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ORIGINAL PAPER

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The cold-regulated transcriptional activator *Cbf3* is linked to the frost-tolerance locus *Fr-A2* on wheat chromosome 5A

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Abstract Wheat chromosome 5A plays a key role in cold acclimation and frost tolerance. The major frost tolerance gene *Fr-A1* (formerly *Fr1*) and two loci that regulate the transcription of cold-regulated genes (*Cor*) have previously been mapped on the long arm of this chromosome. In this study we report the discovery of a new locus for frost tolerance designated *Fr-A2*. This new locus was mapped on the long arm of chromosome 5A of diploid wheat (*T. monococcum*), 40 cM from the centromere and 30 cM proximal to the major frost tolerance locus *Fr-A1*. We found also that frost-tolerant and frost-susceptible *T. monococcum* parental lines differed in the transcription level of the cold induced gene *Cor14b* when plants were grown at 15°C. Transcription levels of this gene were measured in each of the recombinant inbred lines and mapped as a QTL that perfectly overlapped the QTL for frost survival at the *Fr-A2* locus. This result suggested that frost tolerance in this cross was mediated by differential regulation of the expression of the *Cor* genes. In a previous study in hexaploid wheat (*T. aestivum*) we had shown that *Cor14b* was regulated by two loci located on chromosome 5A, one in the same chromosome region as the *T. monococcum Fr-A2* locus and the other one closely linked to *Fr-A1*. Since CBF transcriptional activators in *Arabidopsis* regulate *Cor* genes and are involved in frost tolerance, we decided to localize the cold-regulated *CBF*-like barley gene *Cbf3* on the *T. monococcum* map. This gene was mapped on the peak of the *Fr-A2* QTL for frost tolerance. This result suggests that the observed differential regulation of *Cor14b* at the *Fr-A2* locus is due to allelic variation at the *XCbf3* locus, and that this transcriptional activator gene might be a candidate gene for the *Fr-A2* frost tolerance locus on wheat chromosome 5A.

Keywords Frost tolerance · *Cor* genes · CBF transcription factors · Cold acclimation

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Introduction

Freezing temperatures limit the geographical distribution of wheat (*T. aestivum* L.) and often cause severe losses in agricultural productivity. Therefore, increasing frost tolerance has been a major objective for most breeding programs in regions subject to severe winters. Frost-tolerant wheat varieties show an increase in freezing tolerance after exposure to low, non-freezing, temperatures, a phenomenon known as cold acclimation (Sakai and Larcher 1985). During cold acclimation, winter cereals adjust their metabolism to low temperatures and protect critical cell structures against the effect of freezing temperatures.

The genetic control of frost tolerance in wheat is complex, and at least 10 of the 21 pairs of chromosomes are involved in the regulatory gene network. However, the major genes affecting winter hardiness have been mapped on the long arms of homeologous groups 5 (Roberts 1990; Sutka 1994; Sutka and Snape 1989; Veisz and Sutka 1993). The major frost tolerance locus, *Fr-A1* (formerly *Fr1*), was mapped on the long arm of chromosome 5A, 2-cM proximal to the vernalization gene *Vrn-A1* (Galiba et al. 1995). Physical mapping using Chinese Spring deletion lines suggest that *Vrn-A1* and *Fr-A1* are different genes and that *Fr-A1* is located proximal to *Vrn-A1* (Galiba et al. 1995). An additional QTL for frost tolerance, designated Q*Fr.jic-5D*, was

mapped on a colinear region of chromosome 5D, 10 cM proximal to *Vrn-D1* (Snape et al. 1997).

In previous work, we mapped two loci that control the expression of the Cold regulated gene *Cor14b* to the long arm of chromosome 5A (Vágújfalvi et al. 2000). One of these genes was closely linked to *Fr-A1*, whereas the other one was linked to *Xpsr911*, an RFLP marker located approximately 35 cM proximal to *Fr-A1* (Gale et al. 1995). The *Cor* genes are strictly regulated by low temperatures, and are probably involved in the acquisition of frost tolerance (for a review, see Cattivelli et al. 2002).

The well studied *Arabidopsis* gene *Cor15a* encodes a 15-kDa protein that is targeted to the stromal compartment of the chloroplast. Over-expression of this gene in transgenic plants increases the frost tolerance not only of the chloroplasts but also of the protoplasts isolated from leaves of the non-acclimated plants (Artus et al. 1996).

In *Arabidopsis* the *Cor* genes are regulated by three members of the CBF family of transcriptional activators encoded by genes that are organized in a tandem array on chromosome 4 (for a review, see Thomashow et al. 2001). The CBF1, CBF2 and CBF3 proteins are 84% identical, suggesting that the *CBF* genes were duplicated relatively recently (Medina et al. 1999). Genes similar to the Arabidopsis *CBF* genes are present in Triticeae EST databases. One of them, barley *Cbf3*, has been recently mapped on the long arm of chromosome 5H, but in a region that has not been associated with frost tolerance in barley or wheat (Choi et al. 2002). In addition to the transcriptionally regulated *Cor* genes described above, there are other *Cor* sequences which are controlled at the post-transcriptional level (Dunn et al. 1994; Phillips et al. 1997). These *Cor* genes may represent cold-response components that are not regulated by cold-induced transcription factors.

In the present work we report the mapping of a new QTL for frost tolerance in diploid wheat *T. monococcum*) and its linkage with the *Cbf3* gene. We also show that variation at this locus is associated with differences in the threshold induction temperature of the cold-regulated gene *Cor14b*, but does not affect expression of the post-transcriptionally regulated gene *Ao86*. The interaction between this new frost tolerance locus and growth habit is discussed.

Materials and methods

Plant material and frost tolerance tests

Analyses of frost tolerance and of *Cor14b* expression were performed in a single-seed descent population derived from the F_2 population used to construct a *T. monococcum* map that included 335 RFLP loci (Dubcovsky et al. 1996). These 74 RILs were derived from a cross between *T. monococcum* ssp. *monococcum* DV92 (spring and susceptible to frost) and *T. monococcum* ssp. *aegilopoides* G3116 (winter and tolerant to frost). DNA was extracted from F_5 plants as described by Dvorak et al. (1988) and frost tolerance and *Cor14b* expression studies were performed on the F_5-derived F_6 plants.

For the *Cor14b* expression studies plants were grown in modified Hoagland solution (Nagy and Galiba 1995) for 2 weeks, with 16 h illumination per day (260 μmol/m^{2}s), at different temperatures: 5, 10, 15, 20 and 25°C. Plants were then kept at −80°C prior to Northern analysis.

For the frost tolerance tests, seeds were potted in wooden boxes in a randomized block design arrangement. Seedlings were grown in phytotronic chambers at 15/10°C (day/night), 75% relative humidity, and the light intensity was 260 μmol/m^{2}s. Hardening was initiated when the temperature was reduced to 10/5°C for 2 weeks, than to 5/0°C for another 2 weeks, and to +2/−2°C for 1 week. Then the temperature was gradually lowered to the freezing temperature, −13°C, and maintained there for 1 day. After freezing, the temperature was gradually increased to 17/16°C. At this temperature the leaves were cut several cm above the soil. Frost tolerance was estimated by assessing the re-growth of the plants, which was scored on a scale running from 0 (death) to 5 (undamaged). Twenty five individual F_6 plants were evaluated for each of 51 RILs in the first experiment in 1998, and 35 individual plants for each of 58 RILs were evaluated in the second experiment in 1999.

Northern and Southern analyses

Frozen shoots from each of the RILs were ground in liquid N_2, and total RNA was extracted using Trizol reagent (GibcoBRL Cat. No. 15596-026). Samples (20 μg) of total RNA were fractionated by electrophoresis on denaturing formaldehyde-agarose gel, and than blotted to Hybond membranes. Radioactively labeled *Cor14b* and *Ao86* barley cDNA probes (Cattivelli and Bartels 1990) were prepared by the random-primer method (Feinberg and Vogelstein 1983) and hybridization was carried out overnight. The barley gene *Ao86*, a member of the best characterized post-transcriptionally regulated *Cor* gene family *Blt14* (Grossi et al. 1998), was chosen to represent a sequence that is putatively not regulated by cold-induced transcription factors. Filters were washed three times at 65°C in 2xSSC-0.1% SDS solution, then exposed to Kodak Scientific film. To adjust the *Cor14b* hybridization signal for differences in RNA loading, filters were hybridized with a barley DNA probe coding for protein 12 of the ribosomal large subunit (RPL12), whose expression is not affected by low temperature (Baldi et al. 2001). Developed films were scanned and the expression levels of *Cor14b* and ribosomal probes in each line were quantified using Bio-Rad Molecular Analyst software (version 1.5). Southern blots and hybridizations were performed as described previously (Dubcovsky et al. 1994).

Mapping

The first whole-genome scan for frost tolerance QTLs was performed with the marker information inferred from the homozygous lines available from the F_2 generation (Dubcovsky et al. 1996). Marker information was then completed for the regions where significant QTLs for frost tolerance were detected. Ten markers previously mapped on chromosome 5A in the *T. monococcum* F_2 population were remapped in the 74 F_5 RILs, and the QTL analysis was repeated with the complete mapping data matrix. Sources of these probes have been described before (Dubcovsky et al. 1996). In addition, probes for RFLP loci *Xpsr426, Xpsr2021* (Gale et al. 1995), *Xmwg2062* (Graner et al. 1991), *Xbcd926* (Anderson et al. 1992) and the microsatellite markers *Xgwm639* and *Xgwm186* (Röder et al. 1998) were added to the map to facilitate comparisons with previous maps that include QTLs for frost tolerance (Snape et al. 2001) or genes that control the expression of *Cor14b* genes (Vágújfalvi et al. 2000). The probe from the barley *Cbf3* gene was kindly provided by Jeff Skinner in the Chen and Hayes lab (Oregon State University). This probe was cloned from the barley variety Dicktoo, and is allelic to the *Cbf3* gene from barley variety Morex (Accession No. AF298231). Maps were constructed with the software Mapmaker/EXP 3.0 and MapmakerQTL (Lander et al. 1987) using the Kosambi function (Kosambi 1943). QTL confidence intervals were calculated based on a difference of 1 LOD score with the peak of the QTL.

Nomenclature

The frost tolerance gene on the long arm of chromosome 5A has traditionally been referred to as *Fr-1* (McIntosh et al. 1998), whereas the QTL for frost tolerance mapped on chromosome 5D has been referred to as *Fr-2* (Snape et al. 1997) or *QFr.jic-5D* (Snape et al. 2001). The presence of more that one frost tolerance locus on the long arm of chromosome 5A (results from this study) and of genes with similar effects on chromosomes 5B and 5D would make the use of the sequential number gene nomenclature complicated. Therefore, we propose to use a homoeologous system of nomenclature similar to the one currently in use for the vernalization genes in wheat (McIntosh et al. 1998). Hereafter, we will refer to *Fr1* as *Fr-A1,* to the new locus described in this study as *Fr-A2,* and to the locus on 5DL as *QFr.jic-5D* until the orthology with *Fr-A1* or *Fr-A2* has been more clearly demonstrated.

For the mapped CBF locus we use the name *XCbf3* previously published by Choi et al (2002). However, this does not necessarily indicate orthology between barley clone AF298231 and the Arabidopsis *Cbf3* gene. The proteins predicted from the three closely related Arabidopsis *Cbf* genes are more similar to each other than to the protein predicted from the barley *Cbf3* sequence (data not shown).

Results

QTL analyses for frost tolerance

The molecular characterization of the 74 F_5 RILs with 10 RFLP markers from the QTL regions showed 7.8% residual heterozygosity, a slightly higher value than the theoretical 3% expected in F_5. Heterozygous markers were scored as missing data for all the QTL analyses.

Winter *T. monococcum* accession G3116 was significantly more frost tolerant than spring accession DV92 ($P < 0.0001$) after freezing at −13°C. Average frost tolerance, evaluated as survival capability on a 0 to 5 scale, was 1.9 for G3116 (95% confidence interval 1.7 to 2.1) and 0.2 for DV92 (95% confidence interval 0.0 to 0.4).

Segregation for frost tolerance scores was observed among the RILs in the 1998 (0–2.9) and 1999 (0–1.5) experiments. A QTL analysis of the seven *T. monococcum* chromosomes at a LOD threshold of 3, showed only one QTL on the long arm of chromosome 5A for both experiments and for the average frost tolerance (Fig. 1). In the 1998 experiment, a QTL with a LOD score of 7.5 was mapped in a 7-cM confidence interval, which encompassed the *Xbcd508* locus (Fig. 1). This QTL explained 48% of the variation in frost survival in this cross. A similar location was found for the QTL for frost tolerance mapped in the 1999 experiment (Fig. 1). This QTL with a LOD score of 5.0 explained 40% of the variation in frost tolerance and was mapped into a 9 cM confidence interval around locus *Xbcd508*. The combined data for both years showed a QTL with a LOD score of 8.9 with a confidence interval for the peak centered on *Xbcd508* (7 cM confidence interval). This QTL explained 49% of the variation in frost tolerance in the DV92 x G3116 cross (Fig. 1).

QTL analyses for *Cor14b* transcription levels

In our previous study in hexaploid wheat (Vágújfalvi et al. 2000) we found that that at 18/13°C (day/night temperatures) the *Cor14b* gene was transcribed in the frost-tolerant genotypes, but not in the frost sensitive

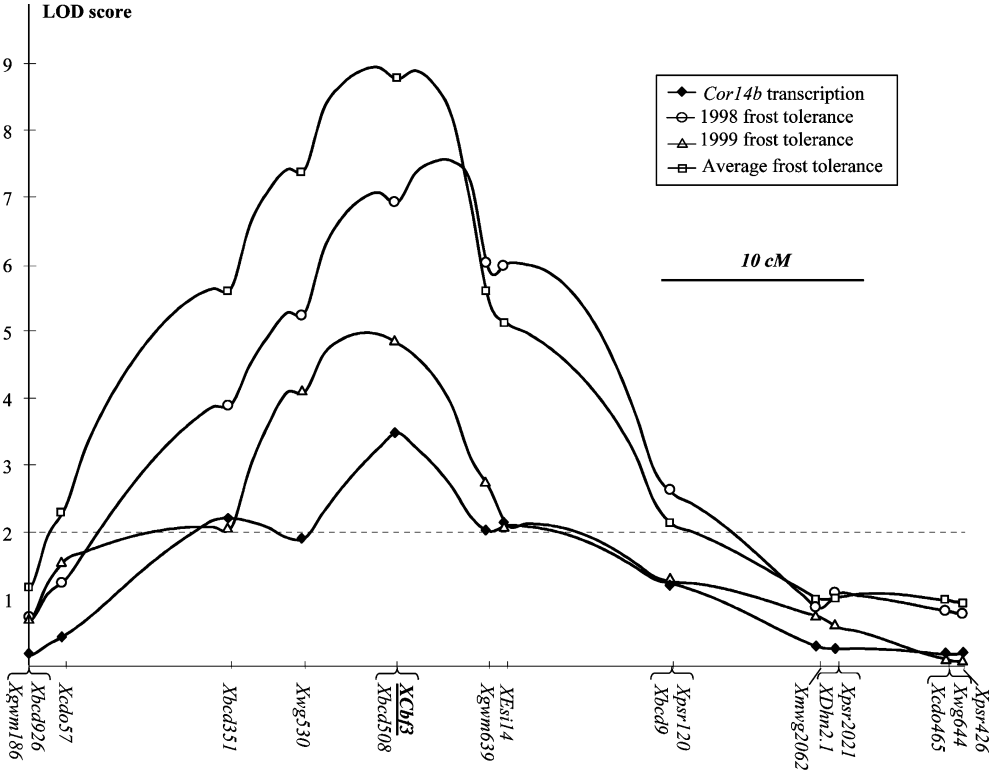

Fig. 1 QTL analysis for frost tolerance (open circles, 1998 experiment; *open squares*, 1999 experiment, *triangles*, average from 1998 and 1999 experiments) and *Cor14b* transcript levels at 15°C (*filled diamonds*). Distances between markers on the X-axis are proportional to the genetic distances on the RIL map

ones. All genotypes, independently of their frost tolerance, showed high levels of transcription at 2°C but non-detectable levels of transcripts at 25°C. To determine the optimum differential threshold temperature in *T. monococcum,* the level of transcription of *Cor14b* gene was determined in plants from the parental lines DV92 and G3116 grown at 5, 10, 15, 20 and 25°C. Northern analysis of these samples showed that G3116 had a higher level of transcription of *Cor14b* than DV92 when plants were grown at 15 or 20°C. The largest difference between parental lines was observed at 15°C (Fig. 2A). When the same filter was hybridized with a probe for the cold-regulated gene *Ao86* (Grossi et al. 1998) similar expression profiles were observed from both genotypes (Fig. 2B), demonstrating that the cold-induced expression of *Cor14b* and *Ao86* is controlled by two different mechanisms.

Plants from 62 RILs were grown at 15°C, and the total RNA extracted from each line was blotted onto a single membrane to facilitate comparisons. Hybridization of this membrane with a *Cor14b* probe showed different levels of transcripts among the RILs. The same membrane was hybridized with a wheat ribosomal probe to normalize the hybridization signal of *Cor14b* for putative differences in total RNA loaded in the gel (Fig. 2C). The normalized *Cor14b* values were analyzed using Mapmaker QTL (Fig. 1). A significant QTL with a LOD score of 3.5 was mapped with a peak on RFLP marker *Xbcd508*. This locus explained 24% of the variation in intensity in the normalized *Cor14b* values in this segregating population. The QTL for differential transcription level of *Cor14b* overlapped with the QTL for frost survival (Fig. 1).

Mapping of the *Cbf3* gene

Barley clone *Cbf3* hybridized with multiple RFLP fragments (Fig. 3) in *T. monococcum,* suggesting the presence of additional genes with at least partial sequence similarity to *Cbf3*. The RFLP fragment with the strongest hybridization signal (Fig. 3, arrows) was mapped to the *XCbf3* locus on the long arm of chromosome 5A, and showed complete linkage to RFLP locus *Xbcd508*. This RFLP locus is located at the center of the confidence intervals for the QTLs for frost tolerance and for *Cor14b* transcription levels at 15°C (Fig. 1).

Two additional restriction fragments that give low hybridization signals (Fig. 3, arrowheads) cosegregate with the previous RFLP fragments, suggesting the possibility of multiple CBF-like genes at this locus. A similar result was obtained in barley (Choi et al. 2002). The other RFLP fragments that gave low hybridization signals were not polymorphic or did not cosegregate with the *XCbf3* locus (e.g. the fragments numbered 3, 4, and 6 in Fig. 3).

Effect of the vernalization requirement locus *Vrn-A2* on frost tolerance

The 74 RILs were grown in the greenhouse without vernalization and growth habit was determined for each line. Growth habit showed complete linkage to the RFLP marker *Xbcd402*, which was previously shown to be tightly linked to the *Vrn-A2* locus in the F_2 population (Dubcovsky et al. 1998). This marker is not shown in Fig. 1 because it is 90 cM distal to the *XCbf3* locus.

The *Vrn-A2* locus showed only a marginal LOD score of 2 in the initial QTL analysis for frost tolerance,

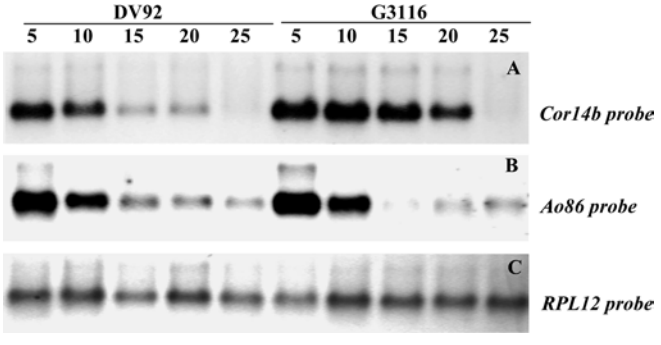

Fig. 2 Northern blot of RNAs from G3116 and DV92 plants grown at the indicated temperatures (°C), hybridized with *Cor14b* (*upper panel*) *Ao86* (*middle panel*) and with the ribosomal probe RPL12 (*lower panel*)

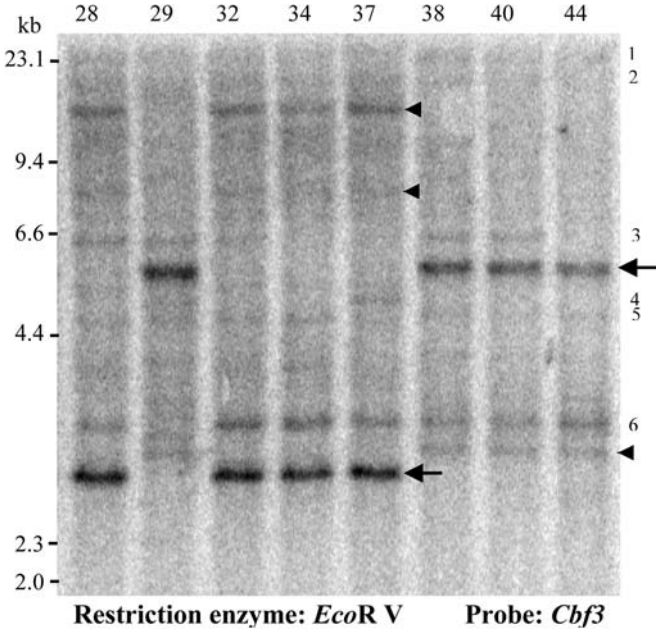

Fig. 3 Southern blot of genomic DNAs from eight RILs from the DV92 x G3116 mapping population. DNAs were digested with *Eco*RV, and hybridized with the complete barley *Cbf3* cDNA as a probe. The *arrows* indicate the RFLP fragments that give strong hybridization signals, which mapped at the *XCbf3* locus. The *arrowheads* indicate additional RFLP fragments that cosegregate with the previous ones. The *numbers* on the *right* indicate additional RFLP fragments that give low hybridization signals; these fragments were not mapped

because of the simultaneous segregation of the *Fr-A2* locus in this population. To increase our ability to detect the effect of the *Vrn-A2* locus on frost tolerance, a factorial analysis of variance was performed using the *Fr-A2* and *Vrn-A2* alleles as classification variables (heterozygous loci were coded as missing data). Significant differences in frost tolerance scores were detected for both *Fr-A2* and *Vrn-A2* in the 1998 and 1999 tests and in the combined results (Table 1). The two-gene model explained 58% of the variation in average frost tolerance scores in this population ($R^2 = 0.58$). Plants carrying the G3116 allele for winter growth habit showed significantly higher frost tolerance scores (average 1.08) than plants carrying the DV92 alleles (average 0.65).

The levels of *Cor14b* transcripts at 15°C showed significant differences when plants were grouped by the *Fr-A2* alleles but no significant differences when they were grouped by the *Vrn-A2* alleles.

The interactions between the two loci on frost tolerance were not significant in any of the analyses (Table 1). However, this result should be interpreted with caution, given the limited sample size used and the possibility that the developmental stage of the shoot apexes at the time of the frost tolerance test (which was not determined in this study) may influence the effect of this locus on frost tolerance.

Discussion

Frost tolerance

Chromosome 5A from *T. monococcum* is colinear with chromosome 5A from wheat (Dubcovsky et al. 1996; Gale et al. 1995), facilitating comparison of the results from this study with previous studies of frost tolerance in polyploid wheats. The major gene for frost tolerance, *Fr-A1*, was mapped in hexaploid wheat on the long arm of chromosome 5A – closely linked to RFLP marker *Xwg644* (Galiba et al. 1995; Sutka et al. 1999). The same marker showed no association with frost tolerances in this *T. monococcum* mapping population, indicating that the parental lines DV92 and G3116 did not differ at the *Fr-A1* locus. This lack of segregation at the *Fr-A1* locus increased the power of the QTL analysis to detect the new *Fr-A2* locus.

The new frost tolerance locus *Fr-A2* was mapped in diploid wheat 30 cM proximal to RFLP marker *Xwg644*, which is known to be tightly linked to *Fr-A1* in polyploid wheat. Although no frost tolerance locus has been described before in the *Fr-A2* region in polyploid wheats, it is possible that allelic variation at the *Fr-A2* locus might have been obscured by simultaneous variation at a linked *Fr-A1* locus. The presence of two loci for frost tolerance on chromosome 5A was suggested more than 10 years, ago based on inheritance studies using crosses between 'Winalta' and 'Winalta'-'Rescue'-5A chromosome substitution lines (Roberts 1990). The discovery of a locus controlling the expression of the *Cor14b* gene in the *Fr-A2* region on *T. aestivum* chromosome 5A (*Rcg1*, for Regulator for cor14b gene) suggests that allelic variation at the *Fr-A2* locus is also present in polyploid wheats (Vágújfalvi et al. 2000).

The *Rcg1* locus was linked to *Xpsr911* on the long arm of chromosome 5A of polyploid wheat (Vágújfalvi et al. 2000). This RFLP marker was mapped 36 cM from the centromere and 35 cM proximal to *Xpsr426*, a marker that is tightly linked to *Fr-A1* on chromosome 5A (Gale et al. 1995; Galiba et al. 1995). The *Fr-A2* locus, which was also associated with the control of the expression of *Cor14b* (Fig. 1) showed complete linkage in this study to *Xbcd508*, which is 35 cM from the centromere and 35 cM proximal to *Xpsr426* in *T. monococcum* (Dubcovsky et al. 1996). Furthermore, RFLP marker *Xksu8*, located 17 cM proximal to the *Xpsr911* (*Rcg1*) locus on chromosome 5B (Gale et al. 1995), was mapped 18 cM proximal to the *Xbcd508* locus on chromosome 5D (Gill et al. 1996). Similar distances to common reference markers suggest that the *Rcg1* locus in *T. aestivum* and the *Fr-A2* locus in *T. monococcum* are located in the same chromosome region.

Studies with chromosome substitution lines have shown that all three chromosomes of homoeologous group 5 carry major genes for frost tolerance (Sutka 2001). Since chromosomes 5A, 5B and 5D are colinear in the *Fr-1–Fr-2* region, it is possible for each chromosome to carry different allelic variants for frost tolerance at each of the two loci. The complex segregation patterns resulting from these six genes would limit the success of breeding efforts to combine the best alleles for frost tolerance. The new molecular markers generated in this study for the *Fr2* locus will facilitate the characterization and selection of optimal allele combinations for frost tolerance. RFLP markers mapped in the *Fr-A2* region can be also used to develop isogenic lines for the 5B and 5D *Fr2* region to test for the presence of allelic differences in *Cor14b* regulation or frost tolerance in these chromosomes.

The presence of two loci for frost tolerance on the same chromosome arm can also complicate the interpretation of orthologous relationships among frost tolerance loci on chromosomes from homoeologous group 5. For example, it was suggested that a QTL for frost tolerance designated Q*Fr.jic-5D*, which has been mapped on the long arm of chromosome 5D, was homeoallelic to *Fr-A1* (Snape et al. 1997, 2001). However, these authors also pointed out that no clear

Table 1 Factorial analysis of variance for frost tolerance scores and transcription levels of *Cor14b* at 15°C using *Fr-A2* and *Vrn-A2* as classification variables

Parameter	*Fr-A2*	*Vrn-A2*	Interaction
Frost tolerance 1998	$P < 0.0001$	$P = 0.0007$	$P = 0.3605$
Frost tolerance 1999	$P = 0.0001$	$P = 0.0395$	$P = 0.1625$
Average frost tolerance	$P < 0.0001$	$P = 0.0003$	$P = 0.8589$
Cor14b transcription level	$P < 0.0001$	$P = 0.9466$	$P = 0.8728$

segregational patterns were apparent in their mapping population, and that the peak of the Q*Fr.jic-5D* fell a more proximal position (10 cM proximal to *Vrn-D1*) than *Fr-A1* (2 cM proximal to *Vrn-A1*). This data should be re-examined for the possibility of simultaneous segregation at both *Fr-D1* and *Fr-D2* before deciding on the allelic relationship between Q *Fr.jic-5D* and the two frost tolerance genes on chromosome 5A.

Effects of vernalization requirement on frost tolerance

The DV92 x G3116 mapping population segregates simultaneously for frost tolerance at the *Fr-A2* locus and for vernalization requirement at the *Vrn-A2* locus (Dubcovsky et al. 1998). The parental lines from this population have the same recessive *vrn-A1* allele for winter growth habit and do not segregate at this locus, and the spring growth habit of DV92 is determined by the infrequent recessive *vrn-A2* allele (Dubcovsky et al. 1998; Tranquilli and Dubcovsky 1999). The *Vrn-A2* locus has been mapped at the end of the long arm of chromosome 5A (in the region translocated from chromosome arm 4AL), approximately 60 cM distal to the *Vrn-A1* locus and 90 cM distal to *Fr-A2* (Dubcovsky et al. 1998). The independent segregation of *Fr-A2* and *Vrn-A2* in this mapping population provides a unique opportunity to study simultaneously the effects of frost tolerance and vernalization requirement on survival following exposure to freezing temperatures.

The higher frost tolerance observed in winter RILs compared to the spring RILS, indicates that growth habit plays a significant role in the determination of frost tolerance. The delay in the differentiation of the vegetative apex into the more sensitive reproductive apex might be involved in the increased frost tolerance of the plants with winter growth habit. A delay in the transition from the vegetative to the reproductive stage in barley plants grown under short-day conditions has been shown to increase the expression of cold-induced genes and the level of frost tolerance, compared to plants grown under long-day conditions (Fowler et al. 2001).

The significant effect of growth habit on frost tolerance suggests that some caution is necessary in the interpretation of the magnitude of the effect of the *Fr-A1* locus on frost tolerance in polyploid wheats. Because of its close linkage with *Vrn-A1* (2 cM), the effect of growth habit is difficult to separate from the effect of *Fr-A1* in polyploid wheats segregating simultaneously at both loci. The *T. monococcum* mapping population used in this work may provide a better tool to study the interaction between growth habit and frost tolerance.

Cor14b transcript level

In our mapping population, the non-significant differences in *Cor14b* regulation between the spring and winter RILs indicates that the frost tolerance conferred by the allele for winter growth habit was not mediated by differences in the threshold temperature for induction of the *Cor* genes. On the other hand, the overlap between the QTLs for frost tolerance and for *Cor14b* transcript levels suggests that frost tolerance conferred by the *Fr-A2* locus might be mediated by the differential regulation of the *Cor* genes.

The *Cor14b* gene itself has been mapped on chromosome 2A, but was regulated by two genes located on chromosome 5A and linked to the *Fr-A1* and *Fr-A2* loci in polyploid wheat (Vágújfalvi et al. 2000). Similarly, different expression levels of the cold-regulated gene *wcs120* between frost-resistant and susceptible wheat and rye cultivars were associated with variation at the *Vrn-A1*/*Fr-A1* region (Fowler et al. 1996). These observations suggest the possibility that the molecular basis for cold tolerance could be a regulatory gene that is able to control the simultaneous expression of many cold-related genes. After the discovery of the *Arabidopsis* CBF transcription factors it was suggested that the cereal loci controlling frost tolerance could represent cereal *Cbf* homologs, but no direct evidence for this hypothesis was presented (Sarhan and Danyluk 1998).

Association of *Cbf3* transcription factors with frost tolerance and differential *Cor14b* regulation

The *Arabidopsis* C*BF* gene products bind to regulatory CRT (C-repeat)/DRE (dehydration responsive element) sequences present in the promoters of many *Cor* genes, activating a regulon of genes involved in cold acclimation (Medina et al. 1999). The expression of the three *Arabidopsis* C*BF* genes is regulated by cold but not by abscisic acid or dehydration, suggesting that they control the level of *Cor* gene expression and promote cold acclimation through an abscisic acid-independent pathway. It was also demonstrated that *CBF1* overexpression in *Arabidopsis* induced coordinated *Cor* gene expression without a low-temperature stimulus (Jaglo-Ottosen et al. 1998). Constitutive expression of the cold-regulated *Arabidopsis Cor15a* gene increases both chloroplast and protoplast freezing tolerance (Artus et al. 1996).

Similar results to those reported in *Arabidopsis* have been found in different *Triticeae* species. The CRT/DRE core motif CCGAC recognized by the CBF transcription factors is also present in the promoter sequences of cold-regulated genes from wheat (Ouellet et al. 1998) and barley (Dunn et al. 1998). Northern blot studies using a rye *Cbf* gene as a probe have shown that CBF-like transcripts accumulated rapidly (within 15–30 min) in both wheat and rye in response to low temperatures (Jaglo et al. 2001). More specific expression studies using quantitative PCR and *Cbf3*-specific primers have shown that the barley *Cbf3* gene is transiently up-regulated within 15 min of exposure to cold (Choi et al. 2002). The cold induction of barley *Cbf3*, and the presence of CRT

(C-repeat)/DRE (dehydration responsive element) regulatory sequences in the promoter sequences of cold-regulated genes from wheat, make the *Cbf3* gene an attractive candidate gene for frost tolerance. Although the promoter sequences of *Cor14b* have not yet been published, the genetic evidence reported in the present work suggest that the transcription of *Cor14b*, but not of *Ao86*, might be under the control of CBF-like proteins. Notably, *Ao86* is a member of the *Blt14* gene family (Grossi et al. 1998), a class of sequences whose expression is up-regulated in response to cold only through post-transcriptional mechanisms (Dunn et al. 1994; Phillips et al. 1997).

Choi et al. (2002) mapped the *Cbf3* gene in barley in a similar location to the one reported in this study, but, as in previous studies, they did not detect a frost tolerance effect associated with the *Cbf3* region. Our study provides evidence for the first time for cosegregation of a *Cbf* gene, differences in the threshold temperatures for induction of *Cor14b*, and frost tolerance in wheat. However, the relationship between *Cbf3* and frost tolerance needs further investigation because of the presence of multiple copies of *Cbf* like genes in cereals, some of them linked to *Cbf3* (Fig. 3). Any of the *Cbf* like genes tightly linked to *Cbf3* has a similar probability to be responsible for the observed differences in frost tolerance in this segregating population. We have recently initiated a systematic characterization of all the members of this family in *T. monococcum* to address these questions.

In summary, the *Cbf3* transcription factor has been mapped on the long arm of chromosome *5A* in *T. monococcum* at the peak of the QTLs for frost tolerance and differnetial regulation of *Cor14b* transcription (Fig. 1). This association suggests the possibility that CBF-like transcriptional activators play a major role in the determination of frost tolerance in wheat. Demonstration of this relationship would be a fundamental step towards the biotechnological improvement of frost tolerance in wheat.

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