RESEARCH PAPER

Regulation by Vrn-1/Fr-1 chromosomal intervals of CBF-mediated Cor/Lea gene expression and freezing tolerance in common wheat

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Abstract

Vrn-1/Fr-1 chromosomal regions of common wheat possess major QTLs for both winter hardiness (Fr) and vernalization requirement (Vrn). The Vrn-1/Fr-1 intervals are assigned to long arms of the homoeologous group 5 chromosomes. To investigate the role of the Vrn-1/Fr-1 intervals on the low-temperature (LT) inducibility of wheat Cor/Lea genes and its putative transcription factor gene Wcbf2, LT response of these genes was monitored using near-isogenic lines (NILs) for the Vrn-1 loci. The Wcbf2 transcript accumulated rapidly after LT treatment and remained at a high level in lines without any dominant Vrn-1 alleles. By contrast, the Wcbf2 transcript level was greatly reduced in lines carrying the Vrn-1 alleles. The Vrn-1 NILs accumulated much lower amounts of Cor/Lea transcripts and COR/LEA proteins than the non-carrier line. The observed patterns and levels of gene expression, particularly in the Vrn-A1 NIL, agreed with the higher sensitivity to freezing damage in this line than in the non-carrier line. Up-regulation of the expression of the WAP1 gene, a candidate of the Vrn-1 loci, was much delayed in the non-carrier line than all the NILs carrying the Vrn-1 loci. Neither positive nor negative relationships were found between the WAP1 expression and the Cbf2/Cor/Lea expression. These results support the intimate relationship between the Cbf2/Cor/Lea expression and the level of freezing tolerance, and suggest that a functional Fr-A1 allele linked to the vrn-A1 allele, instead of the vernalization gene itself, plays a major role in regulating the CBF-mediated Cor/Lea gene expression in wheat.

Key words: Cor/Lea genes, freezing tolerance, homoeologous group 5 chromosomes, Triticum aestivum L., vernalization, WAP1, Wcbf2.

Introduction

Major loci and QTLs controlling cold/freezing tolerance have been mapped and their chromosomal syntenies have been determined among Triticeae species (Cattivelli et al., 2002). It has long been known that the homoeologous group 5 chromosomes exert a major effect on freezing tolerance in Triticeae. In common wheat (Triticum aestivum L.), the most significant loci for freezing tolerance (Fr) have been mapped on the long arms of 5A, 5B, and 5D chromosomes that, respectively, carry Fr-A1 (formerly Fr1), Fr-B1, and Fr-D1 (Fr2) (Galiba et al., 1995; Snape et al., 1997; Tóth et al., 2003). A new locus for freezing tolerance, Fr-A2, has been identified and mapped on a distal region of the long arm of chromosome 5A in diploid wheat, Triticum monococcum (Vágújfalvi et al., 2003).

Vernalization requirement, which necessitates certain periods of exposure of overwintering plants to low temperature (LT) for ensuring transition from the vegetative phase to the reproductive phase, is another critical trait for cold adaptation. Common wheat has three major loci for vernalization requirement, Vrn-A1 (formerly Vrn1), Vrn-B1 (Vrn2), and Vrn-D1 (Vrn3), which are all linked with the Fr-1 loci, i.e. Vrn-A1 with Fr-A1, Vrn-B1 with Fr-B1, and Vrn-D1 with Fr-D1 (Galiba et al., 1995; Snape et al., 1997, 1998; Iwaki et al., 2002; Tóth et al., 2003). Galiba et al.

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(1995) estimated the genetic distance of the Vrn-A1/Fr-A1 interval to be c. 2 cM. By contrast, the genetic distance of Vrn-D1/Fr-D1 was much larger at c. 10 cM (Snape et al., 1997) and that of Vrn-B1/Fr-B1 was c. 40 cM (Tóth et al., 2003). Homozygous deletion lines for the long arm of 5A (5AL), which were generated by taking advantage of the gametocidal gene system in the standard wheat cv. ‘Chinese Spring’ (CS) (Endo and Gill, 1996), were tested for flowering time without vernalization and for freezing tolerance after cold acclimation (Sutka et al., 1999). Their result confirmed that the Vrn-A1 and Fr-A1 loci are closely linked, but physically separated, on chromosome 5AL. Many spring-type wheat accessions, which allow the phase transition without vernalization, carry dominant Vrn-A1 alleles. By contrast, winter-type accessions, which require vernalization to promote floral development, carry recessive vrn-A1 alleles. Winter-type wheat should possess at least one dominant, ‘winter-type’ Fr-A1 allele that guarantees winter survival, but such an allele is unnecessary for spring-type wheat (Thomashow, 1999). Several studies demonstrated a significant relationship between the genotype of at least the Vrn-A1/Fr-A1 interval and the degree of cold/freezing tolerance in wheat. According to the freezing tolerance test in the 5AL chromosome deletion lines, lines possessing the functional Fr-A1 locus showed a 13% higher survival rate than the lines lacking it (Sutka et al., 1999).

Recently, Yan et al. (2003) isolated the VRN1 (Vrn-A″1) gene of T. monococcum by positional cloning and suggested that a wheat homologue of the AP1 (APETALA1) MADS-box gene is a candidate gene for VRN1. VRN1 is now considered to be an orthologue of WAP1 (wheat AP1) in common wheat (Murai et al., 1998, 2002). WAP1 was assigned to the region near the Vrn-A1 and Vrn-D1 loci by deletion mapping, and was shown to function as a key gene in the transition from the vegetative phase to the reproductive phase in cereals (Danyluk et al., 2003; Murai et al., 1998; Trevaskis et al., 2003). These results suggest that WAP1 is a possible candidate gene for Vrn-1 in common wheat. Another major gene, Ppd, regulating wheat flowering time reduces the delay of heading time under short-day conditions, and the expression level of the WAP1 gene is regulated independently of the Ppd allele under long-day conditions (Murai et al., 2003). Despite many efforts to map the Vrn-1/ Fr-1 intervals in common wheat, isolation of the key Fr-1 gene on this chromosomal region has yet to be realized.

Plants have evolved adaptive systems to various climatic conditions. Cold/freezing tolerance is an important trait for the adaptation of overwintering crops to LT conditions. Cold/freezing tolerance is acquired through the cold acclimation process that is triggered in response to LT (Hughes and Dunn, 1996; Thomashow, 1999). LT induces and/or enhances expression of a number of cold-responsive (Cor) late-embryogenesis-abundant (Lea) genes, and the accumulated COR/LEA proteins are believed to promote and sustain the development of freezing tolerance (Thomashow, 1999). It is also known that the LT inducibility of the Cor/Lea genes is regulated by the CBF/DREB1 protein family (Thomashow, 2001; Shinozaki and Yamaguchi-Shinozaki, 2000). In Arabidopsis, three CBF/DREB1 genes, CBF1/ DREB1A, CBF2/DREB1C, and CBF3/DREB1A, are induced by LT through binding of the CBF/DREB1 proteins to the cis-acting elements, CRT (C-repeat)/DRE (dehydration responsive element), in the promoter region of Cor/Lea genes. According to Jaglo-Otto et al. (1998), the over-expression of CBF1 not only leads to strong expression of Cor/Lea genes, but also to improved freezing tolerance.

In common wheat, a considerable number of LT responsive Cor/Lea genes have been characterized (Hughes and Dunn, 1996; Kobayashi et al., 2004). Recently, wheat CBF/DREB homologues such as TaCBF and TaDREB1 have been isolated and characterized (Jaglo et al., 2001; Shen et al., 2003). Two cDNAs (accession nos AB178166 and AB178167) of a wheat CBF orthologue named as Wcbf2 (Takumi et al., 2003a) have also been isolated and characterized. In barley, three CBF/ DREB1 homologues, HvCBF3, HvCBF4 and HvCBF8, were mapped on the chromosome 5H (Choi et al., 2002; Francia et al., 2004). Although wheat CBFs have not yet been mapped, they are probably located on chromosome 5 based on the synteny with the barley CBFs. A regulatory effect of 5AL on Cor/Lea gene expression and freezing tolerance was suggested by the observation that the chromosome 5A substitution line of CS, in which chromosome 5A of CS is replaced by that of a winter cv. ‘Cheyenne’, showed an increased freezing tolerance and accumulation of COR/LEA protein (Limin et al., 1997; Danyluk et al., 1998). Relationships between the vernalization response, Cor/Lea gene expression, and freezing tolerance were also shown in near-isogenic lines of wheat cv. ‘Norstar’ and ‘Manitou’ for the Vrn-A1 locus (Danyluk et al., 2003).

Despite significant progress, the role of the Vrn-1/Fr-1 loci on the CBF-mediated Cor/Lea gene expression remains unclear. The Vrn-1 near-isogenic lines (NILs) of a spring cv. ‘Triple Dirk’ were used here to investigate the effects of the Vrn-1/Fr-1 intervals on the LT inducibility of Wcbf2 and Cor/Lea genes and the development of freezing tolerance in wheat. The expression profile of the WAP1 gene was compared with that of the Wcbf2 and Cor/Lea genes after LT treatment. The results supported that cold/ freezing tolerance in wheat was not directly associated with the Vrn-1 loci. The possible role of the Fr-A1 locus linked to the Vrn-A1 locus on CBF-mediated Cor/Lea gene expression and freezing tolerance in wheat was discussed.

Materials and methods

Plant materials and bioassay conditions for freezing tolerance

Near-isogenic lines for the Vrn-1 genes of a spring-type common wheat (Triticum aestivum L.) cv. ‘Triple Dirk’ (Pugsley, 1971, 1972) were used in this study. A winter-type non-carrier line TD was bred.
by eliminating all of the dominant Vrn-1 alleles from ‘Triple Dirk’. TD and two Vrn-1 NILs, designated TD(A1) and TD(B1) carrying dominant Vrn-A1 and Vrn-B1 alleles, respectively, were examined in the bioassay for freezing tolerance and the gene expression studies. TD(A1B1) carrying both Vrn-A1 and Vrn-B1 alleles and TD (A1B1ppd) carrying ppd (a recessive allele of the photoperiod-responsive Ppd locus) in addition to Vrn-A1 and Vrn-B1, were also used in the gene expression analysis.

Seeds were imbibed under tap water for 5 h and kept at 4 °C for 15 h to promote synchronized germination. Twenty imbibed seeds from each of the lines (TD versus TD(A1) and TD versus TD(B1)) were planted in pots (25×12×12 cm in soil. The pots were 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 μmol photons m−2 s−1 (at the pot height) provided by cool white fluorescent lamps. The seedlings were watered every other day with 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 μmol photons m−2 s−1 (at the pot height) provided by cool white fluorescent lamps. The seedlings were watered every other day with

RT-PCR analysis
First strand cDNA was synthesized from 1 μg of DNase I-treated total RNA with the oligo-dT primer using Rever Tra Ace (Toyobo, Osaka, Japan). For PCR amplification, primer sets were designed from the sequences of WAP1 (formerly TaMADS#11, AB007504) and Wcbf2 (AB178166); 5'-ATCGACCTACGGTCTCAAAACA-3' and 5'-TAGAGCAGGTTATCATGGAA-3' for WAP1, and 5'-CTGAAACCAACCTGCAAC-3' and 5'-AAGCCTTTTGCCAT-TACATTA-3' for Wcbf2. As an internal control, a fragment from the wheat ubiquitin gene was amplified with the primer set: 5'-GCA-TGCGATATTGTGAA-3' and 5'-GGAGCTTACTGGCCAC-3'. The RT-PCR conditions were carefully manipulated to measure transcripts at the exponential phase of amplification. The PCR products in the exponential range of amplification were separated on 1.2% agarose gel and stained with ethidium bromide.

Northern blot analysis
Transcript accumulation of five wheat Cor/Lea genes were studied by northern blot analysis using the corresponding cDNA clones as probes. These cDNA clones (Wcor14, Wcor15, Wdhn13, Wlt10, and Wrah17) were previously characterized (Tsvetanov et al., 2000; Tsuda et al., 2000; Ohno et al., 2001, 2003; Takumi et al., 2003; Kobayashi et al., 2004). For RNA extraction, 2-week-old seedlings of the TD and Vrn-1 NILs grown under the standard conditions were previously constructed (Ohno et al., 2001; Kobayashi et al., 2004). For RNA extraction, 2-week-old seedlings of the TD and Vrn-1 NILs grown under the standard conditions were transferred to 4 °C and kept for the periods indicated. RNA extraction and northern blot were performed according to Kobayashi et al. (2004).

Immunoblot analysis
Polyclonal antibodies against WCOR14, WCOR15, and WDHIN13 were previously constructed (Ohno et al., 2003; Kobayashi et al., 2004). For immunoblot analysis, soluble proteins were extracted from the second leaves of TD and Vrn-1 NILs that were treated under the same LT condition as for plants used in RNA extraction. Protein extraction, immunoblotting, and signal detection were performed according to Kobayashi et al. (2004).

Results
Comparison of freezing tolerance among TD and the Vrn-1 NILs
To examine the effects of the Vrn-1/Fr-1 intervals on freezing tolerance, the levels of freezing tolerance of the non-carrier line TD and the two Vrn-1 NILs, Vrn-A1 (TD(A1)) and Vrn-B1 (TD(B1)) were compared, using 3-week-cold-acclimated (4 °C) seedlings. Freezing tolerance was evaluated based on the recovery rate (%) of seedlings from freezing damage at −15 °C for 6 h. This simple bioassay could effectively detect line differences: the seedling recovery rate in the Vrn-A1 NIL (5.5%) was significantly (<0.1%) lower than that of TD (51.3%) (Fig. 1). The Vrn-B1 NIL also showed a lower mean recovery rate (31.9%) than TD (53.4%), although the difference was not statistically significant (>5%). The results showed that at least the NIL carrying a dominant Vrn-1 allele was more sensitive to the freezing stress than the non-carrier line.

Transcript levels of WAP1, Wcbf2, and Cor15 genes in TD and the Vrn-1 NILs during early stages of LT treatment
WAP1, which is a candidate gene for Vrn-1, is considered as a key gene in the regulatory pathway controlling the phase transition from vegetative to reproductive growth in wheat and barley (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003). WAP1 expression was previously reported to be induced within 7 d of LT treatment in the TD NILs with the dominant Vrn-A1 allele, while the appearance of transcript was delayed until a much later stage (detected at day 35) in TD (Murai et al., 2003). WAP1 gene expression was monitored during the early stages of LT treatment (within 24 h) by RT-PCR analysis. In TD and the Vrn-A1 NIL (TD(A1)), the WAP1 transcript began to

Fig. 1. Bioassay for freezing tolerance in TD and NILs for the Vrn-1 loci. Two-week-old wheat seedlings were cold-acclimated at 4 °C for 3 weeks, frozen at −15 °C for 6 h, and returned back to the standard temperature conditions. Comparisons of freezing tolerance of the non-carrier line (TD) with (A) the Vrn-A1 line (TD(A1)) and (B) the Vrn-B1 line (TD(B1)). Recovery rates are presented as mean percentages of surviving seedlings ± standard errors (n=4–6). An asterisk indicated statistical significance at the 1% level (Student’s t-test).
accumulate after 24 h of LT treatment, while all other NILs showed constitutive expression of the WAP1 gene (Fig. 2). The NIL (TD(A1B1ppd)) carrying ppd in addition to Vrn-A1 and Vrn-B1 showed similar levels of accumulation of the WAP1 transcripts to those in the NILs carrying Vrn-A1, Vrn-B1, and Vrn-A1B1, suggesting no effects of the Ppd locus on WAP1 expression.

Arabidopsis CBF/DREB1 transcripts begin accumulating within 15 min after exposure of plants to low temperature (Jaglo et al., 2001). Rapid induction and/or enhancement of CBF/DREB1 homologues was also reported in other plant species including barley (Choi et al., 2002; Xue, 2003). The patterns of induction/enhancement of Wcbf2 gene expression were compared among TD and the Vrn-1 NILs using RT-PCR analysis. Low amounts of Wcbf2 transcript were detected in the untreated seedlings of all the lines, confirming that the Wcbf2 gene is constitutively expressed at a low level under normal temperature conditions. In TD, the transcript level increased rapidly to reach a maximum within 2–4 h of LT treatment (Fig. 2). Although the time attaining the maximum level appeared to be the same in the Vrn-A1 and Vrn-B1 NILs as in TD, the magnitude of transcript accumulation was much lower in these NILs than in TD. The inhibitory effect of the chromosomal regions each carrying Vrn-A1 and Vrn-B1 (respectively Vrn-A1 and Vrn-B1 regions) appeared to be additive, because fewer Wcbf2 transcripts accumulated in the NILs carrying both Vrn-A1 and Vrn-B1 (TD(A1B1) and TD(A1B1ppd)). It has previously been predicted that a wheat transcription factor gene Wcbf2 regulates the LT inducibility of a wheat Cor gene, Wcor15, which possesses CRT/DRE-like sequence motifs in its promoter region (Takumi et al., 2003a, b). Therefore, the Wcor15 gene expression was examined by northern blot analysis. In all the lines, the Wcor15 transcript was detected after 6–8 h of LT treatment (Fig. 3A), which was much later than the time of accumulation of the Wcbf2 transcript (Fig. 2), indicative that the expression of Wcbf2 can lead to the expression of Wcor15. A comparison after 24 h showed that the NILs with dominant Vrn-1 loci accumulated lower amounts of Wcor15 transcript than TD. The observation suggested that the negative regulation of the transcript accumulation of Wcbf2 and Wcor15 by the Vrn-1/Fr-1 intervals were apparent within 1 d of LT treatment.

Transcript levels of WAP1, Wcbf2, and Cor/Lea genes in TD and the Vrn-1 NILs during long-term LT treatment

WAP1 gene expression was monitored at the later stages of LT treatment (up to 35 d) by RT-PCR analysis. During long-term LT treatment, the WAP1 gene was LT-inducible and the amount of its transcript markedly increased in TD (Fig. 4), and the amount of WAP1 transcript either increased in TD(A1) and TD(A1B1ppd) or remained fairly constant in...

Fig. 2. An early phase of the WAP1 and Wcbf2 gene expression in response to LT treatment (4 °C) in TD and the Vrn-1 NILs having different combinations of alleles in the Vrn-1/Fr-1 intervals. Transcript accumulation of the WAP1 and Wcbf2 genes within 24 h of LT treatment was monitored by RT-PCR analysis using the gene-specific primers. The ubiquitin gene (Ubi) was used as an internal standard. PCR cycles are indicated at the right side of each panel. TD, a non-carrier line possessing recessive vrn-1 alleles at all three Vrn-1 homoeologous loci; TD(A1), a Vrn-A1 NIL with the Vrn-A1 allele; TD(B1), a Vrn-B1 NIL with the Vrn-B1 allele; TD(A1B1), a NIL carrying both Vrn-A1 and Vrn-B1 alleles; TD(A1B1ppd), a NIL carrying both Vrn-A1 and Vrn-B1 alleles in addition to the recessive ppd allele.
It was reported that different genotypes of the Vrn-A1 region affected differently the expression of two wheat Cor genes, Wcs120 and Wcs19, in the reciprocal NILs of a winter cultivar ‘Norstar’ and a spring cultivar ‘Manitou’ (Danylyuk et al., 2003). The expression levels of five Cor/Lea genes (Wcor14, Wcor15, Wlt10, Wdhn13, and Wrab17) in response to the prolonged LT treatment were compared by northern blot analysis. The five Cor/Lea genes were isolated from a cDNA library of cold-acclimated winter-hardy common wheat cultivar ‘Mironovskaya 808’ (M808) and their cold-responsiveness had been proved in previous studies (Tsvetanov et al., 2000; Ohno et al., 2001, 2003; Tsuda et al., 2000; Takumi et al., 2003b; Kobayashi et al., 2004). No Cor/Lea transcripts were detected under the normal temperature condition in all the NILs (Fig. 5). After LT treatment, the amount of Cor/Lea transcripts increased and reached maximum levels within 3–14 d, depending on the genes in TD. Although the observed patterns of expression differed among the Cor/Lea genes, the amounts of transcripts were generally much lower in the Vrn-1 NILs than in TD until day 28. At day 35, the Vrn-B1 NIL alone showed varying amounts of Cor/Lea transcripts. No reasonable explanation could be found for this, but some instability in the amounts of Cor/Lea transcripts was repeatedly observed at this stage in this NIL. Additive and suppressive effects of the Vrn-A1 and Vrn-B1 regions was observed, particularly for Wdhn13 throughout the period, suggesting some differential effects of the Vrn-1/Fr-1 intervals on the expression of different Cor/Lea genes. Together, these gene expression data showed that, in the Vrn-1 NILs, expression of the Cor/Lea genes was not maintained during the prolonged LT treatment, which was correlated with the reduced levels of the Wcbf2 gene expression and the higher freezing sensitivity in these NILs than in TD. The NIL (TD(A1B1ppd)) carrying the recessive ppd allele in addition to Vrn-A1, Vrn-B1 showed similar levels of accumulation of the Cbf2 and Cor/Lea transcripts to those in the Vrn-A1 and Vrn-B1 NILs, suggesting that the Ppd locus has little effect on the expression levels of these LT-inducible genes. The observed correlation between the Wcbf2 and Cor/Lea gene expression suggested that the Vrn-1/Fr-1 intervals effectively suppressed the expression of Cor/Lea genes through suppression of the upstream transcription factor Wcbf2 in the LT signal transduction pathway in wheat.

Levels of COR/LEA proteins in TD and the Vrn-1 NILs during long-term LT treatment

It has been previously been shown that accumulation of the COR/LEA proteins followed that of the Cor/Lea transcripts with some time lags after LT treatment (Ohno et al., 2003; Kobayashi et al., 2004). Western blot analysis using polyclonal antibodies showed that the levels of WCOR14 and WCOR15 proteins increased steadily after LT treatment in TD, while that of WDHN13 showed some fluctuations...
The amounts of WCOR14 and WCOR15 proteins in TD continued to increase even after the transcript levels showed substantial decreases after day 7, suggesting their stability under the LT condition. All of the Vrn-1 NILs showed decreased amounts of WCOR14 compared with TD throughout the LT treatment, and the Vrn-A1 and Vrn-B1 regions showed an additive effect on the reduction in protein accumulation. WCOR15 accumulated at a much higher level than that of WCOR14 throughout the period. Although not as clear as in WCOR14, perhaps due to the higher expression level, WCOR15 showed similar changes in the Vrn-1 NILs compared with TD. The level of WDHN13 was much lower than the two COR proteins and became barely detectable after day 21 in all the Vrn-1 NILs. No apparent differences...
were observed in the effect of the NIL carrying the *Vrn-A1*, *Vrn-B1* and *ppd* alleles (TD(A1B1ppd)) compared with the NIL carrying the *Vrn-A1* and *Vrn-B1* alleles (TD(A1B1)).

**Discussion**

The patterns and levels of expression of the *Cor/Lea* genes and their putative transcription factor gene *Wcbf2* differed significantly between the non-carrier line TD and the *Vrn-1* NILs during the cold acclimation period. In general, expression of the *Wcbf2* gene was up-regulated in all the lines under the LT condition, and its magnitude was much greater in TD than in the *Vrn-1* NILs (Figs 2, 4). In the NILs carrying the dominant *Vrn-1* allele, the steady-state levels of the *Wcbf2* transcript was much lower than in TD during the early response phase and further decreased or became undetectable during the later stage until day 28. The suppressive effect of the *Vrn-1* region on *Wcbf2* gene expression was much greater than that of the *Vrn-B1* region and their effect appeared to be additive. The *Cor/Lea* transcripts showed co-ordinated patterns of accumulation with that of the *Wcbf2* transcript in all the lines (Figs 3, 5). The lower levels of *COR/LEA* proteins in the *Vrn-1* NILs than in TD reflected their lower levels of *Cor/Lea* transcript accumulated after LT treatment (Fig. 6). According to previous studies, a winter-type wheat cv. M808 acquired much higher levels of freezing tolerance than a spring-type cv. CS during the cold acclimation period. In the cold acclimation process, the *Cor/Lea* genes were up-regulated and the *COR/LEA* proteins were accumulated more in M808 than in CS, indicating that most members of the *Cor/Lea* regulon participate in developing freezing tolerance (Ohno et al., 2000; Kobayashi et al., 2004). The *Vrn-A1* NIL showed a significantly lower level of freezing tolerance than TD in the bioassay performed here after 21 d of LT treatment (Fig. 1). The *Vrn-B1* NIL also showed a lower mean value of freezing tolerance, although its statistical significance could not be shown at the 5% level. At the time of bioassay, the *Cor/Lea* transcripts were hardly detectable in the *Vrn-1* NILs (Fig. 5) and their levels of *COR/LEA* proteins were lower than those in TD (Fig. 6). Thomashow (1999) hypothesized that the *Vrn-A1/Fr-A1* interval possibly encodes a protein(s) involved in regulating the expression of cold-inducible genes that have roles in freezing tolerance. This bioassay and gene expression study strongly suggests that at least the *Vrn-A1/Fr-A1* interval plays a critical role in cold acclimation and freezing tolerance through the CBF/DREB1-mediated signal pathway in wheat. This result also suggests that the *Vrn-A1/Fr-A1* interval exerts a larger negative effect on cold acclimation and freezing tolerance than the *Vrn-B1/Fr-B1* interval.

WAPI is a likely candidate of *Vrn-1* in winter wheat (Yan et al., 2003; Trevaskis et al., 2003; Danyluk et al., 2003; Murai et al., 2003). Danyluk et al. (2003) showed that accumulation of the TaVRT-1 (*WAP1*) transcript was associated with the down-regulation of *Cor* genes, and thus they suggested that *Vrn-A1* was a major allele suppressing *Cor* gene expression and thereby lowering freezing tolerance in wheat. To examine if the *WAPI* gene is involved in the regulation of *Wcbf2* and *Cor/Lea* gene expression, RT-PCR analysis of the *WAPI* transcript in the *Vrn-1* NILs after LT treatment was performed (Figs 2, 4). WAPI gene expression was LT-inducible in TD and also in the *Vrn-A1* NIL, but constitutive in the other *Vrn-1* NILs. Danyluk et al. (2003) showed that the *WAPI* transcript was associated with the down-regulation of *Cor/Lea* genes, and thus they suggested that at least the *Vrn-A1/Fr-A1* interval exerts a larger negative effect on cold acclimation and freezing tolerance than the *Vrn-B1/Fr-B1* interval.
higher in the Vrn-1 NILs than in TD throughout the period studied. No apparent relationship was found between the expression pattern and level of WAP1 and those of Wcbf2 and Cor/Lea genes under the LT condition in the Vrn-1 NILs, indicating that WAP1 is not directly involved in the down-regulation of the CBF/DREB1-mediated Cor/Lea gene expression.

The Vrn-A1 locus is known to be tightly linked with the Fr-A1 locus on the long arm of chromosome 5A in wheat (Galiba et al., 1995). It is generally expected that spring-type wheat possesses a ‘spring-type’ Fr-A1 allele linked with a dominant Vrn-A1 allele in the Vrn-A1/Fr-A1 interval. By contrast, winter-type wheat should have a ‘winter-type’ Fr-A1 allele linked with a recessive vrn-A1 allele, reflecting the necessity of winter-type wheat to be equipped with the ‘winter-type’ Fr-A1 allele for cold adaptation. The higher levels of Wcbf2 and Cor/Lea gene expression and freezing tolerance in the non-carrier line TD than in the Vrn-A1 NIL could be ascribed to the possession of the effective ‘winter-type’ Fr-A1 allele in TD and the lack of it in the Vrn-A1 NIL. Considering the construction process of TD and the Vrn-1 NILs, the Vrn-A1 NIL might possess ‘winter-type’ Fr-B1/Fr-D1, while the Vrn-B1 NIL might possess ‘winter-type’ Fr-A1/Fr-D1. Since the Vrn-B1 NIL showed a higher freezing tolerance than the Vrn-A1 NIL, the Fr-A1 locus probably provoked a greater effect on the Wcbf2 and Cor/Lea gene expression and freezing tolerance in wheat. These results also suggest that the Fr-1 loci are additive in promoting the development of freezing tolerance. The exact conditions of the Vrn-1 and Fr-1 alleles in the Vrn-1/Fr-1 intervals on the homoeologous group 5 chromosomes in wheat should be clarified.

With respect to the Ppd locus, no detectable effect was observed on WAP1, Wcbf2, and Cor/Lea gene expression under the long-day condition (16 h light). It was reported that the expression of Ppd reduces the delay of heading time in wheat under short-day conditions, and no differences were observed in the level of WAP1 gene expression between the TD NILs, with and without the Ppd allele under long-day conditions (Murai et al., 2003). This result thus supports that the WAP1 and Ppd genes act on different pathways in the promotion of the phase transition from vegetative to reproductive growth.

In barley, three CBF/DREB1 homologues, HvCBF3, HvCBF4, and HvCBF8, were mapped on the long arm of chromosome 5H (Choi et al., 2002; Francia et al., 2004) and the existence of a multi-locus cluster of the HvCBF loci was demonstrated (Von Zitzewitz et al., 2003). It is clearly demonstrated in barley that a mapped HvCBF locus is the best candidate to explain a QTL of frost tolerance and is colinear with the Fr-A2 locus, thus named Fr-H2 (Francia et al., 2004). The Fr-H1 locus was also mapped as a QTL by these authors, far from any HvCBF loci, but in a tight linkage with the Vrn-H1 locus. 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 (Vágújfali et al., 2003). Chromosomal location of the CBF/DREB1 homoeologues of wheat, including Wcbf2, remains unknown, but the high homology of the Wcbf2 homoeologues to the barley HvCBF genes suggests that Wcbf2 is probably located on the homoeologous group 5 chromosomes in the wheat genome. This expression study suggests that the Fr-1 loci can control Wcbf2 gene expression and thus up-regulation of the downstream Cor/Lea gene expression, at least partly through the CBF transcription factor. Expression of Wcor14, Wcor15, and Wht10 is induced specifically by LT in wheat (Tsvetanov et al., 2000; Takumi et al., 2003b; Ohno et al., 2000; Kobayashi et al., 2004), while Wrab17 is induced by both LT and ABA treatment (Tsuda et al., 2000; Kobayashi et al., 2004). The LT-inducible expression pattern of Wdhn13 differs from those of other Cor/Lea genes (Fig. 2; Kobayashi et al., 2004). Although the 5’ upstream regions of Wrab17 and Wdhn13 possess putative CRT/DRE-like motifs (F Kobayashi et al., unpublished results) similar to Wcor15 (Takumi et al., 2003b), regulatory networks of the LT-responsive expression of the wheat Cor/Lea genes might be controlled, not only by the CBF/DREB1 pathway, but also by other pathways such as ABA-dependent and/or MYC/MYB-pathways. It seems to be reasonable that at least the Fr-A1 locus, or another unknown linked gene, instead of vrn-A1 (WAP1), is a master locus functioning upstream of cold-signal pathways that are mediated through transcription factors and that lead to the expression of Cor/Lea genes and thus to freezing tolerance in wheat.

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References


