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## **Fine mapping at the frost resistance locus *Fr-H2* in barley**



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## Fine mapping of a HvCBF gene cluster at the frost resistance locus *Fr-H2* in barley \*

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**Abstract:** Barley is an economically important model for the *Triticeae* tribe. We recently developed a new resource: the ‘Nure’ x ‘Tremois’ mapping population. Two low temperature QTLs were found to segregate on the long arm of chromosome 5H (*Fr-H1*, distal; *Fr-H2*, proximal). With the final aim of positional cloning of the genetic determinants of *Fr-H1* and *Fr-H2*, a large segregating population of 1,849 F<sub>2</sub> plants between parents ‘Nure’ and ‘Tremois’ was prepared. These two QT loci were first validated by using a set of F<sub>3</sub> families, marker-selected to harbor pairs of reciprocal haplotypes, with one QTL fixed at homozygosity and the alternate one in heterozygous phase. The study was then focused towards the isolation of the determinant of *Fr-H2*. Subsequent recombinant screens and phenotypic evaluation of F<sub>4</sub> segregants allowed us to estimate ( $P \leq 0.01$ ) a refined genomic interval of *Fr-H2* (4.6 cM). Several barley genes with the CBF transcription factor signature had been already roughly mapped in cluster at *Fr-H2*, and they represent likely candidate genes underlying this QTL. Using the large segregating population (3,698 gametes) a high-resolution genetic map of the HvCBF gene cluster was then constructed, and after fine mapping, six recombinations between the HvCBFs were observed. It was therefore possible to genetically divide seven HvCBF subclusters

in barley, in a region spanning 0.81 cM, with distances among them varying from 0.03 to 0.32 cM. The few recombinants between the different *HvCBF* sub-clusters are being marker-selected and taken to homozygosity, to phenotypically separate the effects of the single *HvCBF* genes.

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## Introduction

The ability of *Triticeae* species to survive low winter temperatures is a phenomenon entailing a number of factors, including freezing duration and severity, alternation of freeze and thaw periods, synthesis of toxic substances affecting recovery capacity, plant growth stage during the stress, and the duration of the hardening period prior to freezing temperatures. Because the traditional breeding strategies to improve frost resistance in winter cereals have been rather inefficient (Limin and Fowler 1993), increased attention has been given to understand the molecular genetic mechanisms that plants have evolved to tolerate this environmental stress (Pecchioni et al. 2002). In this view, studies undertaken to unravel the molecular basis of cold acclimation in model plants and in crops have led on one hand to the isolation of many cold-regulated (*COR*) genes, and on the other hand to the identification of genomic regions which exert a major measurable effect on the tolerance, i.e. quantitative trait loci – QTLs (Cattivelli et al. 2002).

In the *Triticeae* genome, the long arm of chromosome group 5 is the region most frequently associated with two traits critical for overwinter survival: low temperature tolerance and vernalization requirement (Hayes et al. 1993; Vagujfalvi et al. 2003; Francia et al. 2004; Reinheimer et al. 2004). In particular, in barley, the position of the two major low-temperature tolerance QTLs, *Fr-H1* and *Fr-H2*, was reported in the ‘Nure’ (winter) x ‘Tremois’ (spring) – NxT cross (Francia et al. 2004). They are located, approximately 25 cM apart, on the long arm of chromosome 5H. The NxT *Fr-H2* QTL is orthologous to the frost tolerance QTL *Fr-A<sup>m</sup>2* found in diploid wheat (*Triticum monococcum* L.) by

Vagujfalvi et al. (2003); in both species these QTL also cosegregate with *COR* gene product accumulation.

The two loci of NxT represent the most complete QTL architecture for frost tolerance of group 5 segregating in a single population (Galiba et al. 2005), with respect both to wheat 5A (*Fr-A1*, Galiba et al., 1995; *Fr-A<sup>m</sup>2*, Vagujfalvi et al. 2003) and to other barley mapping populations (e.g., *Fr-H1* in the 'Dicktoo' x 'Morex' – DxM population, Hayes et al. 1993; Skinner et al. 2006).

Vernalization is a period ranging from about one to eight weeks of cold-temperature exposure required to induce reproductive development during a normal, annual growing season life cycle (Takahashi and Yasuda 1971). The underlying genetic effects have been reported as QTLs because they show complex, rather than Mendelian, inheritance. The cold tolerance *Fr-H1* QTL is coincident with *Vrn-H1* (Hayes et al. 1993; Laurie et al. 1995; Francia et al. 2004). Limin and Fowler (2006) hypothesized a direct role of *Vrn-A1* locus in frost tolerance of winter type wheats, however results of Sutka et al. (1999) indicate that *Vrn-A1* and *Fr-A1* are physically separated. In barley, and in particular in the model population NxT, it remains to be determined whether linkage or pleiotropic effects of *Vrn-H1* are the molecular basis behind *Fr-H1*.

*Arabidopsis thaliana* L. is an excellent model plant for studying the low-temperature response (Thomashow 1999). However a question that arises is whether discoveries made in *Arabidopsis* will have related or biotechnology applications in *Triticeae* and other crops. Importantly, not only are structural proteins conserved between *Arabidopsis* and crop plant but also regulatory proteins such as transcriptional regulators are conserved (Jaglo et al. 2001). This was especially true for the C-

repeat Binding Factor (*CBF*) “regulon”, since in barley and diploid wheat several pieces of experimental evidence suggest that the cold responsive pathway triggered by these transcription factors is conserved between *Arabidopsis* and the *Triticeae*, and that a set of *COR* eVector genes is trans-activated by barley *HvCBFs* (Xue 2002, 2003; Skinner et al. 2005).

In a previous report we showed that *HvCBF4* is the marker peak of *Fr-H2* in the NxT population (Francia et al. 2004), and more recently, that five additional *HvCBFs* are tightly linked to *HvCBF4* and *Fr-H2* (Tondelli et al. 2006). At the same position in the DxM barley population, Skinner et al. (2006) mapped eleven *HvCBF* genes into two clusters (approximately 1 cM apart), while at the homoeologous *Fr-A<sup>m</sup>2* region in *Triticum monococcum* Miller and co-workers identified eleven *TmCBF* genes (Miller et al. 2006). The cluster of group 5 *CBFs* is currently the most likely set of candidate genes to explain cold tolerance at *Fr-2* in *Triticeae*, rather than their regulator *ICE1* (Tondelli et al. 2006). Whether the QTL effect of *Fr-H2* in barley and *Fr-A<sup>m</sup>2* in diploid wheat is the result of a single *CBF* gene, the combined effect of a subset (or all) of the *CBFs*, or independent of the *CBF* genes remains to be determined.

With the final aim to positionally clone the genetic determinants of *Fr-H1* and *Fr-H2*, our present objectives were to: (1) develop a new large segregating population from the cross ‘Nure’ x ‘Tremois’, for both *Fr-H1* and *Fr-H2*, (2) validate *Fr-H1* and *Fr-H2* in this population, (3) refine the position of *Fr-H2*, and (4) construct a high-resolution genetic map of this locus, based on a cluster of seven *HvCBF* candidate genes.

## **Materials and methods**

### *Plant material and DNA extraction*

A 'Nure' (winter, frost tolerant) x 'Tremois' (spring, frost susceptible) cross was used to generate a population of 1,256 F<sub>2</sub> plants (Fig. 1). Using marker-assisted selection, a first set of 36 F<sub>2</sub> recombinants was chosen and their corresponding F<sub>3</sub> progeny phenotyped for frost tolerance to independently validate *Fr-H1* and *Fr-H2*. A second set of 389 F<sub>3</sub> individuals was obtained by field sowing the seed of 49 F<sub>2</sub> plants that were expected to be recombinant at the *Fr-H2* locus based on their marker haplotype. Twenty-nine homozygous F<sub>4</sub> progeny were selected, checked again with molecular markers, and phenotyped for frost tolerance (Fig. 1). Twenty-eight had a single recombination event above or below *Fr-H2*, whereas one F<sub>4</sub> plant (Fo3702\_361.8) was a rare double recombinant, carrying the 'Nure' *Fr-H2* allele introgressed into a 'Tremois' background. To increase the number of recombinant genotypes and for high-resolution mapping, 593 F<sub>2</sub> plants were added to the first F<sub>2</sub> population for a total of 1,849 recombinant plants – i.e. 3,698 gametes (Fig. 1). This set of 593 recombinants was obtained by crossing doubled-haploid lines NT-6 and NT-105 to 'Tremois' (NT-6 and NT-105 harbour the 'Nure' alleles at both *Fr-H* loci, Francia et al. 2004). The resulting F<sub>1</sub> lines were then selfed to obtain 296 and 297 plants, respectively.

Genomic DNA (gDNA) of the 136 NxT DH lines maintained at the CRA - Istituto Sperimentale per la Cerealicoltura, was used for preliminary mapping of the newly developed PCR-based marker MWG583 (see below). gDNA of the remaining plant material (i.e. F<sub>2</sub>, F<sub>3</sub>, and F<sub>4</sub> lines) was isolated by placing leaf tissues from field grown plants in 96-well microtube plates. Plant material was

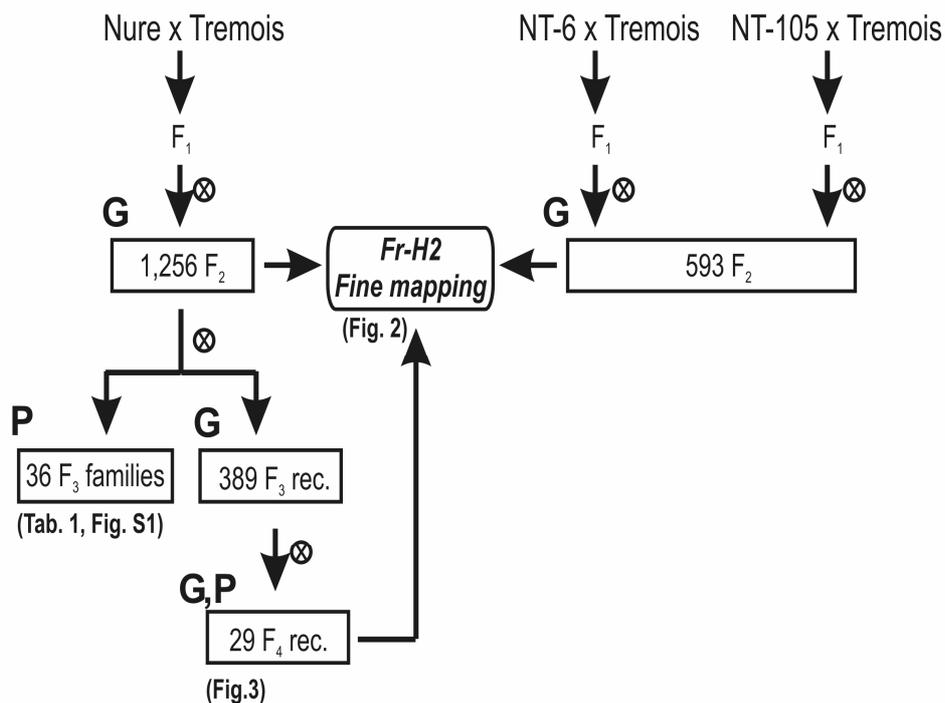
ground using the Retsch<sup>®</sup> MM300 Mixer Mill and DNA was extracted using the Wizard<sup>®</sup> Magnetic 96 DNA Plant System (Promega) following manufacturer's instructions. All DNA for PCR reactions was at 20 ng/μl.

#### Molecular marker analysis

Fluorescently labeled SSR markers Bmag0223, Hv635P2.4, and Bmag0222 were PCR-amplified as described in Francia et al. (2004) and genotyped using the ABI Prism<sup>®</sup> 3100 Genetic Analyzer (Applied Biosystem). HvBM5A, Zcct-H and HvMYB1 CAPS markers were analyzed as reported by von Zitzewitz et al. (2005) and Tondelli et al. (2006). Nomenclature of the barley CBFs follows that of Skinner et al (2005) where HvCBFs designated with the same number but different letters (e.g. A and B) describe highly identical although distinct genes. Specific markers targeting the genes HvCBF2B, 3A, 4, 6, 10B, and 12 were genotyped according to Francia et al. (2004) and Tondelli et al. (2006). To classify HvCBF4 with respect to previously characterized HvCBF4A, 4B, and 4D genes (AY785849, AY785850, and AY785852 respectively – Skinner et al. 2005), nucleotide sequence comparisons were performed by using both local and global alignments (data not shown). Accordingly, hereafter we will refer to the previously mapped HvCBF4 gene (Francia et al. 2004) as HvCBF4B.

For HvCBF13, allele specific PCR primers (Nure-F 5'-ATGCCAATTACTCCCTC-3', Nure-R 5'-TCATGGCACATGAG-3'; Tremois-F 5'-ATGATTAGGAGGACTGTG-3', Tremois-R 5'-TCACCTCGTCGATCTTGA-3') were designed. Amplification was performed in a 15μl final volume containing: 40

ng of genomic DNA as template, 1X Qiagen PCR buffer, 1.5 mM of MgCl<sub>2</sub>, 1X Q-solution, 0.25 mM of each dNTP, 0.4 μM of each primer, and 1U of *Taq* DNA Polymerase (Qiagen). Reactions were incubated for 2 min at 94°C, followed by 40 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 7 min. PCR products were then separated on a standard 1.5% agarose gel.



**Figure 1:** Plant materials developed, and outline of *Fr-H2* fine mapping strategy. P, phenotyping; G, genotyping.

For MWG583, specific oligonucleotide primers were designed on the RFLP probe sequence AJ234522 using Primer3 (Rozen and Skaletsky 2000): MWG583-F 5'-CTCGTCTCGTGAGTGAG- C-3' and MWG583-R 5'-

TGTACGCAGGGAAACAGGTC-3'. Based on the SNP polymorphisms found in sequenced products, a new CAPS marker was developed. Amplification was as described above except that 55°C annealing temperature was used. Digestion was performed with the enzyme *BsrI* according to manufacturer's instructions, 10 µl of PCR product were incubated for 1.5 h with 2U of restriction enzyme, 1X reaction buffer and 0.1 mg/ml of BSA, and then separated on a standard 2% agarose gel.

For all the tested markers doubtful scoring were confirmed by repeating the amplification reaction for at least three times.

#### Linkage analysis and genetic distance

The software MAPMAKER 3.0 (Lander et al. 1987) was employed to determine the most likely order of markers and to perform linkage analyses of the DH and F<sub>2</sub> populations. The Kosambi mapping function was used to convert recombination frequencies into centimorgans (cM). The genetic distance between all HvCBF markers was instead calculated as the number of recombinants between two markers divided by the number of gametes screened, multiplied 100.

#### Phenotypic evaluation and data analysis

Frost tolerance of selected F<sub>3</sub> and F<sub>4</sub> recombinants was evaluated under controlled conditions (growth chamber) on plants in the first leaf stage that were cold acclimated for 4 weeks (3°C, 8 h light and 2°C, 16 h dark). In the freezing treatments, plants were placed at -11°C for 18 h. The frost-induced damage was measured in the leaves as a decrease in the photochemical capacity of photosystem II (PSII), using the chlorophyll fluorescence parameter F<sub>v</sub>/F<sub>m</sub>. The F<sub>v</sub>/F<sub>m</sub> values were determined

using a Pulse Amplitude-Modulated fluorometer (PAM 2000, Walz, Effeltrich, Germany) 24 h after a recovery period at 20°C, according to Francia et al. (2004). Testing of 36 F<sub>3</sub> progeny derived from F<sub>2</sub> recombinants was performed in 2004; phenotyping of the 29 F<sub>4</sub> recombinants was performed in 2005. In both tests the parents 'Nure' and 'Tremois' were included as checks, plants were arranged in a randomized block design with six replications, and the experiments were repeated twice. Data were analyzed performing General Linear Model ANOVA (SYSTAT 9, SPSS Inc. 1999, Chicago, IL, USA) and differences between reciprocal classes of recombinant-derived F<sub>3</sub> families were tested using Tukey's test ( $P \leq 0.05$ ). The confidence limits ( $L_2$ ) for *Fr-H2* were obtained for the two genetic intervals encompassing the HvCBF gene cluster as a fraction of interval length following exact computations  $L_2 = 1 - (\alpha/2)^{1/n}$ , where  $\alpha = 0.01$ , and  $n$  is the number of observations (Zar 1999).

## Results

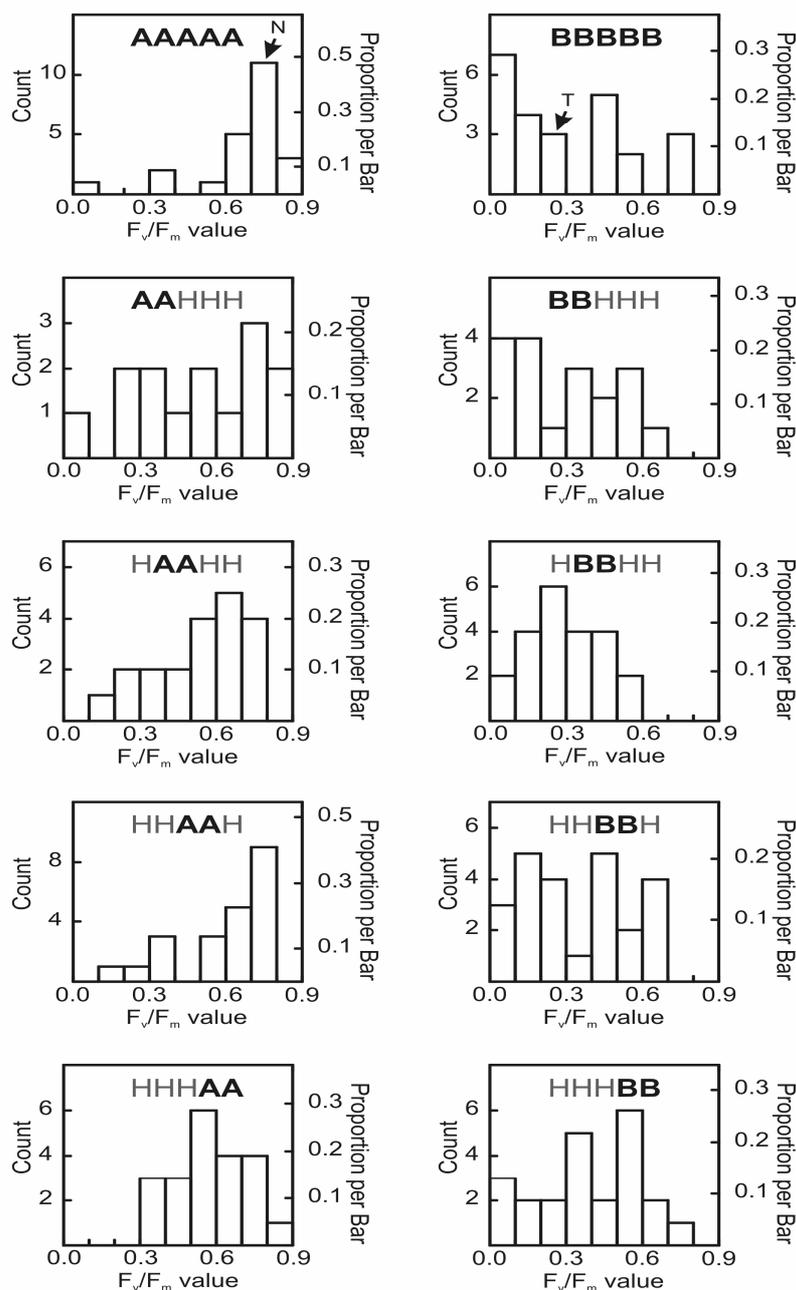
### Validation of *Fr-H1* and *Fr-H2* loci in the NxT cross

As an initial step in the development of a high-resolution genetic map of barley chromosome 5HL (Figure 3a), 1,256 'Nure' x 'Tremois' F<sub>2</sub> plants were screened with molecular markers encompassing the *Fr-H* loci, including: Bmag0223, HvCBF4B, HvMYB1, Hv635P2.4, and Bmag0222. HvCBF4B and Hv635P2.4 were previously mapped to the peaks of *Fr-H2* and *Fr-H1*, respectively. The observed order and genetic distances between these five loci in the F<sub>2</sub> population (Figure 3b) were consistent with those reported in our previous studies on the NxT DH population (Francia et al. 2004) and on a consensus map (Tondelli et al. 2006). To validate the phenotypic effects of both *Fr-H1* and *Fr-H2*, thirty-six F<sub>3</sub> families derived from marker-selected individual F<sub>2</sub> recombinants were analyzed for frost tolerance. The selected progeny represented five pairs of reciprocal haplotype classes, with one QTL (*Fr-H* locus) fixed at homozygosity for either the 'Nure' or the 'Tremois' allele, and the second *Fr-H* locus in heterozygous phase, plus homozygous (non-recombinant) individuals representing the parental allele classes (Table 1). Freezing plants to -11°C clearly discriminated phenotypic differences amongst the haplotype classes. The observed F<sub>v</sub>/F<sub>m</sub> indices in the F<sub>3</sub> families ranged from 0.273 (high frost damage) to 0.653 (moderate frost damage), in comparison to the parents 'Nure' (0.725) and 'Tremois' (0.222) (Table 1). Phenotypic class mean values are listed along with the difference between reciprocal classes, whereas a summary of the observed phenotypic frequency distributions is given in Figure 2.

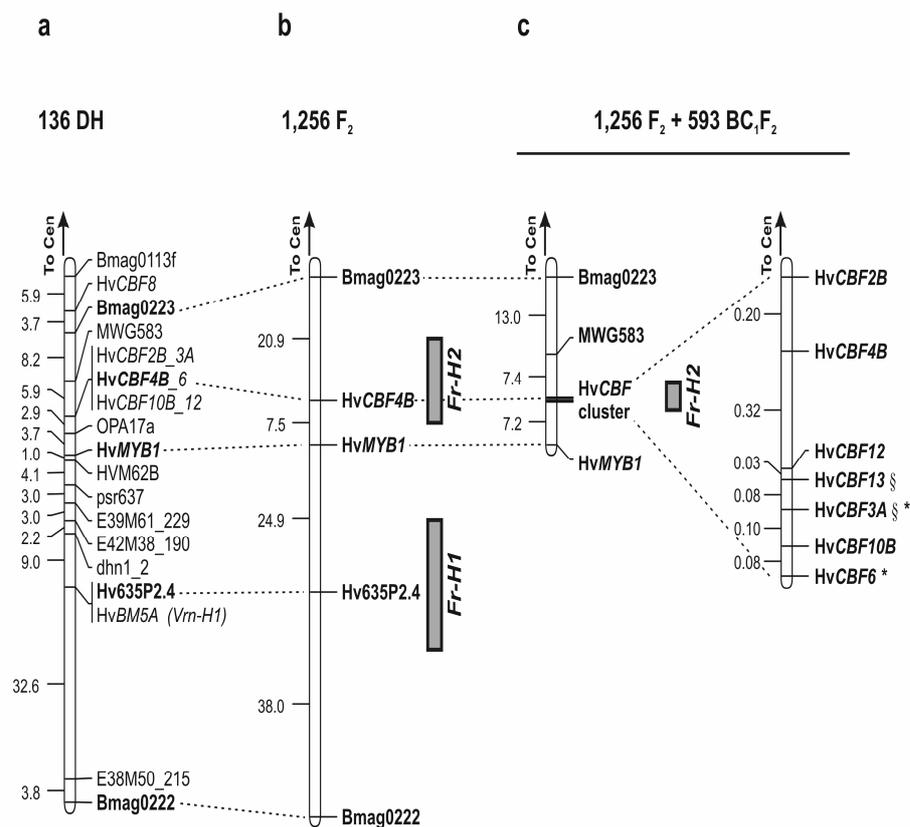
Haplotype of F <sub>3</sub> families		Phenotypic class mean		Difference (F <sub>v</sub> /F <sub>m</sub> )	P
Bmag0223- <b>HvCBF4B</b> -HvMYB1- <b>Hv635P2.4</b> -Bmag0222		(F <sub>v</sub> /F <sub>m</sub> )			
<u>AAAAA</u> (3)	<u>BBBBB</u> (3)	0.653	0.331	0.322	<0.001
<u>AAHHH</u> (3)	<u>BBHHH</u> (3)	0.524	0.273	0.251	0.018
H <u>AAHH</u> (4)	H <u>BBHH</u> (4)	0.532	0.292	0.240	0.005
H <u>HAAH</u> (4)	H <u>HBBH</u> (4)	0.589	0.338	0.251	0.001
H <u>HHA</u> A (4)	H <u>HHB</u> B (4)	0.580	0.374	0.206	0.026
<u>AAAAA</u> (N)	<u>BBBBB</u> (T)	0.725	0.222	0.503	0.001

**Table 1:** Frost tolerance of reciprocal classes of 36 marker-selected F<sub>3</sub> families and parents (N='Nure' and T='Tremois'). Number of genotypes phenotyped for each class is in parenthesis. Statistical significance (P) for each comparison is given in the last column. "A" and "B" indicate 'Nure' and 'Tremois' allelic state at the five tested markers, "H" indicates heterozygous at the marker loci. *Fr-H1* (Hv635P2.4) and *Fr-H2* (HvCBF4B) QTL peak markers and their allelic states are shown in bold and larger font in the table. Homozygous regions are underlined.

For all comparisons, these differences were statistically significant ( $P \leq 0.05$ ) at both *Fr-H* loci; a clear positive effect of the 'Nure' ("A") allele on the level of frost-tolerance in comparison to the 'Tremois' ("B") allele was detected. The positive effect of the 'Nure' allele was detected without significant confounding effects of the alternate QTL in heterozygous phase (Table 1 and Figure 2), and the two QTLs confirm their centre at previously identified peak markers (Table 1). The updated genomic regions of *Fr-H1* and *Fr-H2*, based on the intervals and distances between the five markers used for the screening of the large F<sub>2</sub>, are shown in Figure 3b.



**Figure 2:** Phenotypic frequency distributions for frost tolerance of 'Nure' x 'Tremois'  $F_3$  progeny.  $F_3$  families are grouped in classes on the basis of their haplotype at the five loci *Bmag0223*, *HvCBF4B*, *HvMYB1*, *Hv635P2.4*, and *Bmag0222* (see Table 1). "A", "B" and "H" indicate 'Nure', 'Tremois' and 'Heterozygous' allelic state at these markers, respectively. Phenotypic values of the two parents (N='Nure', T='Tremois'), included in the "all A" and "all B" classes respectively, are indicated by arrowheads.



**Figure 3:** Consecutive stages of high-resolution mapping of *Fr-H2*. **a** Linkage map of barley chromosome 5HL constructed using the ‘Nure’ x ‘Tremois’ DH population of 136 individuals. Segregation data are from Tondelli et al. (2006), and includes the STS marker MWG583 added in this study. **b** Genetic map of chromosome 5HL markers encompassing *Fr-H2* and *Fr-H1*. This map was constructed by using the five markers and a population of 1,256 ‘Nure’ x ‘Tremois’  $F_2$  plants. Phenotypic effect confirmation of the two *Fr-H* QTLs found by Francia et al. (2004) is indicated on the right, approximately in the middle of the intervals flanking the peak markers, following the frost tolerance test of the 36  $F_3$ s described in the text. **c** Refinement of the *Fr-H2* locus and high-resolution genetic map of the HvCBF gene cluster, after screening of 1,849 plants (1,256 + 593  $F_2$ ); confidence interval ( $P \leq 0.01$ ) for *Fr-H2* around the HvCBF4B marker was estimated after phenotyping results of 29  $F_4$ s summarized in Figure 4. Genetic distances (in Kosambi cM) calculated for specific intervals are shown on the left of each bar. All maps are drawn in the same scale, except for the high-resolution map of the HvCBF cluster. HvCBF3A and HvCBF6 have been labeled with an asterisk because they were found by Skinner et al. (2005) to be located on a single ‘Morex’ BAC clone: 804E19. HvCBF13 and HvCBF3A are located on the same  $\lambda$  clone (Stockinger et al. 2006), and have been labeled accordingly.

A refined genomic interval of *Fr-H2* QTL

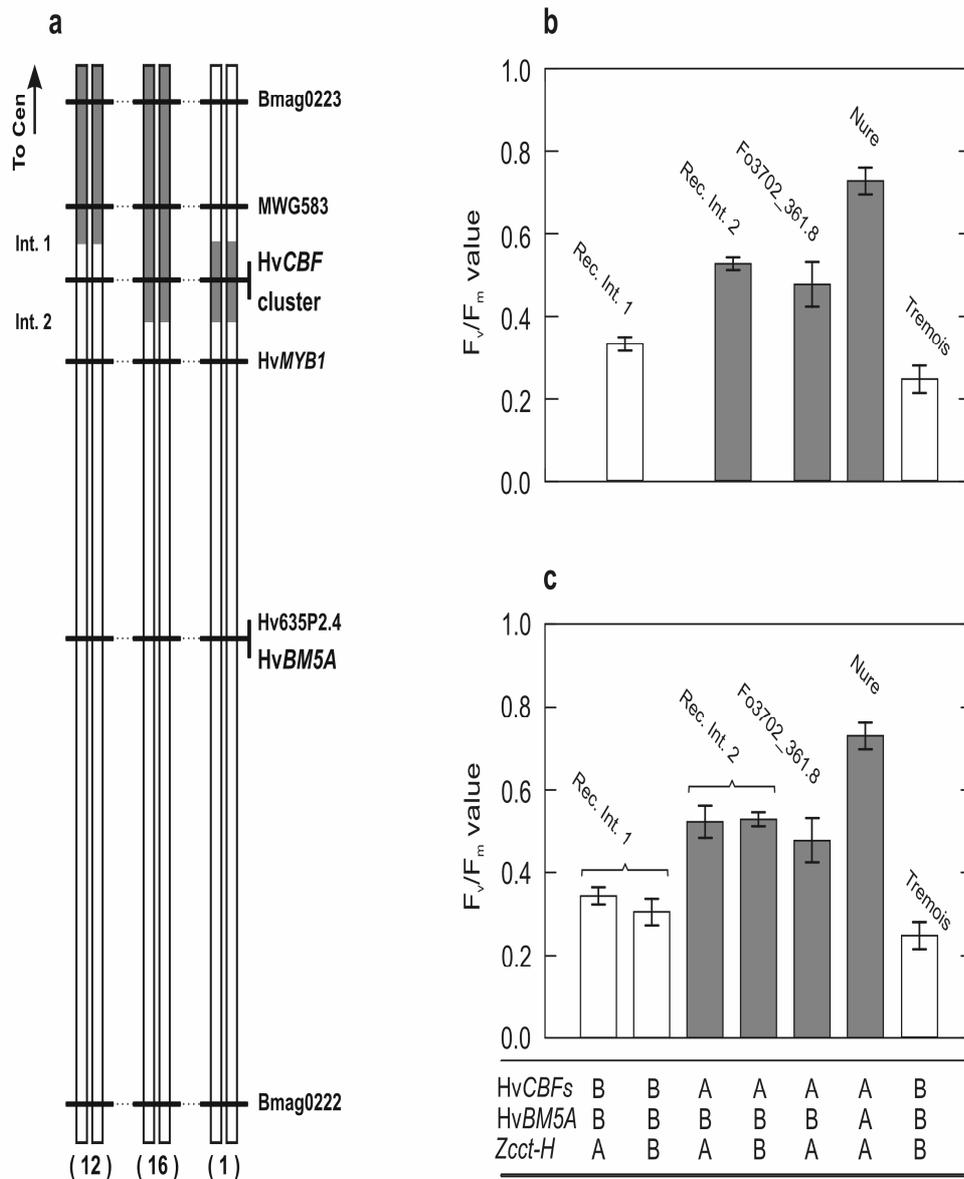
Validation of *Fr-H1* and *Fr-H2* in the NxT F<sub>2</sub>, led us to concentrate efforts on further refining *Fr-H2* with respect to molecular markers. An additional 593 recombinants were generated from two additional F<sub>2</sub> populations derived from the cross NT-6 x 'Tremois' and NT-105 x 'Tremois' (see methods). Inclusion of these F<sub>2</sub> individuals in the mapping population yielded a total of 1,849 recombinant plants – i.e. 3,698 gametes (Figure 1). Mapping Bmag0223, MWG583, *HvCBF4B*, and *HvMYB1* in the expanded population of 1,849 F<sub>2</sub> individuals (1,256 + 593), allowed us to refine the genetic distances around *Fr-H2*. In particular, adding the new CAPS marker for MWG583 (developed by re-sequencing the RFLP probe AJ234522 in 'Nure' and 'Tremois'), the resolution of the *Fr-H2* region improved, now targeting a 14.6 cM interval (Figure 3c). Informative F<sub>2</sub> recombinants were marker-selected, field-sown, and 389 F<sub>3</sub> plants were selfed to produce F<sub>4</sub> seed (Figure 1). Following genotypic evaluation, homozygous individuals were chosen and divided in two classes carrying a recombination either between MWG583 and *HvCBF4B* ('Interval 1'), or between *HvCBF4B* and *HvMYB1* ('Interval 2') (Figure 4a). All these recombinants carried the 'Nure' allele above the recombination event, and the 'Tremois' allele below the recombination event (Figure 4a), thus also eliminating any effects of *Fr-H1* on further phenotypic analyses. A double recombinant F<sub>4</sub> line Fo3702\_361.8 was found, carrying a 'Nure' *Fr-H2* allele in homozygous state introgressed into the 'Tremois' background, and it was included for phenotypic tests (Figure 4a). These 29 F<sub>4</sub> lines were tested for frost resistance in controlled conditions by freezing to –11°C and then measuring F<sub>v</sub>/F<sub>m</sub>. As shown in Fig. 3b, all 16 F<sub>4</sub> recombinant lines of the

group in Interval 2 had a significantly higher level of frost tolerance ( $F_v/F_m=0.527$ ) than the 12  $F_4$  lines in which a recombination event occurred in Interval 1 ( $F_v/F_m=0.333$ ). Additionally, no difference was observed between the recombinants in Interval 2 and the double recombinant line Fo3702\_361.8 (Figure 4b). Applying the binomial distribution to the proportion of recombinant genotypes between MWG583 and *HvCBF4B*, or between *HvCBF4B* and *HvMYB1*, a confidence interval ( $P \leq 0.01$ ) for *Fr-H2* was then calculated, which refined *Fr-H2* to a 4.6 cM segment around *HvCBF4B* (Figure 3c).

In accordance with what observed in the previous experiment of  $F_3$  families phenotypic testing (Table 1 and Figure 2), after phenotyping the  $F_4$  recombinants harbouring the 'Nure' ("A") allele at the *HvCBF* cluster (i.e. at *Fr-H2*) and the 'Tremois' ("B") allele at *Hv635P2.4/HvBM5A* (i.e. at *Fr-H1/Vrn-H1*) reached an intermediate level of resistance, compared with the frost tolerance of the resistant parent 'Nure' ( $F_v/F_m=0.728$ ) and of the susceptible parent 'Tremois' ( $F_v/F_m=0.248$ ) (Fig 3b). In this view, it is further confirmed that there is a reduced, although highly significant contribution of *Fr-H2 per se* to frost tolerance in the NxT crosses, independently by the allelic state at *Fr-H1/Vrn-H1* region (Figure 4a and 4b).

To test if the allelic state at the second vernalization requirement locus segregating in NxT, *Vrn-H2*, on chromosome 4H, contributed or not to low-temperature tolerance, all the  $F_4$  recombinants were screened and classified according to their alleles at *Zcct-H* candidate gene of *Vrn-H2* (von Zitzewitz et al. 2005). As shown in Fig. 3c, no significant effects were found between allelic classes according to *Zcct-H* and level of frost

tolerance for the assayed F<sub>4</sub> Interval 1 and Interval 2 recombinants.



**Figure 4:** Graphic genotyping and frost tolerance of 'Nure' x 'Tremois' F<sub>4</sub> recombinants above (Interval 1) and below (Interval 2) the HvCBF cluster. **a** Graphical representation of chromosome 5HL region of the phenotyped F<sub>4</sub> plants. Chromosomal segments with 'Nure' and 'Tremois' marker alleles are in gray and white, respectively. The number of F<sub>4</sub> plants for each recombinant class is in parenthesis, and distances are in scale with those reported in Fig. 2a. **b** Phenotypic F<sub>v</sub>/F<sub>m</sub> values of the three F<sub>4</sub> recombinant classes are presented in comparison with the two parents. **c** Effect of the allelic state at HvCBF cluster (*Fr-H2*), HvBM5A (*Fr-H1/Vrn-H1*), and *Zcct-H* (*Vrn-H2*) on frost tolerance of F<sub>4</sub> recombinants and parents. "A" and "B" indicate 'Nure' and 'Tremois' allelic state, respectively. Vertical bars in **b** and **c** represent standard errors.

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*Construction of a high-resolution genetic map of a cluster of seven HvCBF candidate genes at Fr-H2 locus*

In a previous work, we mapped HvCBF4B to the peak of *Fr-H2* in the NxT population (Francia et al. 2004). Subsequently, we placed five additional HvCBFs at the same locus (Tondelli et al. 2006). To determine their genetic order at *Fr-H2*, a high-resolution genetic map of the HvCBF cluster on 5H including these six genes and the newly tagged HvCBF13 was generated. The large segregating population of 1,849 individuals (1,256 + 593 F<sub>2</sub>) was screened for recombinants between each of the HvCBF genes polymorphic between 'Nure' and 'Tremois'. This allowed us to produce the fine map drawn in Figure 3c. The HvCBF cluster spans in barley a total genetic distance of 0.81 cM, with the largest distance between HvCBF4B and HvCBF12 (0.32 cM, 12 recombinants), and the shortest recombination interval between HvCBF12 and HvCBF13 (0.03 cM, 1 recombinant).

## Discussion

The genetic material derived from the 'Nure' (winter) x 'Tremois' (spring) cross underscores the advantage of having different agronomic traits segregating in a single population, e.g. yield stability in droughted Mediterranean environments, malting quality and beta-glucan content, and in particular low-temperature tolerance (Francia et al. 2004). Despite the large number of segregating populations developed to date to study frost tolerance in the *Triticeae* (Hayes et al. 1997; Tuberosa et al. 1997; Baum et al. 2003; Vagujfalvi et al. 2003; Reinheimer et al. 2004; Skinner et al. 2006), the 'Nure' x 'Tremois' cross where both the *Fr-H* loci are segregating in a colinear order and size respect to diploid and hexaploid wheats showed to be the most complete model for dissecting the trait in the tribe (Galiba et al. 2005). In this view, the phenotypic effects of the two major quantitative trait loci controlling frost resistance in barley (*Fr-H1* and *Fr-H2*, Francia et al. 2004) has been independently validated in  $F_2$ -derived  $F_3$  families and results summarized in Table 1 and Figure 2. Since the difference in  $F_v/F_m$  between reciprocal classes of  $F_3$  families can be considered as a rough measure of the allelic substitution effect at *Fr-H1* and *Fr-H2*, present data suggest again an additive effect for the two *Fr-H* genes in the 'Nure' x 'Tremois' system, although incomplete. Therefore, the large population of 1,849  $N \times T$  recombinants developed in this study not only represents a useful advance towards isolating the genetic determinants of the *Fr-H* loci, but is also a valuable tool to study their genetic and molecular interactions.

The recombinant screens and phenotypic evaluation allowed us to narrow the *Fr-H2* interval, moving from 27.2 cM (Francia et al.

2004) to 4.6 cM. This 5.9-fold position refinement around the *HvCBF4B* peak marker once again indicated that the *HvCBFs* are at present the best positional and functional candidate genes for the *Fr-2* QTL in the *Triticeae* genomes. Beside this,  $F_v/F_m$  testing of  $F_3$ s and  $F_4$ s still highlighted small differences in frost tolerance/susceptibility between progeny carrying homozygous alleles at both *Fr-H1* and *Fr-H2* and the parents 'Nure' and 'Tremois' (Table 1 and Figure 4b), at least significant for the  $F_4$ s. This could be explained by the action of minor loci affecting the trait, carried by cultivar 'Nure' in other chromosomal regions that have not been detected yet in the NxT population, most likely for the predominant effect of the two 5H QTLs. Some evidence in fact exists that other chromosome regions, in addition to *Fr-H1* and *Fr-H2*, are associated with cold tolerance (Tuberosa et al. 1997). Once again, the large  $F_2$  population that has been developed could be used to test this hypothesis, by selecting segregating material with fixed 'Tremois' alleles at *Fr-H1* and *Fr-H2*.

The CBF/DREB proteins are transcriptional activators that bind to the CRT/DRE DNA regulatory elements present in the promoter region of many cold-induced genes. They are a key component in configuring the low temperature transcriptome of the dicot *Arabidopsis*, which results in increased freezing tolerance (Novillo et al. 2004). The "CBF regulon" includes the most highly expressed *COR* genes (Vogel et al. 2005). Moreover, the AtCBF cold response pathway has a prominent role in determining the composition of the low-temperature metabolome (Cook et al. 2004). Orthologs of AtCBFs have now been isolated from a variety of crop plants, including cereals (Gao et al. 2002; Zhang et al. 2004; Qin et al. 2004; Ito et al. 2006; El Kayal et al. 2006). In particular, it was demonstrated that barley contains a

large family of at least 20 *CBF* genes (Skinner et al., 2005) and similar observations were recently done by Miller et al. (2006) in diploid wheat (*Triticum monococcum*) and by Badawi et al. (2007) in hexaploid wheat (*Triticum aestivum*). Barley *HvCBFs* were shown to specifically bind monocot and dicot *COR* gene CRT elements *in vitro* (Skinner et al. 2005) leading to the conclusion that in the *Triticeae* – as in *Arabidopsis* – members of the *CBF* gene family function as fundamental components of the winter hardiness regulon.

Of the more than 20 *HvCBF* genes identified in the barley genome (Skinner et al. 2005), there are at least 12 that map to *Fr-H2*. These *CBFs* at *Fr-H2* cosegregated as a single unit in the NxT population (136 DHs, Tondelli et al. 2006), and as two units (a single recombinant) in the DxM population (236 DHs, Skinner et al. 2006). Importantly, our large segregating population (3,698 gametes) allowed us to resolve genetically the map position of seven of these genes. Thus, we expect this population will be a critical resource to resolve each of the *HvCBF* genes relative to one another, and most importantly, relative to *Fr-H2*. Due to the absence of polymorphisms in the coding sequences of *HvCBF9* and *HvCBF14* between 'Nure' and 'Tremois', we were unable to order these two genes relative to the other *HvCBFs*. However, as additional sequences are obtained in the *HvCBF9* and *HvCBF14* flanking genomic regions, we anticipate this issue will be cleared up. Because the *HvCBF4* subgroup genes (*CBF2-4-9-14*) and the *HvCBF3* subgroup genes (*CBF3-6-10-12-13*) appear to co-cluster in both barley (Skinner et al. 2006) and *T. monococcum* (Miller et al. 2006), *HvCBF9* may be closer to *HvCBF2B* and *HvCBF4B*, than it is to the *HvCBF3* subgroup genes, and *HvCBF14* is probably somewhere between *HvCBF4B* and *HvCBF12*.

At the physical level, *HvCBF2A* resides on the same bacteriophage  $\lambda$  genomic clone as *HvCBF4B* (Stockinger et al. 2006). Similarly, *HvCBF10A* and *HvCBF10B* reside on a single  $\lambda$  genomic clone as do *HvCBF3A* and *HvCBF13* (Stockinger et al. 2006). *HvCBF2B* and *HvCBF12* on the other hand are the sole CBFs on single  $\lambda$  genomic clones (Stockinger et al. 2006). All of these physical relations are established in the 'Dicktoo' genotype. Preliminary data suggest that the physical map of 'Nure' is identical to 'Dicktoo', but that 'Morex' and 'Tremois' are quite different (EJS, AK Knox, and H Cheng, unpublished data). For example in 'Morex', *HvCBF3*, *HvCBF10A*, and *HvCBF6* reside on a single BAC clone (804E19), yet this 'Morex' clone does not harbor *HvCBF10B* (Skinner et al. 2005). This clone is part of a contig (ctg5873) of 620.1 kb by the Barley Physical Mapping Database (<http://phymap.ucdavis.edu:8080/barley/>). It is also noteworthy that our high resolution map of the *HvCBF* gene cluster (Figure 3c) spans a genetic distance of 0.81 cM that is essentially identical to the 0.8 cM distance that *Fr-2* spans in the A genome of wheat (Miller et al. 2006). Also the genetic order of our linkage map seems to match that reported by Miller et al. (2006), with the only exception of *TmCBF3* and *TmCBF10*, which however were not ordered for certainty in relation to the other genes. We can thus state that the recombinant genotypes used to finely map the *HvCBF* gene cluster represent the appropriate tool in the dissection of the genomic region which harbours the genetic determinant of *Fr-H2*. As next aim following this work, the recombinants at CBF cluster are being taken to homozygosity for contrasting ('Nure' and 'Tremois') alleles, in order to test the hypothesis whether the effect of *Fr-H2* is due to a "CBF number

game” – i.e. to the action of only one HvCBF, or to a particular and critical number of HvCBFs acting in a coordinated manner.

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