvCBF genes suggests that Wcbf2 is probably located on the homoeologous group 5 chromosomes in the wheat genome. QTLs determining the cultivar difference of freezing tolerance have not yet been identified in a M808/CS mapping population, but the Fr-1 exerts a major effect on the cultivar difference between spring- and winter-type accessions in Triticeae (Cattivelli et al., 2002). The difference of freezing tolerance between M808 and CS, therefore, is expected to be in large part caused by the Fr-1 allelic difference. Our recent study using near isogenic lines of ‘Triple Dirk’ showed that the Vrn-1/Fr-1 chromosomal intervals regulated the Cor/Lea expression through Wcbf2 and freezing tolerance after cold acclimation in wheat (Kobayashi et al., 2005). This strongly suggests that the signal transduction from Fr-1 to Wcbf2 is one of central pathways in the development of freezing tolerance in wheat. Further studies in wheat and barley should address the most important question on the mechanisms of the Wcbf2 regulation via the Fr loci.

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Expression profiles of wheat CBF and Cor/Lea genes


