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ORIGINAL PAPER

Fine mapping of a Hv*CBF* 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' × '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_2 plants between parents 'Nure' and 'Tremois' was prepared. These two QT loci were first validated by using a set of F_3 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_4 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 Hv*CBF* gene cluster was then constructed, and after fine mapping, six recombinations between the Hv*CBF*s were observed. It was therefore possible to genetically divide seven Hv*CBF* 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 Hv*CBF* subclusters are being marker-selected and taken to homozygosity, to phenotypically separate the effects of the single Hv*CBF* 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) × 'Tremois' (spring)—N × T cross (Francia et al. 2004). They are located, approximately 25 cM apart, on the long arm of chromosome 5H. The N × T *Fr-H2* QTL is orthologous to the frost tolerance QTL *Fr-A^{m}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 N × T 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^{m}2*, Vagujfalvi et al. 2003) and to other barley mapping populations (e.g., *Fr-H1* in the 'Dicktoo' × 'Morex'—D × M population, Hayes et al. 1993; Skinner et al. 2006).

Vernalization is a period ranging from about 1 to 8 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 N × T, 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 plants, 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* effector genes is trans-activated by barley Hv*CBF*s (Xue 2002, 2003; Skinner et al. 2005).

In a previous report we showed that Hv*CBF4* is the marker peak of *Fr-H2* in the N × T population (Francia et al. 2004), and more recently, that five additional Hv*CBF*s are tightly linked to Hv*CBF4* and *Fr-H2* (Tondelli et al. 2006). At the same position in the D × M barley population, Skinner et al. (2006) mapped 11 Hv*CBF* genes into two clusters (approximately 1 cM apart), while at the homoeologous *Fr-A^{m}2* region in *Triticum monococcum* Miller and co-workers identified 11 Tm*CBF* genes (Miller et al. 2006). The cluster of group 5 *CBF*s 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^{m}2* in diploid wheat is the result of a single *CBF* gene, the combined effect of a subset (or all) of the *CBF*s, 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' × '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 Hv*CBF* candidate genes.

Materials and methods

Plant material and DNA extraction

A 'Nure' (winter, frost tolerant) × 'Tremois' (spring, frost susceptible) cross was used to generate a population of 1,256 F_2 plants (Fig. 1). Using marker-assisted selection, a first set of 36 F_2 recombinants was chosen and their corresponding F_3 progeny phenotyped for frost tolerance to independently validate *Fr-H1* and *Fr-H2*. A second set of 389 F_3 individuals was obtained by field sowing the seed of 49 F_2 plants that were expected to be recombinant at the *Fr-H2* locus based on their marker haplotype. Twenty-nine homozygous F_4 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_4 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_2 plants were added to the first F_2 population for a total of 1,849 recombinant

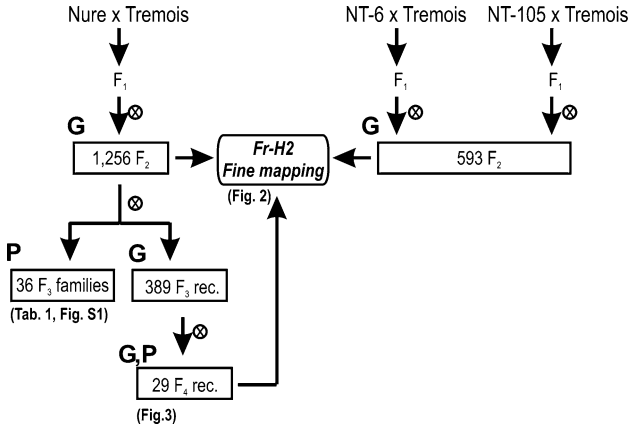

Fig. 1 Plant materials developed, and outline of *Fr-H2* fine mapping strategy. P, phenotyping; G, genotyping

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 harbor the 'Nure' alleles at both *Fr-H* loci, Francia et al. 2004). The resulting F_1 lines were then selfed to obtain 296 and 297 plants, respectively.

Genomic DNA (gDNA) of the 136 N × T 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_2, F_3, and F_4 lines) was isolated by placing leaf tissues from field grown plants in 96-well microtube plates. Plant material was ground using the Retsch® MM300 Mixer Mill and DNA was extracted using the Wizard® 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® 3100 Genetic Analyzer (Applied Biosystem). Hv*BM5A*, *Zcct-H* and Hv*MYB1* CAPS markers were analyzed as reported by von Zitzewitz et al. (2005) and Tondelli et al. (2006). Nomenclature of the barley *CBF*s follows that of Skinner et al (2005) where Hv*CBF*s designated with the same number but different letters (e.g. A and B) describe highly identical although distinct genes. Specific markers targeting the genes Hv*CBF2B*, *3A*, *4*, *6*, *10B*, and *12* were genotyped according to Francia et al. (2004) and Tondelli et al. (2006). To classify Hv*CBF4* with respect to previously characterized Hv*CBF4A, 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 Hv*CBF4* gene (Francia et al. 2004) as Hv*CBF4B*.

For Hv*CBF13*, allele specific PCR primers (Nure-F 5′-ATGCCAATTACTCCCTC-3′, Nure-R 5′-TCATGGCACATGAAG-3′; Tremois-F 5′-ATGATTAGGAGGACTGTG-3′, Tremois-R 5′-TCACCTCGTCGATCTTGA-3′) were designed. Amplification was performed in a 15 µl final volume containing: 40 ng of genomic DNA as template, 1× Qiagen PCR buffer, 1.5 mM of $MgCl_2$, 1× 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.

For MWG583, specific oligonucleotide primers were designed on the RFLP probe sequence AJ234522 using Primer3 (Rozen and Skaletsky 2000): MWG583-F 5′-CTCGTCTCGTGTGAGTGAGC-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 *Bsr*I according to manufacturer's instructions, 10 µl of PCR product were incubated for 1.5 h with 2U of restriction enzyme, 1× 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_2 populations. The Kosambi mapping function was used to convert recombination frequencies into centimorgans (cM). The genetic distance between all Hv*CBF* 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_3 and F_4 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_v/F_m. The F_v/F_m 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_3 progeny derived from F_2 recombinants was performed in 2004; phenotyping of the 29 F_4 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_3 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 Hv*CBF* 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 N × T cross

As an initial step in the development of a high-resolution genetic map of barley chromosome 5HL (Fig. 2a), 1,256 'Nure' × 'Tremois' F_2 plants were screened with molecular markers encompassing the *Fr-H* loci, including: Bmag0223, Hv*CBF4B*, Hv*MYB1*, Hv635P2.4, and Bmag0222. Hv*CBF4B* 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_2 population (Fig. 2b) were consistent with those reported in our previous studies on the N × T 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*, 36 F_3 families derived from marker-selected individual F_2 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_v/F_m indices in the F_3 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 Electronic Supplementary Material (Fig. S1). 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 Fig. S1), and the two QTLs confirm their center 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_2, are shown in Fig. 2b.

A refined genomic interval of *Fr-H2* QTL

Validation of *Fr-H1* and *Fr-H2* in the N × T F_2, 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_2 populations derived from the cross NT-6 × 'Tremois' and NT-105 × 'Tremois' (see Methods). Inclusion of these F_2 individuals in

Table 1 Frost tolerance of reciprocal classes of 36 marker-selected F_3 families and parents (N = 'Nure' and T = 'Tremois')

Haplotype of F_3 families		Phenotypic class mean (F_v/F_m)		Difference (F_v/F_m)	*P*
Bmag0223-**Hv*CBF4B***-Hv*MYB1*-**Hv635P2.4**-Bmag0222					
AA**A**A**A** (3)	**B**B**B**B**B** (3)	0.653	0.331	0.322	<0.001
AA**H**H**H** (3)	**B**B**H**H**H** (3)	0.524	0.273	0.251	0.018
H**A**A**H**H (4)	H**B**B**H**H (4)	0.532	0.292	0.240	0.005
H**H**A**A**H (4)	H**H**B**B**H (4)	0.589	0.338	0.251	0.001
H**H**H**A**A (4)	H**H**H**B**B (4)	0.580	0.374	0.206	0.026
AA**A**A**A** (N)	**B**B**B**B**B** (T)	0.725	0.222	0.503	0.001

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* (Hv*CBF4B*) QTL peak markers and their allelic states are shown in bold in the table. Homozygous regions are underlined

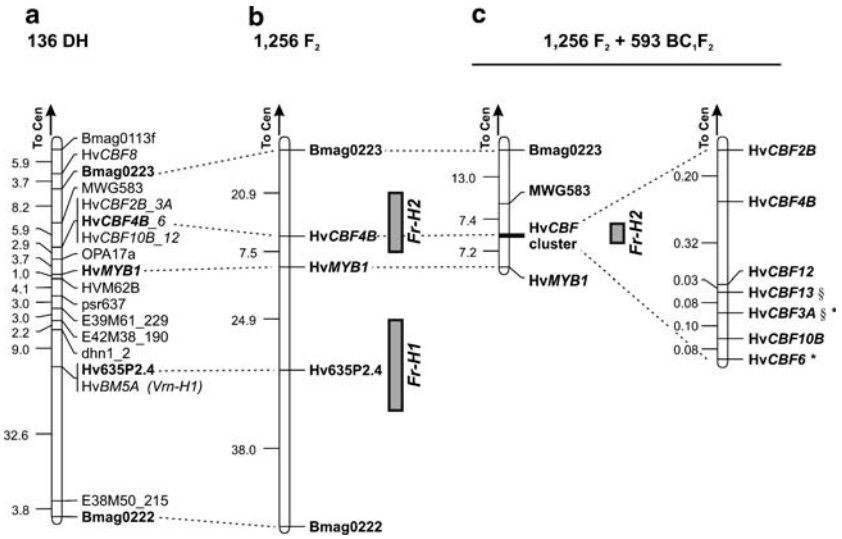

Fig. 2 Consecutive stages of high-resolution mapping of *Fr-H2*. (**a**) Linkage map of barley chromosome 5HL constructed using the 'Nure' × 'Tremois' DH population of 136 individuals. Segregation data are from Tondelli et al. (2006), and includes the STS marker MWG583 added in this study. (**b**) Genetic 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' × '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 drawn in the middle of the intervals flanking the peak markers, following the frost tolerance test of the 36 F_3s described in the text. (**c**) Refinement of the *Fr-H2* locus and high-resolution genetic map of the Hv*CBF* gene cluster, after screening of 1,849 plants (1,256 + 593 F_2); confidence interval ($P \leq 0.01$) for *Fr-H2* around the Hv*CBF4B* marker was estimated after phenotyping results of 29 F_4s summarized in Fig. 3. 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 Hv*CBF* cluster. Hv*CBF3A* and Hv*CBF6* 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. Hv*CBF13* and Hv*CBF3A* are located on the same λ clone (Stockinger et al. 2006), and have been labeled accordingly

the mapping population yielded a total of 1,849 recombinant plants—i.e. 3,698 gametes (Fig. 1). Mapping Bmag0223, MWG583, Hv*CBF4B*, and Hv*MYB1* in the expanded population of 1,849 F_2 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 (Fig. 2c). Informative F_2 recombinants were marker-selected, field-sown, and 389 F_3 plants were selfed to produce F_4 seed (Fig. 1). Following genotypic evaluation, homozygous individuals were chosen and divided in two classes carrying a recombination either between MWG583 and Hv*CBF4B* ('Interval 1'), or between Hv*CBF4B* and Hv*MYB1* ('Interval 2') (Fig. 3a). All these recombinants carried the 'Nure' allele above the recombination event, and the 'Tremois' allele below the recombination event (Fig. 3a), thus also eliminating any effects of *Fr-H1* on further phenotypic analyses. A double recombinant F_4 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 (Fig. 3a). These 29 F_4 lines were tested for frost resistance in controlled conditions by freezing to −11°C and then measuring F_v/F_m. As shown in Fig. 3b, all 16 F_4 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 (Fig. 3b). Applying the binomial distribution to the proportion of recombinant genotypes between MWG583 and Hv*CBF4B*, or between Hv*CBF4B* and Hv*MYB1*, a confidence interval ($P \leq 0.01$) for *Fr-H2* was then calculated, which refined *Fr-H2* to a 4.6 cM segment around Hv*CBF4B* (Fig. 2c).

In accordance with what observed in the previous experiment of F_3 families phenotypic testing (Table 1 and Fig. S1), after phenotyping the F_4 recombinants harboring the 'Nure' ("A") allele at the Hv*CBF* cluster (i.e. at *Fr-H2*) and the 'Tremois' ("B") allele at Hv635P2.4/Hv*BM5A* (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 N × T crosses, independently by the allelic state at *Fr-H1/Vrn-H1* region (Fig. 3a, b).

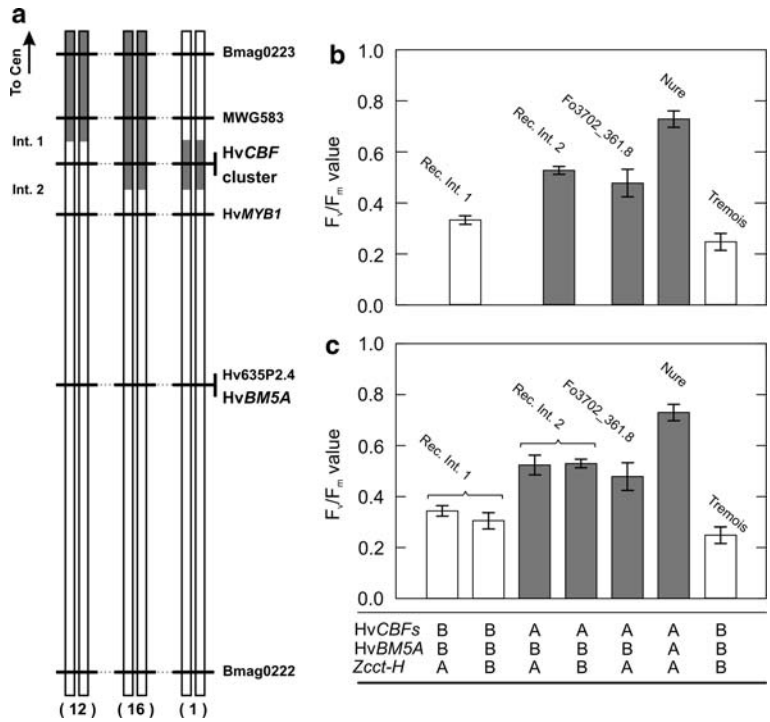

Fig. 3 Graphic genotyping and frost tolerance of 'Nure' × 'Tremois' F_4 recombinants above (Interval 1) and below (Interval 2) the Hv*CBF* cluster. (**a**) Graphical representation of chromosome 5HL region of the phenotyped F_4 plants. Chromosomal segments with 'Nure' and 'Tremois' marker alleles are in gray and white, respectively. The number of F_4 plants for each recombinant class is in parenthesis, and distances are in scale with those reported in Fig. 2a. (**b**) Phenotypic F_v/F_m values of the three F_4 recombinant classes are presented in comparison with the two parents. (**c**) Effect of the allelic state at Hv*CBF* cluster (*Fr-H2*), Hv*BM5A* (*Fr-H1/Vrn-H1*), and *Zcct-H* (*Vrn-H2*) on frost tolerance of F_4 recombinants and parents. "A" and "B" indicate 'Nure' and 'Tremois' allelic state, respectively. Vertical bars in (**b**) and (**c**) represent standard errors

To test if the allelic state at the second vernalization requirement locus segregating in N × T, *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_4 Interval 1 and Interval 2 recombinants.

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

In a previous work, we mapped Hv*CBF4B* to the peak of *Fr-H2* in the N × T population (Francia et al. 2004). Subsequently, we placed five additional Hv*CBF*s at the same locus (Tondelli et al. 2006). To determine their genetic order at *Fr-H2*, a high-resolution genetic map of the Hv*CBF* cluster on 5H including these six genes and the newly tagged Hv*CBF13* was generated. The large segregating population of 1,849 individuals (1,256 + 593 F_2) was screened for recombinants between each of the Hv*CBF* genes polymorphic between 'Nure' and 'Tremois'. This allowed us to produce the fine map drawn in Fig. 2c. The Hv*CBF* cluster spans in barley a total genetic distance of 0.81 cM, with the largest distance between Hv*CBF4B* and Hv*CBF12* (0.32 cM, 12 recombinants), and the shortest recombination interval between Hv*CBF12* and Hv*CBF13* (0.03 cM, 1 recombinant).

Discussion

The genetic material derived from the 'Nure' (winter) × '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' × '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 Fig. S1. 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' × 'Tremois' system, although incomplete. Therefore, the large population of 1,849 N × 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 (Francia et al. 2004) to 4.6 cM. This 5.9-fold position refinement around the *HvCBF4B* peak marker once again indicated that the Hv*CBF*s 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_3s and F_4s 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 Fig. 3b), at least significant for the F_4s. 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 N × T 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 At*CBF*s 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 Hv*CBF*s 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 Hv*CBF* 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 N × T population (136 DHs, Tondelli et al. 2006), and as two units (a single recombinant) in the D × M 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 Hv*CBF* genes relative to one another, and most importantly, relative to *Fr-H2*. Due to the absence of polymorphisms in the coding sequences of Hv*CBF9* and Hv*CBF14* between 'Nure' and 'Tremois', we were unable to order these two genes relative to the other Hv*CBF*s. However, as additional sequences are obtained in the Hv*CBF9* and Hv*CBF14* flanking genomic regions, we anticipate this issue will be cleared up. Because the Hv*CBF4* subgroup genes (CBF2-4-9-14) and the Hv*CBF3* 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), Hv*CBF9* may be closer to Hv*CBF2B* and Hv*CBF4B*, than it is to the Hv*CBF3* subgroup genes, and Hv*CBF14* is probably somewhere between Hv*CBF4B* and Hv*CBF12*.

At the physical level, Hv*CBF2A* resides on the same bacteriophage λ genomic clone as Hv*CBF4B* (Stockinger et al. 2006). Similarly, Hv*CBF10A* and Hv*CBF10B* reside on a single λ genomic clone as do Hv*CBF3A* and Hv*CBF13* (Stockinger et al. 2006). Hv*CBF2B* and Hv*CBF12* on the other hand are the sole CBFs on single λ 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', Hv*CBF3*, Hv*CBF10A*, and Hv*CBF6* reside on a single BAC clone (804E19), yet this 'Morex' clone does not harbor Hv*CBF10B* (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 Hv*CBF* gene cluster (Fig. 2c) 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 Tm*CBF3* and TmCBF*10*, 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 Hv*CBF* gene cluster represent the appropriate tool in the dissection of the genomic region which harbors the genetic determinant of *Fr-H2*. As next aim following this work, the recombinants at CBF cluster are being taken to homozygosis 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 Hv*CBF*, or to a particular and critical number of Hv*CBF*s acting in a coordinated manner.

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