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(Citation)

Journal of Experimental Botany, 56(413):887-895

(Issue Date)

2005-01

(Resource Type)

journal article

(Version)

Accepted Manuscript

(URL)

<https://hdl.handle.net/20.500.14094/90000348>



**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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Date of submission: 27 May 2004

Date of submission of the revised form: 1 Oct. 2004

Date of submission of the final form: 29 Oct. 2004

Number of Figures: 6

Running title: Regulation of wheat *CBF* and *Cor/Lea* expression by *Vrn-1/Fr-1*

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. Our 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.

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

Introduction

Major loci and QTLs controlling cold/freezing tolerance have been mapped and their chromosomal syntenic has 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; Tóth et al., 2003; Snape et al., 1997). 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 over-wintering 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; Iwaki et al., 2002; Tóth et al., 2003; Snape et al., 1997, 1998). Galiba et al. (1995) estimated the genetic distance of the *Vrn-A1/Fr-A1* interval to be ca. 2-cM. In contrast, the genetic distance of *Vrn-D1/Fr-D1* was much larger at ca. 10-cM (Snape et al., 1997) and that of *Vrn-B1/Fr-B1* was ca. 40-cM (Toth et al., 2003). Homozygous deletion lines for the long arm of 5A (5AL), which were generated by taking advantages 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. In 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 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 13% higher survival rate than the lines lacking it (Sutka et al., 1999).

Recently, Yan et al. (2003) isolated the *VRN1* (*Vrn-A^m1*) gene of *T. monococcum* by positional cloning and suggested that a wheat homolog of the *API* (*APETALA1*) MADS-box gene is a candidate gene for *VRN1*. *VRN1* is now considered to be an ortholog of *WAP1* (wheat *API*) 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., 2003; Trevaskis et al., 2003). These results suggest that the *WAP1* is likely a 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 much efforts for mapping the *Vrn-1/Fr-1* intervals in common wheat, however, 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 adaptation of over-wintering 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 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/DREB1B*, *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-Ottosen 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). We also have isolated and characterized two cDNAs (accession nos. AB178166 and AB178167) of a wheat CBF ortholog named as *Wcbf2* (Takumi et al., 2003a). In barley, three *CBF/DREB1* homologs, *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 likely located on chromosomes 5 based on the synteny with the barley *CBFs*. A regulatory effect of 5AL on the *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 COR/LEA protein accumulation (Limin et al., 1997; Danyluk et al., 1998). Relationships among vernalization response, *Cor/Lea*

gene expression and freezing tolerance were further shown in near-isogenic lines of wheat cv. 'Norstar' and 'Manitou' for the *Vrn-A1* locus (Danyluk et al., 2003).

Despite the significant progress, the role of the *Vrn-1/Fr-1* loci on the CBF-mediated *Cor/Lea* gene expression remains unclear. We herein used the *Vrn-1* near-isogenic lines (NILs) of a spring cv. 'Triple Dirk' to investigate 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. We compared the expression profile of the *WAP1* gene with that of the *Wcbf2* and *Cor/Lea* genes after LT treatment. Our results supported that cold/freezing tolerance in wheat was not directly associated with the *Vrn-1* loci. We discussed 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.

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(*Al*) and TD(*Bl*) carrying dominant *Vrn-Al* and *Vrn-Bl* alleles, respectively, were examined in the bioassay for freezing tolerance and the gene expression studies. TD(*AlBl*) carrying both *Vrn-Al* and *Vrn-Bl* alleles and TD(*AlBlppd*) carrying *ppd* (a recessive allele of the photoperiod-responsive *Ppd* locus) in addition to *Vrn-Al* and *Vrn-Bl* 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 vs TD(*Al*) and TD vs TD(*Bl*)) were planted in pots (25 cm x 12 cm in width and 12 cm in depth) with 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 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (at the pot height) provided by cool white

fluorescent lamps. The 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 transferred to 4°C and cold-acclimated for 3 weeks, and then treated with a single freezing temperature at -15°C for 6 h in the darkness. The frozen seedlings were thawed overnight at 4°C and transferred back to the standard temperature condition. On the fifth day after the transfer, the number of seedlings showing growth recovery was recorded. The experiment was repeated four to six times and the data were analyzed using the Student's *t*-test. This bioassay method was previously proven to be effective to evaluate the levels of freezing tolerance of winter- and spring-type cultivars of wheat (Ohno et al., 2001; Kobayashi et al, 2004).

RT-PCR analysis

First strand cDNA was synthesized from 1 µg of DNaseI-treated total RNA with 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'-ATCAGACTCAGCCTCAAACA-3' and

5'-TAGAGACGGGTATCATGGAA-3' for *WAP1*, and
5'-CTCAAACCAACCTGCAAC-3' and 5'-AAGCGTTTTTGACATTACATTA-3' for
Wcbf2. As an internal control, a fragment from the wheat ubiquitin gene was
amplified with the primer set; 5'-GCATGCAGATATTTGTGAA-3' and
5'-GGAGCTTACTGGCCAC-3'. We carefully manipulated the RT-PCR conditions 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 *Wrab17*) were previously characterized
(Tsvetanov et al., 2000; Tsuda et al., 2000; Ohno et al., 2001, 2003; Takumi et al.,
2003b; 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 indicated periods. RNA extraction and northern blot were performed according to Kobayashi et al. (2004).

Immunoblot analysis

Polyclonal antibodies against WCOR14, WCOR15 and WDHN13 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 effects of the *Vrn-1*/*Fr-1* intervals on freezing tolerance, we compared 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*)), 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-A1* 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 day of LT treatment in the TD NILs with the dominant *Vrn-A1* allele, while the appearance of transcript was delayed until much later stage (detected at day 35) in TD (Murai et al., 2003). We monitored the *WAP1* gene expression at 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 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*, *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 the *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* homologs was also reported in other plant species including barley (Choi et al., 2002; Xue, 2003). We compared the patterns of induction/enhancement of the *Wcbf2* gene expression among TD and the *Vrn-1* NILs employing 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 to 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 less *Wcbf2* transcripts accumulated in the NILs carrying both *Vrn-A1* and *Vrn-B1* (TD(*A1B1*) and TD(*A1B1ppd*)). We have previously 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; 2003b). We therefore studied the *Wcor15* gene expression by northern blot analysis. In all the lines, the *Wcor15* transcript was detected after 6 to 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 the 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 a day of LT treatment.

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

We monitored the *WAP1* gene expression at the later stages of LT treatment (up to 35 day) 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 (Fig4), and the amount of *WAP1* transcript either increased in TD(*AI*) and TD(*AIB1ppd*) or remained fairly constant in TD(*BI*) and TD(*AIB1*). The time of up-regulation in the *Vrn* NILs, however, was much earlier than TD and they accumulated more *WAP1* transcript than TD throughout the studied period. The *Wcbf2* gene expression was also monitored during the long-term LT treatment. The rapid increase until 24 h (Fig. 2) was followed by a temporal decline in the amount of *Wcbf2* transcript but thereafter it increased

steadily until day 21 to 28 in TD (Fig. 4). The *Wcbf2* gene expression was thus up-regulated by the prolonged LT treatment in the non-carrier line TD. In the three NILs carrying the *Vrn-A1* allele, (TD(*A1*), TD(*A1B1*) and TD(*A1B1ppd*)), however, the amount of *Wcbf2* transcript decreased after day 3 and became barely detectable until day 28. A similar expression profile was observed in the *Vrn-B1* NIL (TD(*B1*)), although the suppressive effect of the *Vrn-B1* region was lower than that of the *Vrn-A1* region. The suppressive effect of the *Vrn-A1* and *Vrn-B1* regions appeared to be additive on the *Wcbf2* gene expression.

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’ (Danyluk et al., 2003). We compared the expression levels of five *Cor/Lea* genes (*Wcor14*, *Wcor15*, *Wlt10*, *Wdhn13* and *Wrab17*) in response to the prolonged LT treatment 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 was proven in our previous studies (Tsvetanov et al., 2000;

Takumi et al., 2003b; Ohno et al., 2001, 2003; Tsuda et al., 2000; Kobayashi et al., 2004).

No *Cor/Lea* transcripts were detected under the normal temperature condition in all the lines (Fig. 5). After LT treatment, the amount of *Cor/Lea* transcripts increased and reached maximum levels within 3 to 14 days 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 we repeatedly observed some instability in the amounts of *Cor/Lea* transcripts at this stage in this NIL. Additive and suppressive effect 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, our 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

We have previously 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 (Fig. 6). 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 the 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 and 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-A1* region on the *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 coordinated patterns of accumulation with that of the *Wcbf2* transcript in all the lines (Figs. 3 and 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 our 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* reguron 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 our bioassay performed after 21 day 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. Our 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. Our 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.

WAP1 is a likely candidate of *Vrn-1* in 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 the *Cor* gene expression and thereby lowering freezing tolerance in wheat. To examine if the *WAP1* gene is involved in the regulation of the *Wcbf2* and *Cor/Lea* gene expression, we performed RT-PCR analysis of the *WAP1* transcript in the *Vrn-1* NILs after LT treatment (Figs. 2 and 4). The *WAP1* 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 *WAP1* was inducible only in winter types of wheat and rye, whereas it was constitutively expressed in spring types of wheat and barley. Trevaskis et al. (2003) using the same *Vrn-1* NILs of TD showed that the expression of *WAP1* was constitutive in the *Vrn-A1* NIL and at a higher level than in the *Vrn-B1* under the normal temperature condition. Our observation that *WAP1* expression in the *Vrn-A1* NIL was LT-inducible and up-regulated just like in TD seems to disagree with the above results. Our result however is consistent with that of Murai et al. (2003) showing that the *WAP1* transcript was barely detectable in the *Vrn-A1* NIL under normal temperature condition but its level was greatly increased after 7 day of LT treatment. Murai et al. (2003) also suggested that the *WAP1* gene expression is developmentally regulated, i.e. the *WAP1*

transcript was detected only after 7-leaf stage in non-vernalized wheat plants under long-day conditions. The discrepancy in the non-treated condition might be due to the different plant age used (7-day-old in our experiment, 19-day-old in Danyluk's and 14-days-old in Trevaskis's) or to other unknown reason. An important point is that the magnitude of up-regulation of the *WAP1* gene expression by LT treatment was much greater in TD than in the *Vrn-1* NILs and conversely that the *WAP1* transcript level was higher in the *Vrn-1* NILs than in TD throughout the studied period. 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 the *WAP1* is not directly involved in the down-regulation of the CBF/DBRED1-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. Oppositely, 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 likely provoke a greater effect on the *Wcbf2* and *Cor/Lea* gene expression and freezing tolerance in wheat. Our 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, we did not observe any detectable effect on the *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 the *WAP1* gene

expression between the TD NILs with and without the *Ppd* allele under long-day conditions (Murai et al., 2003). Our result thus support 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* homologs, *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 co-linear 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 (Vágújfalvi et al., 2003). Chromosomal location of the *CBF/DREB1* homologs of wheat including *Wcbf2* remains unknown, but the high homology of the *Wcbf2* homoeologs to the barley *HvCBF* genes suggests that *Wcbf2* is likely located on the

homoeologous group 5 chromosomes in the wheat genome. Our expression study suggests that the *Fr-1* loci can control the *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 *Wlt10* 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 (our 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.

Acknowledgements

The work was supported by a 'grant-in-aid' from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to CN, no. 13306002). Contribution no. 166 from the Laboratory of Plant Genetics, Faculty of Agriculture, Kobe University.

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Legends of Figures

Figure 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 condition.

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 *t*-test).

Figure 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(*AI*), 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.

Figure 3. An early phase of the *Wcor15* gene expression in response to LT treatment in TD and the *Vrn-1* NILs. Northern blots were probed with ³²P-labeled *Wcor15* cDNA. (A) A time-course of the *Wcor15* transcript accumulation within 24 h of LT treatment. (B) A comparison of the *Wcor15* transcript levels after 24 h LT treatment among TD and the *Vrn-1* NILs. The electrophoresis patterns of rRNAs are shown. For the line designations, see the legends to Fig. 2.

Figure 4. A later 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. Accumulation patterns of the *Wcbf2* and *WAP1* transcripts during the prolonged LT treatment in TD and the *Vrn-1* NILs.

Expression of the *Wcbf2* and *WAP1* genes was monitored for 35 days by RT-PCR analysis using the gene-specific primers. The ubiquitin gene (*Ubi*) was used as an internal standard. PCR cycles are indicated at right side of the each panel.

For the line designations, see the legends to Fig. 2.

Figure 5. Accumulation patterns of the *Cor/Lea* transcripts during the long-term LT treatment in TD and the *Vrn-1* NILs. Northern blots were probed with ³²P-labeled cDNAs of *Wcor14*, *Wcor15*, *Wdhn13*, *Wlt10* and *Wrab17*. For the line designations, see the legends to Fig. 2.

Figure 6. Accumulation patterns of the WCOR14, WCOR15 and WDHN13 proteins during the long-term LT treatment in TD and the *Vrn-1* NILs. Polyclonal antibodies against WCOR14, WCOR15 and WDHN13 were used for the immunoblot analysis. For the line designations, see the legends to Fig. 2.

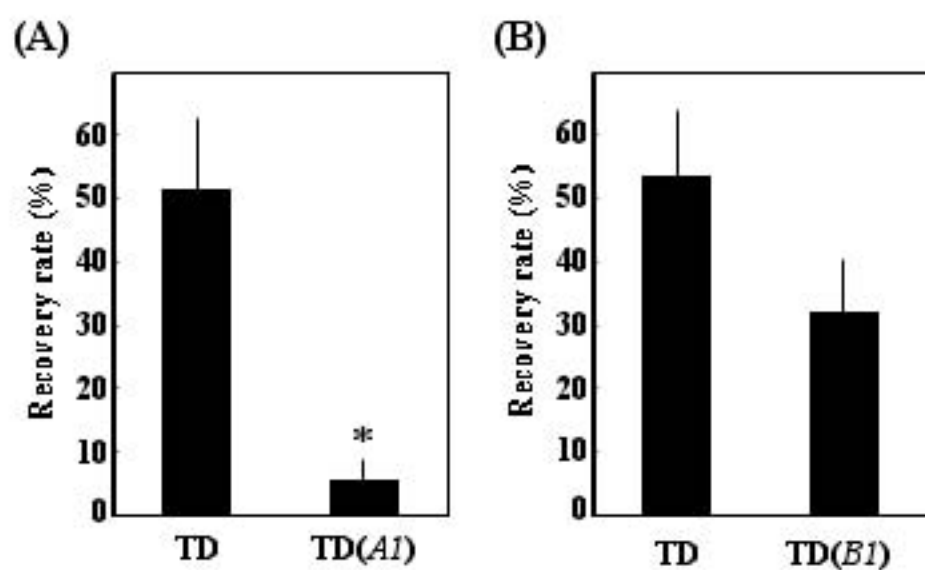


Fig. 1 (Kobayashi et al.)

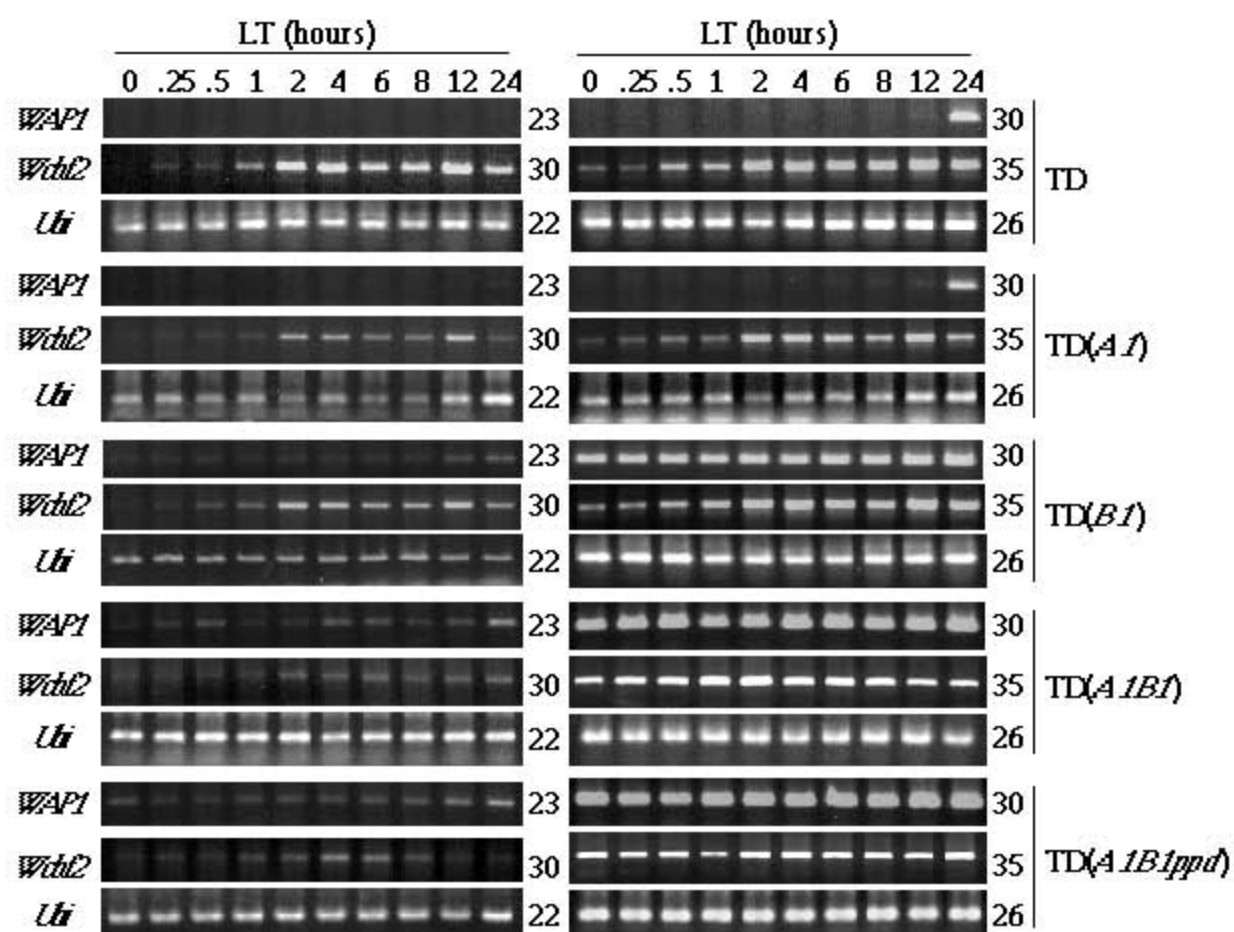


Fig. 2 (Kobayashi et al.)

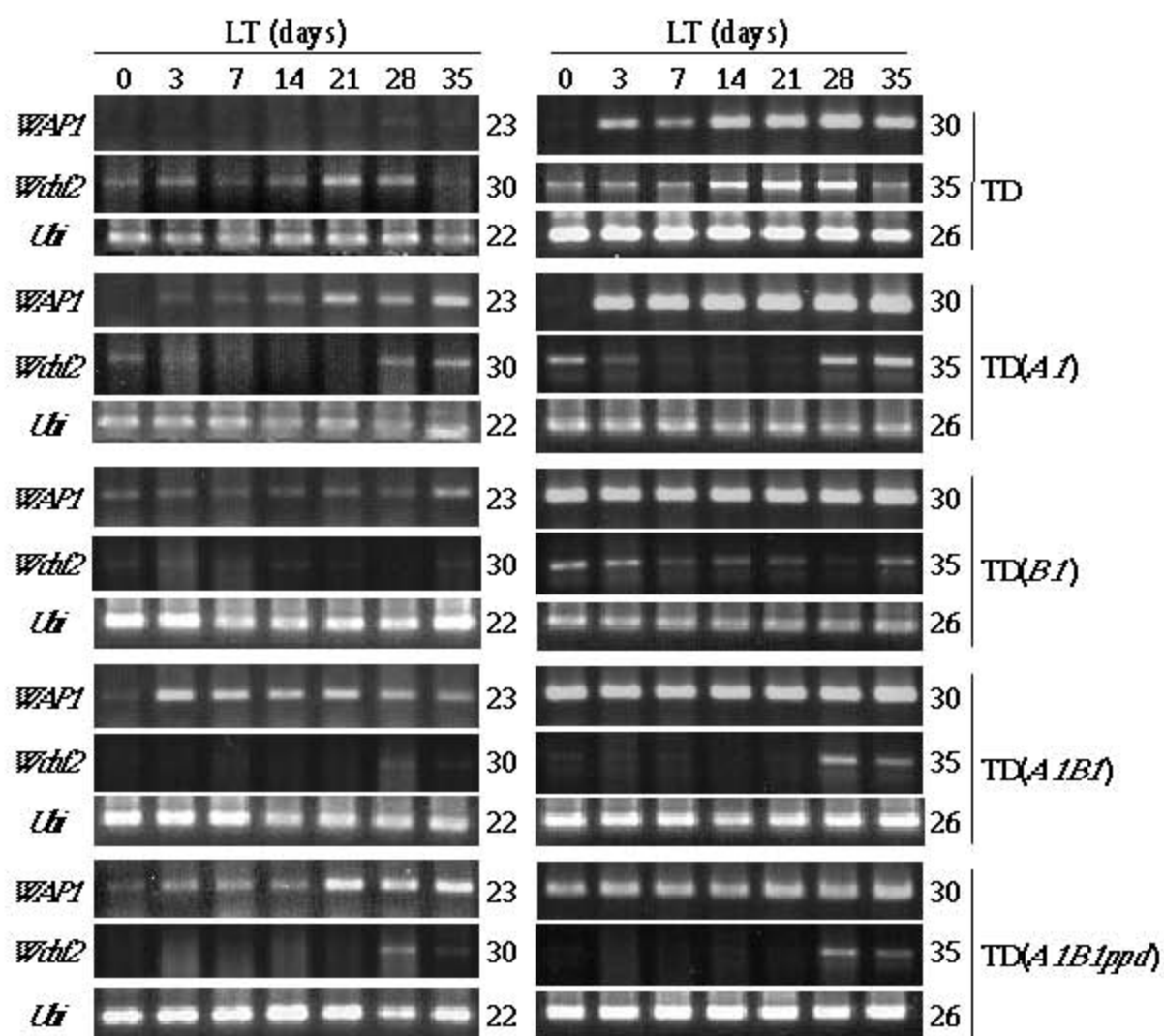


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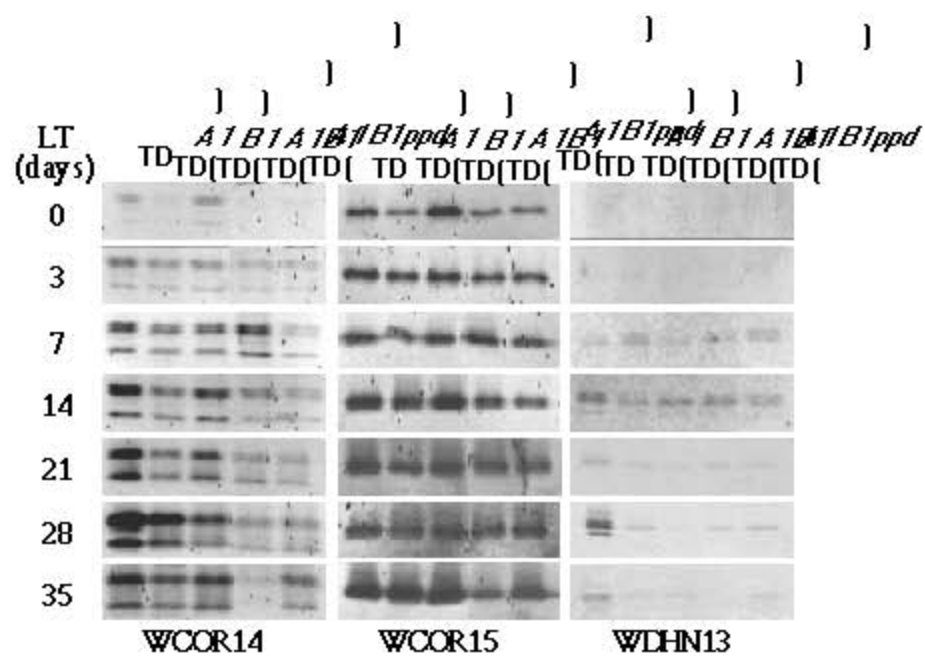


Fig. 6 (Kobayashi et al.)