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ORIGINAL PAPER

E. Francia · F. Rizza · L. Cattivelli · A. M. Stanca · G. Galiba · B. Tóth · P. M. Hayes · J. S. Skinner · N. Pecchioni

Two loci on chromosome 5H determine low-temperature tolerance in a 'Nure' (winter) × 'Tremois' (spring) barley map

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Abstract Barley (*Hordeum vulgare* subsp. *vulgare*) is an economically important diploid model for the *Triticeae*; and a better understanding of low-temperature tolerance mechanisms could significantly improve the yield of fall-sown cereals. We developed a new resource for genetic analysis of winter hardiness-related traits, the 'Nure' × 'Tremois' linkage map, based on a doubled-haploid population that is segregating for low-temperature tolerance and vernalization requirement. Three measures of low-temperature tolerance and one measure of vernalization requirement were used and, for all traits, QTLs were mapped on chromosome 5H. The vernalization response QTL coincides with previous reports at the *Vrn-1/Fr1* region of the *Triticeae*. We also found coincident QTLs at this position for all measures of low-temperature tolerance. Using Composite Interval Mapping, a second proximal set, of coincident QTLs for low-temperature tolerance, and the accumulation of two different COR proteins (COR14b and TMC-Ap3) was identified. The *HvCBF4* locus, or another member of the *CBF* loci clustered in this region, is the candidate gene underlying this QTL. There is a CRT/DRE recognition site in the promoter of *cor14b* with which a CBF protein could interact. These results support the hypothesis that highly conserved regulatory factors, such as members of the *CBF* gene family, may regulate the stress responses of a wide range of plant species.

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E. Francia · F. Rizza · L. Cattivelli · A. M. Stanca · N. Pecchioni (✉)
Istituto Sperimentale per la Cerealicoltura,
29017 Fiorenzuola d'Arda, Italy
e-mail: n.pecchioni@iol.it
Tel.: +39-0523-983758
Fax: +39-0523-983750

G. Galiba · B. Tóth
Agricultural Research Institute
of the Hungarian Academy of Sciences,
2462 Martonvásár, Hungary

P. M. Hayes · J. S. Skinner
Department of Crop and Soil Science
and Department of Horticulture,
Oregon State University, Corvallis, OR 97331, USA

Introduction

A better understanding of the genetics of cold tolerance could have a significant impact on world food supply, since low-temperature-related stresses limit the productivity of many plants of agronomic and horticultural value. Barley (*Hordeum vulgare* subsp. *vulgare*) is an excellent model system for genetic analysis of the molecular basis of low-temperature tolerance in fall-sown cereals. There is indeed abundant genetic variation for this trait within the primary gene pool and an ever-expanding set of tools for genetic analysis, ranging from mapping populations to arrays (Hayes et al. 2003). In barley, as in other members of the *Triticeae*, there is also genetic variation for growth habit, which is broadly described as 'winter' or 'spring'. Winter growth habit is due to the requirement of an external signal to the plant to shift from vegetative to reproductive growth: this signal can be completion of a vernalization requirement (Limin and Fowler 2002) and/or daylength of sufficient duration (Karsai et al. 1999). Resistance to low temperature is necessary for winter-habit genotypes grown in areas with subzero winter temperatures. Maximum low-temperature tolerance is achieved after induction—"hardening", i.e. exposure to moderately low temperatures, and is achieved at vegetative growth stages (Hayes et al. 1997). Vernalization, low-temperature tolerance, and photoperiod sensitivity are inter-related (Limin and Fowler 2002). The same conditions, essential for vernalization of winter genotypes, promote the hardening process in both spring and winter types. Since winter-type plants are generally hardier than spring ones a major debated question is whether vernalization response and low-temperature tolerance are pleiotropic effects of the same genes or determined by separate loci (Limin and Fowler 2002). Nevertheless, in a large sample of barley germplasm the three phenotypes for vernalization, low-temperature tol-

erance and photoperiod sensitivity were observed to occur in all possible combinations (Karsai et al. 2001).

The *Triticeae* form a homogeneous genetic system and comparative genetics studies confirm that the genetic determinants of winter hardiness are conserved, and therefore results from one species are applicable to other members of the tribe (Dubcovsky et al. 1998). Quantitative trait locus (QTL) analysis tools, when applied to the *Triticeae*, converge on a limited number of conserved genome regions as being responsible for the components of winter hardiness. Most consistent is a region on the long arm of chromosome 5H where there are QTL/loci for vernalization response, low-temperature tolerance and photoperiod sensitivity (Pan et al. 1994; Cattivelli et al. 2002). These QTL effects have, in some reports, been described as the effects of single loci. Thus, low-temperature tolerance loci on chromosomes 5A, 5B and 5D have been assigned the locus designations *Fr-A1*, *Fr-B1* and *Fr-D1*, respectively (Sutka and Snape 1989; Snape et al. 1997; Tóth et al. 2003a); and by inference based on molecular markers in common to the various linkage maps, the H genome homoeolog to the *Fr* loci is the low-temperature tolerance QTL first reported in the 'Dicktoo' × 'Morex' population (Hayes et al. 1993). Vernalization loci have been assigned the designation *Vrn*, and *Vrn-1* orthologs map to the group-5 chromosomes (Cattivelli et al. 2002). Dubcovsky et al. (1998) standardized vernalization loci nomenclature with the *Vrn* designation and assigned a more detailed linkage map position to orthologous genes on *Triticeae* homoeolog chromosomes. In barley three *Vrn-H* loci determine the vernalization requirement: *Vrn-H1* on chromosome 5H, *Vrn-H2* on 4H and *Vrn-H3* on 1H. Alleles at these loci interact epistatically, such that a vernalization requirement occurs in plants homozygous recessive at *Vrn-H1* and *Vrn-H3*, and with at least one dominant allele at *Vrn-H2*. The interrelationships of vernalization, photoperiod and low-temperature are most likely attributable to linkage rather than pleiotropy. In the 'Dicktoo' × 'Morex' population, low-temperature tolerance and photoperiod sensitivity QTLs map to the same region as the *Fr-A1* and *Vrn-A1* loci, which Galiba et al. (1995) demonstrated were distinct loci via linkage mapping and Sutka et al. (1999) showed that were distinct loci via physical mapping.

During the physiological processes of cold acclimation, a number of stress-related genes are up-regulated [see Cattivelli et al. (2002) for a recent review]. Among the barley *COR* (COld-Regulated) genes are *cor14b* and *tmc-ap3*. *Cor14b* encodes a soluble protein of unknown function localized in the stroma compartment of the chloroplast (Crosatti et al. 1995), whereas *tmc-ap3* encodes a putative channel protein of the chloroplast outer envelope selective for amino acids (Baldi et al. 1999). Likewise, the existence of genes regulating *COR* genes has been hypothesized. In both wheat and rye, Fowler et al. (1996) hypothesized that a gene, or genes, in the *Vrn-A1/Fr-A1* region were responsible for regulating the *Wcs120* gene-family. Vagujfalvi et al. (2000) hypothesized that two loci (provisionally designated as *Rcg1* and *Rcg2*) on chromosome 5A, regulate the expression of the *cor14b* gene. The first locus, tightly linked to the marker *psr911*, is more proximal on the chromosome, while the second one is slightly distal to the marker *psr2021(ABA2)*, belonging to the region of *Vrn-A1/Fr-A1*.

A notable advance in plant cold-tolerance research was the discovery of the *CBF* (C-repeat Binding Factor) family of genes. In Arabidopsis, these transcription factors have been shown to be key determinants of low-temperature tolerance (Thomashow et al. 2001). Genes with *CBF* signature sequences have been reported in the *Triticeae* and have been characterized in terms of their map location, coding sequence, and expression in barley (Choi et al. 2002; Von Zitzewitz 2003). The mapping of candidate genes, such as transcription factors, can reveal their genetic relationships with previously detected QTLs (Ishimaru et al. 2001); our principal interest is thus to determine the role of the *CBFs* in the cold tolerance of barley and the *Triticeae*, and accordingly a first step is to relate their map locations with QTLs for winter hardiness traits.

In this paper we report the development of a new 'winter' × 'spring' barley map which is a valuable resource for winter hardiness genetics, since the 'Nure' × 'Tremois' (NT) population is segregating for both vernalization requirement and low-temperature tolerance, whereas the 'Dicktoo' × 'Morex' population is segregating for photoperiod sensitivity and low-temperature tolerance (Hayes et al. 1997). With this genetic tool, our objectives were to determine: (1) the number, location and effect of QTLs determining low-temperature tolerance, vernalization response and heading date, (2) the location of *COR* genes relative to these QTLs, (3) the QTLs responsible for two COR proteins accumulation, and (4) the location of representative *CBF* loci relative to these winter hardiness-related traits.

Materials and methods

Plant material and DNA extraction

One hundred and thirty six doubled-haploid (DH) lines were derived by anther culture from the F_1 of the cross of 'Nure' × 'Tremois' at Saaten-Union Resistenzlabor GmbH, Germany. The winter parent—'Nure' [(Fior 40 × Alpha^2) × Baraka]—is a modern, high yielding two-rowed feed-barley cultivar released by the Istituto Sperimentale per la Cerealicoltura Section of Fiorenzuola, Italy. It has a wide range of adaptability and it belongs to the RLT (Recommended-List Trials) of Italy. The spring parent—'Tremois' [(Dram × Aramir) × Berar]—is a modern, high yielding French two-rowed malting variety, also included in the RLT. Genomic DNA was extracted from young leaf tissue of greenhouse-grown plantlets using a modified CTAB method (Saghai-Maroof et al. 1984).

Map construction

The linkage map was built with morphological, biochemical and molecular markers. Linkage map positions of many of these loci have been reported in the literature, allowing us to anchor the maps. Several loci, previously unreported in the literature, were also

mapped, as described below. Four morphological markers—*gsh6* (glossy sheath 6), *eog* (elongated outer glume), *pau* (purple auricle) and *pvc* (purple veined lemma)—were scored using the procedures and nomenclature of Franckowiak and Lundqvist (2002) on field-grown material in the spring of 2000. Two biochemical markers—*Hor1* and *Hor2* (corresponding to the C- and B-hordeins, respectively)—were analyzed by A-PAGE electrophoresis separation of seed-storage proteins as described by Faccioli et al. (1995).

Eighty eight SSR markers were screened for polymorphism using fluoresescently labeled primers and fragment analysis on a ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). Eighty seven of the SSRs were derived from the reports of Becker and Heun (1995) and Ramsay et al. (2000). Forty eight out of the 87 labeled primers were kindly provided by R. Waugh, Scottish Crop Research Institute, Scotland. The SCRI primer sequences described by Ramsay et al. (2000) are available at http://www.genetics.org/cgi/content/full/156/4/1997/DC1. The *Hv635P2.4* SSR primer sequences were kindly provided by J. Von Zitzewitz, Oregon State University, USA: forward 5′-GGCGACCTGTAG-TGGGTATG-3′ and reverse 5′-TGCTCAGCCCCCTCTTATAG-3′. Thirty eight of the 88 SSR loci were polymorphic and selected for mapping. The differentially labeled polymorphic primer combinations were multiplexed for amplification and/or loading. PCR conditions are those reported by Becker and Heun (1995) and Ramsay et al. (2000), except for *Hv635P2.4*. For this SSR, amplification was performed in a 15-μl final volume containing 25 ng of genomic DNA, 1×PCR Gold Buffer (Applied Biosystems, Foster City, Calif.), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of forward and reverse primer, and 0.6 U of *AmpliTaq Gold* DNA polymerase (Applied Biosystems, Foster City, CA).

Four locus-specific, PCR-based, markers were mapped, out of 15 screened for polymorphism, following the published protocols of Kilian et al. (1999) for the telomeric marker *tel3S*, Mano et al. (1999) for *mwg634*, and Toth et al. (submitted) for *psr637* and *wg644*. Three STS markers—*wrlk1*, *wrlk2* and *wrlk4*—were developed at the Istituto Sperimentale per la Cerealicoltura, based on the sequence of the clone dd10 (Cattivelli, unpublished). The forward and reverse primer sequences are:

wrlk1: 5′-GGTTCTTGAGTCGGAGCTACAGCGG-3′;
5′-ATGTTACAACCTCCCTCGCCG-3′;
wrlk2: 5′-ATGGATCCATGGATGGCCAGC-3′;
5′-CTGTGGGGATTAATTGATGTCC-3′, and
wrlk4: 5′-CAGCGGGTGAGCGAGGAGAAC-3′;
5′-ACCACGAGTATGGTCTTGTCC-3′.

Amplifications were performed in a 20-μl final volume containing 100 ng of genomic DNA, 1×PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of forward and reverse primer, and 1 U of *Platinum Taq* DNA polymerase (Invitrogen). After an initial denaturation step of 94°C for 2 min, reactions were subjected to a touch-down amplification: 10 cycles of 94°C for 1 min, 64°C for 1 min (decreasing 1°C/cycle), 72°C for 1 min and subsequent 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min with a final extension step of 72°C for 10 min. Products were separated in a 2% standard agarose gel.

Two RAPD markers (*OPA17a* and *OPA17b*) were scored by amplifying 20 ng of genomic DNA in a 20-μl reaction volume with 1×PCR buffer, 1.5 mM of $MgCl_2$, 0.1 mM of each dNTP, 0.2 μM of the Operon A17 primer and 0.8 units of *Platinum Taq* DNA polymerase (Invitrogen). After an initial denaturation step of 94°C for 2 min reactions were subjected to 45 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min and a final extension of 72°C for 10 min. PCR product separation was as described above for *wrlk* markers.

Four abiotic stress-related genes were mapped as RFLPs (*cor18*, *aba2*, *af93* and *acx1*) and four using PCR [*aba3(psr2020)*, *dhn1*, *dhn3* and *dhn6*]. The cDNA clone for *aba2* was kindly provided by M. Gulli, Università degli Studi di Parma, Italy. For RFLP analysis, digestions were performed with *Dra*I (*af93*) and *Bam*HI (*cor18*, *aba2*, *acx1*). Restricted DNAs (10 μg) were size-fractionated in a 0.8% agarose gel and analyzed via the Southern protocol of Sambrook et al. (1989). The forward and reverse primer sequences for *aba3(psr2020)* were: 5′-CTGGGGTTGGTTCTGG-3′ and 5′-GCCAGCAACATGGCATTC-3′. The primer sequences for amplifying 12 dehydrin genes were kindly provided by T.J. Close, University of California/Riverside, USA. Three polymorphic dehydrin loci were mapped following the amplification and fragment separation conditions described by Choi et al. (2000). The *dhn1* and *dhn3* products were STS mapped, whereas *dhn6* was mapped as a CAPS marker with enzyme *Kpn*I.

Two *CBF* loci—*HvCBF4 and HvCBF8*—were mapped using gene-specific primers. The primers for *HvCBF4* were forward: 5′-ATGGACGTCGCCGACATC-3′, and reverse: 5′-TTAGCA GTC-GAACAAATAGCT-3′ and those for *HvCBF8* were forward: 5′-CAAGTTGCCGGTCGCGAC-3′, and reverse 5′-AACAGTTTCC-TAATTGAGTGATGG-3′. PCR amplification and fragment separation were as described above for *wrlk* markers. For mapping, *HvCBF8* products were digested with enzyme *Dde*I. The *HvCBF4* locus was mapped following the SNP assay procedures of Drenkard et al. (2000). Based on the *HvCBF4* allele sequences of purified PCR products from 'Nure' and 'Tremois', we used PRIMER3 (Rozen and Skaletsky 1998) to design a common reverse primer (5′-TTAGCAGTCGAACAAATAGCT-3′) and two allele-specific forward primers, differing for the SNP in the terminal base. The 'Nure'-specific forward primer sequence was 5′-ACGAGGAG-CAGTGGTTTAGA-3′. The 'Tremois'-specific forward primer sequence was 5′-ACGAGGAGCAGTGGTTTAGC-3′. Polymorphic bands were obtained by amplifying 40 ng of genomic DNA in a 15-μl reaction volume with 1×Qiagen PCR Buffer, 1×Q Solution, 0.25 mM of each dNTP, 0.4 μM of each primer and 1 U of Qiagen *Taq* DNA polymerase. After an initial denaturation step of 94°C for 2 min, reactions were subjected to 30 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 1 min and products were separated on a 1.5% standard agarose gel.

Seven [α^{33}P]-labeled *Eco*RI/*Mse*I AFLP primer combinations (E35M61, E37M38, E38M50, E39M61, E41M38, E42M32 and E42M38) were chosen in order to facilitate map alignment, based on their previous use in the construction of barley linkage maps (Becker et al. 1995; Qi et al. 1998). The combinations E37M38 and E42M32 were in common with both reports; E41M38 and E42M38 were used by Becker et al. (1995); E35M61, E38M50 and E39M61 were used by Qi et al. (1998). The AFLP reactions were carried out following the instructions supplied with the GIBCO BRL Life Technology AFLP kit. The polymorphic AFLP loci were named using the nomenclature proposed by Qi and Lindhout (1997). In addition, AFLP bands mapped in the NT population, that were of the same size and genome location as those reported in the 'Proctor' × 'Nudinka' and the 'L94' × 'Vada' maps, were compared with the AFLP profiles available on GrainGenes (http://wheat.pw.usda.gov/). The eight AFLPs that "bridge" the three barley maps were named using the standard molecular weight nomenclature of Qi and Lindhout (1997), and in addition we included the numerical suffix number assigned by Becker et al. (1995).

Linkage map was constructed using MAPMAKER/EXP 3.0 (Lander and Botstein 1989). Linkage groups were first calculated using a LOD threshold of 10.0 and the best marker order in each group was assessed using the 'COMPARE' command. Linkage groups were extended by dropping the LOD threshold to 3.0 and locus orders were again established using the 'COMPARE' command. Additional markers were placed on the map by means of 'NEAR' and 'TRY' commands. Chromosome designation and polarity were inferred from previously mapped morphological, biochemical and molecular markers.

Phenotyping

The winter survival of the population was rated under field conditions at Fiorenzuola d'Arda (Italy) during the 2001/2002 growing season, as differential winter injury was observed. The minimum and average observed temperatures for the period December 2001/January 2002 were −10.2°C, and −0.3°C respec-

tively. The experiment was seeded in October, 2001 using a three-replicate randomized block design. Each plot measured 4 m^2 (3×1.36 m-long). Winter survival was assessed at the end of February 2002 using a 0–9 scale, where 0 = no survival and 9 = complete survival.

A test of the frost resistance of the NT DH lines plus parentals, was carried out using the procedure described previously by Sutka (1981). Briefly, the plants at the stage of the 3rd leaf were first treated for 5 weeks with a regime of decreasing temperature and light. During the 6th week, hardening was carried out using a day temperature of +2°C and a night temperature of −2°C, with 20 h of daylight. After hardening, the boxes were transferred to a controlled environment where the temperature was reduced by 1°C/h to −4°C. Hardening was continued for a further 2 days in the dark, after which the frost treatment was carried out at −11°C and −13°C. After 24 h of freezing without light, the temperature was raised by 2°C/h to +1°C, and the plants were kept at this temperature for 15 h. The boxes were then transferred to a GB (Conviron) unit for recovery at a day temperature of 16°C and a night temperature of 15°C, with a 14 h/day illumination for 18 days. After recovery, a score was recorded in terms of regrowth on a 0 (dead) to 5 (undamaged) scale.

To evaluate the effect of freezing on the functionality of the Photosystem II (PSII) reaction centers, the maximum quantum yield of the PSII photochemistry was measured by the ratio of variable (F_v) to maximal (F_m) fluorescence in a dark-adapted state, F_v/F_m (Butler and Kitajima 1975), in the NT population and parents. The experiment was repeated 4 times in the growth chamber and was carried out on first-leaf stage plants after 4 weeks of cold acclimation (3°C, 8 h light and 2°C, 16 h dark). For freezing treatments, plants were exposed to −12°C (18 h) and F_v/F_m was measured in leaves at the end of the acclimation period, after stress exposure and after a 24 h recovery time, using a Pulse Amplitude-Modulated fluorometer (PAM 2000, Walz, Effeltrich, Germany) according to Rizza et al. (2001).

Heading date was measured in a fall-sown trial (October 2000) at Fiorenzuola d'Arda (Italy). For vernalization requirement, the same fall-sown trial (October 2000) and a spring-sown one (March 2001, a late-spring sowing season for the North Italian climate) were used. In both experiments a completely randomized block design with two replications was used. Each plot consisted of 2×1 m-long rows. For QTL analysis of heading date (fall-sowing), data were recorded as days to heading, i.e. the number of days from April 1st to where at least 50% of spikes in a plot were completely emerged above the flag leaf. For vernalization requirement (late-spring-sowing) the days to heading after April 1st were recorded, and a score of 100 for days to heading was assigned to those genotypes that did not flower at all. The arithmetical difference in days to heading between the spring and fall-plantings was used for QTL analysis of the vernalization requirement, and this value was termed "heading date delay" (Hd-D).

COR protein accumulation

The accumulation of two COR proteins—COR14b and TMC-Ap3—was assayed in field-grown plant material grown at Fiorenzuola d'Arda, Italy. The population and the parents were sown in October 2000 in 1 m-long rows using a three-replicated randomized complete block design. Leaf samples were collected between 9 and 10 a.m. every 15 days from December 19, 2000, to January 22, 2001. Western blotting analysis was performed as described by Giorni et al. (1999) using COR14b and TMC-Ap3 polyclonal antibodies with the ECL Western kit (Amersham). The tissue samples of the parental lines were analyzed first, and those from the Dec. 19 harvest showed the greatest difference in protein accumulation. Accordingly, Western analysis of the full mapping population was conducted using tissues sampled at this date. The luminescent signal was captured by exposure to Bio-Max Kodak films; signal intensity was then analyzed using Molecular AnalystSoftware vers. 1.5 (Bio-Rad).

Statistical and QTL analyses

ANOVAs were performed using MSTAT-C software (Freed et al. 1988, DOS version 2.10). The functions 'FREQ' and 'STAT' were used to generate phenotypic frequency distributions and to test their normality. For QTL analysis, the winter survival in the field, frost tolerance in the controlled freeze test, frost tolerance measured using the F_v/F_m value and COR protein accumulation data for each DH line, were converted to the corresponding percentage of the 'Nure' parent for QTL mapping. Map density was reduced by eliminating markers closer than 2.0 cM. The resulting map, consisting of 101 markers, were reconstructed using the Kosambi mapping function. The primary tool for QTL analysis was PLABQTL (Utz and Melchinger 1996) using a LOD threshold of 3.0 and a 2.0 cM scan interval. After a preliminary analysis using Simple Interval Mapping (SIM), the markers with the highest LOD value were used as cofactors for Composite Interval Mapping (CIM). As a validation step, the chromosome 5H QTL data were also analyzed using MapQTL version 4.0 (Van Ooijen et al. 2002) using a LOD threshold of 3.0. After a preliminary analysis using SIM, the most statistically significant markers were selected using the backward elimination procedure as cofactors for CIM.

Results

The 'Nure' × 'Tremois' linkage map

One hundred and twenty eight loci were placed on the NT linkage map (Fig. 1). The map spans 1,182 Kosambi cM and has an average density of one non-co-segregating marker every 10 cM, ranging from 16.1 cM for chromosome 4H to 7.4 cM for chromosome 5H. There are several regions where no polymorphisms were detected. The largest of these gaps are on chromosomes 2H and 7H. Clustering of markers is observable at inferred centromeric regions, particularly on 6H. In this region, there was significant segregation distortion in favor of the 'Tremois' parent (mean χ^2=18.10 at $P\leq$0.05). On 2H, markers between *eog* and *pvc* showed significant segregation distortion in favor of the 'Nure' parent (mean χ^2=6.48 at $P\leq$0.05). In general, locus orders and distances are in agreement with previous reports. The morphological and biochemical marker positions coincide with those reported by Franckowiak and Lundqvist (2002). With one exception, the SSRs described by Liu et al. (1996) and Ramsay et al. (2000) mapped to equivalent positions. We mapped EBmac0684 only on chromosome 2H, at a locus in accordance with Costa et al. (2001), and we accordingly called this locus EBmac0684B. We also found that Bmac0273 and HVM62 were multicopy: the additional loci detected with these primers are indicated with letter (A, B or C) suffixes. The map positions of *wg644b*, *tel3S*, *mwg634* and *psr637* are in accordance with previous reports (Langridge et al. 1995; Kilian et al. 1999; Mano et al. 1999; Salvo-Garrido et al. 2001). Three new STS markers were assigned linkage map positions: *wrlk1* and *wrlk2* on 6H, and *wrlk4* on 2H. The *wg644* primers revealed a second locus in the centromeric region of 5H, which we designated *wg644c* because it does not correspond to the reported position of *wg644a* on the long arm of chromosome 5H (http://wheat.pw.usda.

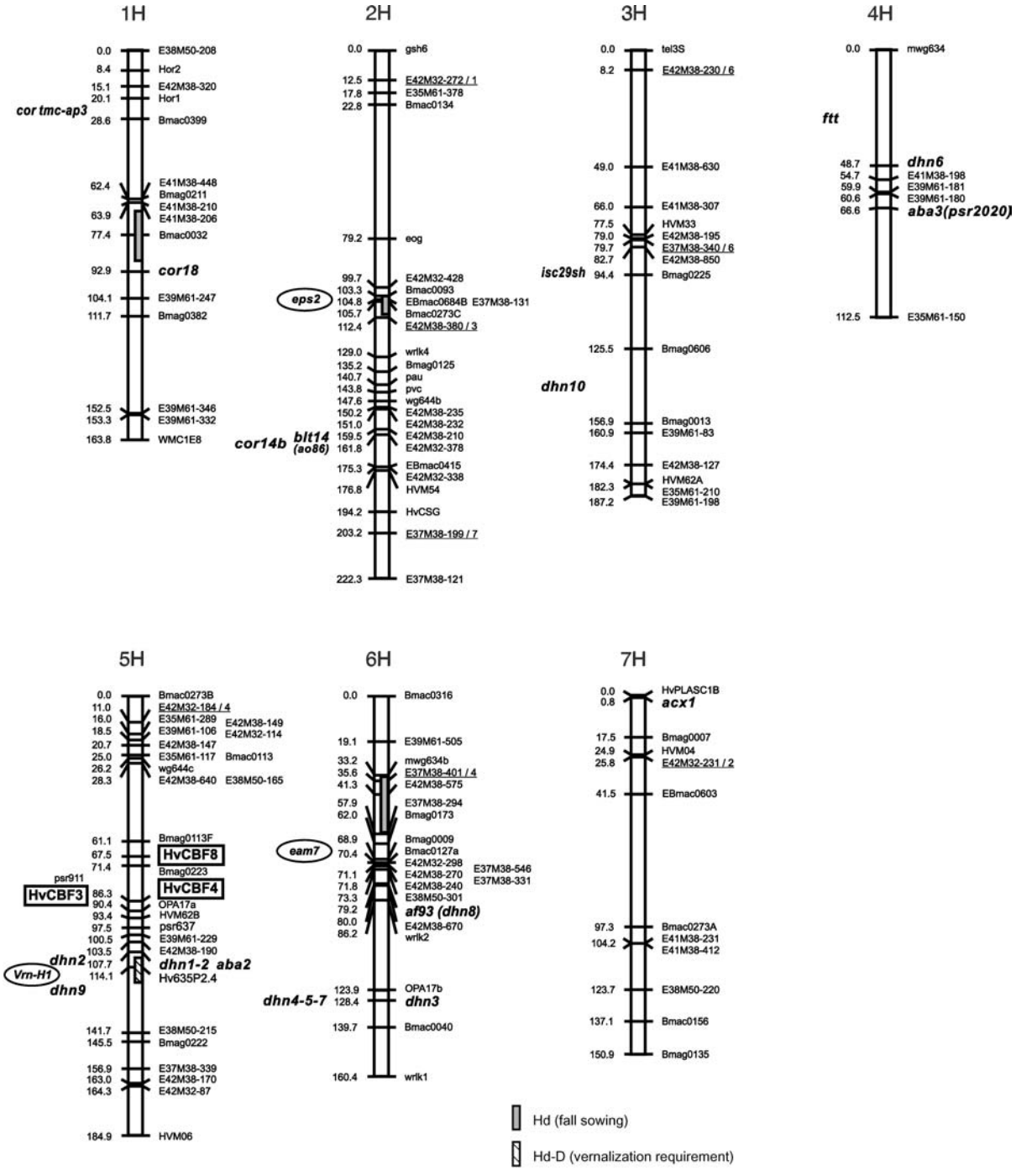

Fig. 1 Linkage map of the 'Nure' × 'Tremois' barley population. Distances are in Kosambi cM and linkage groups are oriented with short arms at the top. Co-segregating markers are placed to the right, and inferred positions of markers mapped in other populations are shown to the left of each cartoon. *COR* genes are in *bold italic*, whereas *CBF* transcription factor genes are in *bold type* highlighted by boxes. AFLP markers are named according to Qi and Lindhout (1997); in addition, AFLP loci that are in common with both the 'Proctor' × 'Nudinka' and the 'L94' × 'Vada' map are *underlined* and have the numerical suffix number assigned by Becker et al. (1995). *Gray boxes* inside chromosomes 1H, 2H and 6H represent heading-date QTLs. The *hatched box* inside chromosome 5H represents a vernalization requirement QTL. *Ellipses* indicate the inferred positions of major genes affecting flowering time (*Vrn-H1*, *eam7* and *eps2*)

gov/). The *mwg634* primers detected an additional locus on chromosome 6H, which we designated as *mwg634b*.

In order to construct a functional map for the study of winter hardiness-related traits, 19 barley *COR* and abiotic stress-induced genes (shown in bold font italics in Fig. 1) were placed on the NT map. Two *CBF* genes—*HvCBF4* and *HvCBF8*—were mapped to the long arm of 5H. The position of *HvCBF4* corresponds to that reported by Von

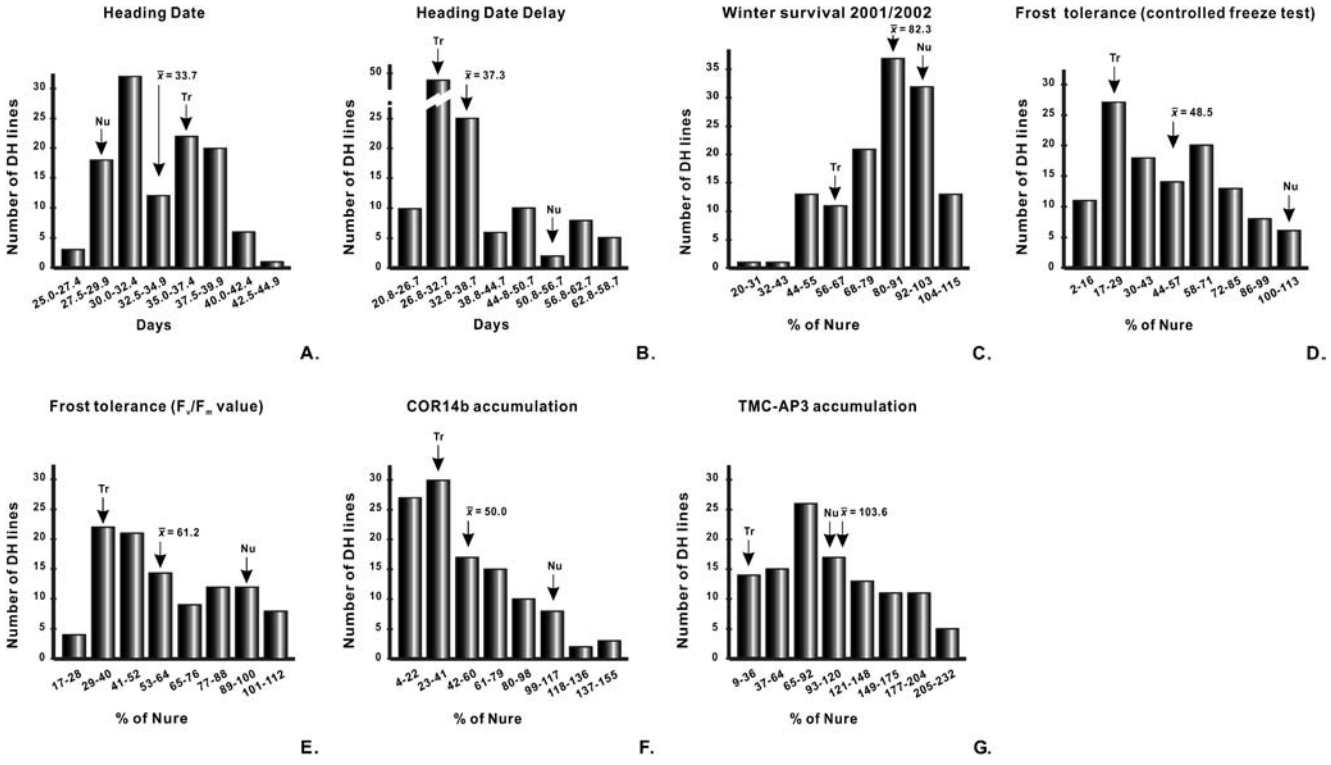

Fig. 2A–G Phenotypic frequency distributions for all the traits on the DH progeny derived from the cross 'Nure' × 'Tremois'. **A** Days to heading (after April 1st) under fall-sown conditions. **B** Heading-date delay (see text for details). **C** Winter survival under field conditions. **D** Controlled environment freezing-test survival. **E** F_v/F_m (see text for detail). **F** COR14b protein accumulation. **G** TMC-Ap3 protein accumulation. Symbol *x* indicates the population average for the considered traits; ''*Nu*'' and ''*Tr*'', respectively, the frequency classes to which parents 'Nure' and 'Tremois' are assigned

Zitzewitz et al. (2003). This is the first reported linkage map position for *HvCBF8*. The position of *HvCBF3* is inferred from Choi et al. (2002) and Von Zitzewitz (2003). Based on the presence of markers in common to the NT map and published maps, we were able to infer the positions of genes related to abiotic stresses in other populations (Fig. 1).

Heading date and vernalization response

The phenotypic frequency distribution for the number of days to heading after April 1st under fall-sown conditions is shown in Fig. 2A. The winter parent was earlier heading in fall-sown conditions than the spring parent ('Nure'=27.7 vs 'Tremois'=37.0). The distribution was bimodal showing a significant kurtosis ($P \leq 0.05$) and there were more late-heading transgressive segregants than early ones. In contrast, as expected (Fig. 2B), under late spring-sown conditions, 'Tremois' was earlier heading than 'Nure' (67.0 vs 79.0). Some DH lines did not flower under these conditions and these were assigned a value of 100. As a measure of vernalization requirement, we calculated heading-date delay (Hd-D) as the difference between heading date under spring-sown and winter-sown conditions. The phenotypic frequency distribution of Hd-D (Fig. 2B) is significantly skewed ($P \leq 0.001$) and shows kurtosis ($P \leq 0.05$).

Three QTLs were found for heading date under fall-sown conditions. The QTL intervals LOD >3.0 on chromosomes 1H, 2H and 6H are shown as grey rectangles within each chromosome cartoon in Fig. 1. Additional details, including the two markers flanking QTL peaks, allele effects, LOD scores and the percentage of variance accounted for, are shown in Table 1. The presence of phenotypic transgressive segregants may be explained by the finding that 'Nure' contributed earliness alleles at two QTLs, including the largest effect QTL on 2H, while 'Tremois' contributed the earliness alleles at the 1H QTL. As shown in Fig. 1, the heading date QTL on 2H maps to the same region as *eps2* (Laurie et al. 1995), and the QTL on 6H is near, but not coincident with, *eam7* (Stracke and Borner 1998). Only one QTL for heading-date delay, a measure of vernalization response, was identified. As shown in Fig. 3 and described in Table 1, this QTL is on 5H and is coincident with the inferred position of *Vrn-H1*. As expected, 'Nure', the winter parent, contributed the higher value allele.

Table 1 Summary of QTLs detected in the 'Nure' × 'Tremois' population using Composite Interval Mapping (CIM) and a LOD threshold = 3.0

Trait	QTL	Interval	Chr	Length[a]	Position[b]	LOD	Allelic[c] effect	R^2(%)[d]
Winter survival (field)	1	*HvCBF4–OPA17a*	5H	4.1 cM	3.1 cM	6.7	10.0	21.5 a
	2	*dhn1–Hv635P2.4*	5H	6.4 cM	6.4 cM	12.6	10.5	36.6 a
								63.5 b
Frost tolerance	1	*HvCBF4–OPA17a*	5H	4.1 cM	1.3 cM	9.2	14.4	30.7 a
(controlled freeze test)	2	*dhn1–Hv635P2.4*	5H	6.4 cM	5.0 cM	9.1	12.6	30.6 a
								65.9 b
Frost tolerance (F_v/F_m value)	1	*HvCBF4–OPA17a*	5H	4.1 cM	1.3 cM	13.7	13.5	46.8 a
	2	*dhn1–Hv635P2.4*	5H	6.4 cM	5.7 cM	13.1	10.9	45.3 a
								80.6 b
COR14b accumulation	1	*HvCBF4–OPA17a*	5H	4.1 cM	1.3 cM	24.3	28.0	63.2 a
	2	*E37M38-401/4–E42M38-575*	6H	5.7 cM	0.6 cM	3.7	10.3	14.1 a
								63.2 b
TMC-Ap3 accumulation	1	*HvCBF4–OPA17a*	5H	4.1 cM	1.3 cM	14.2	37.2	45.3
Heading date	1	*Bmac0032–cor18*	1H	15.5 cM	4.7 cM	5.2	1.3	17.4 a
	2	*Bmac0273C–E42M38-380/3*	2H	6.7 cM	0.3 cM	21.6	−2.8	55.2 a
	3	*E42M38-575–E37M38-294*	6H	16.6 cM	9.6 cM	4.4	−1.4	15.2 a
								66.1 b
Vernalization requirement (*VrnH1*)	1	*dhn1–Hv635P2.4*	5H	6.4 cM	6.4 cM	10.5	6.6	35.0

[a] Length is the distance (cM) of the interval where the QTL is located
[b] Position is the distance (cM) of the QTL peak from the first marker of the interval
[c] Effects on the analyzed traits of the alleles from the parent 'Nure'
[d] R^2(%) is the proportion of phenotypic variance explained by the QTLs; a: Partial R^2(%) value, b: Adjusted model R^2(%) value

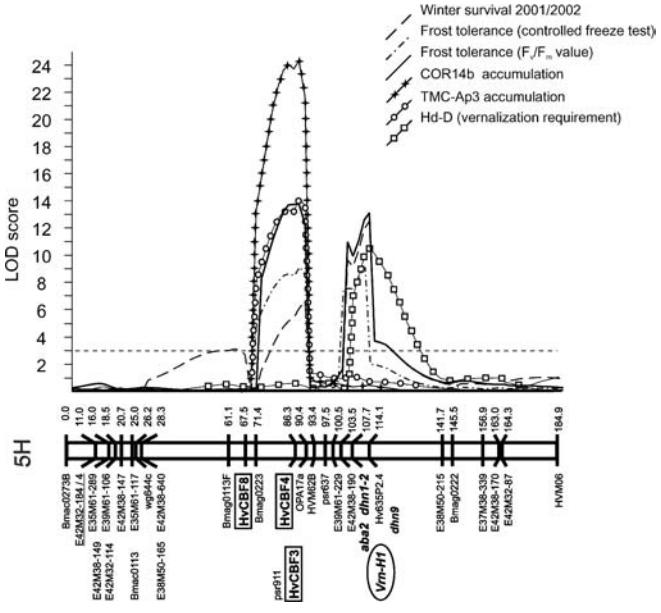

Fig. 3 QTL LOD plots of chromosome 5H obtained with CIM analysis of the indicated traits. The 3.0 LOD score threshold is indicated by a *hatched line*. Chromosome 5H is orientated with short arm at the left; distances are in Kosambi cM; *COR* and *CBF* genes, as well as relevant markers, are highlighted as in Fig. 1

Low-temperature tolerance

The phenotypic frequency distributions for three measures of low-temperature tolerance are shown in Fig. 2C–E. All three confirm the superior low-temperature resistance of the winter parent. The frequency distribution for the controlled freeze-test survival and F_v/F_m measurement are skewed towards the 'Tremois' parent ($P \leq 0.05$), whereas for field survival it was skewed towards the 'Nure' parent ($P \leq 0.05$). For all three traits, there were never phenotypic-significant transgressive segregants with low-temperature tolerance superior to the winter parent, but there were transgressive segregants less cold tolerant than the spring parent.

QTL analysis results are shown in Fig. 3 and detailed in Tables 1 and 2. The CIM QTL plots, shown in Fig. 3, was generated with PLABQTL and the same pattern was observed with MapQTL. The plot shows two distinct peaks for all three measures of low-temperature tolerance. The first (proximal) QTL is bracketed by *HvCBF4* and *OPA17a*, whereas the second (distal) QTL peak is bracketed by *dhn1* and *Hv635P2.4*. In all cases, the higher survival allele is contributed by the winter parent. The resolution of the two peaks is attributable to the use of cofactors in CIM; when the analyses for these three traits are performed with SIM, a single large peak spanning the *HvCBF8—Bmag0222* interval is observed. As shown in Table 1, when cofactors are used, each individual peak has a lower LOD value and R^2, but the multi-locus R^2 is approximately the sum of of the individual R^2 values. In order to further explore this issue of two versus one QTL, we calculated the phenotypic values for DH lines in each of the four alleles classes at the two markers defining the QTL peaks (*HvCBF4* and *Hv635P2.4*). As shown in Table 2, lines with 'Nure' alleles at both markers have the highest low-temperature tolerance values, lines with 'Nure' alleles at

Table 2 Observed mean phenotypic values for DH map lines in 'Nure' × 'Tremois' sorted by allele type at marker loci defining two low-temperature tolerance QTLs on chromosome 5H

Genotype[a]		Winter survival (2001/2002)		Frost tolerance (controlled freeze test)		F_v/F_m value	
HvCBF4	*Hv635P2.4*	Number of DHs	Observed mean[b]	Number of DHs	Observed mean[b]	Number of DHs	Observed mean[b]
A	A	*37*	97.7 a	*33*	77.8 a	*25*	92.9 a
A	B	*13*	81.2 b	*13*	47.1 b	*11*	59.3 b
B	A	*19*	89.1 b	*16*	45.7 b	*15*	57.1 b
B	B	*46*	65.6 c	*41*	24.0 c	*39*	40.0 c

[a] The four genotype classes of two QTLs are based on the alleles of the "peak" markers (*HvCBF4* and *Hv635P2.4*); A and B indicate the 'Nure' and 'Tremois' alleles, respectively

[b] Observed mean value for the trait of each genotype class; values followed by the same letter do not differ significantly according to Tukey's test ($P \leq 0.05$)

only one of the markers have intermediate values, and lines with only 'Tremois' alleles have the lowest values.

COR protein accumulation

The accumulation of two COR proteins—COR14b and TMC-Ap3—in field-grown plant material harvested in midwinter, was measured by Western-blot analysis and the data are expressed as a percentage of the winter ('Nure') parent in the phenotypic frequency distributions shown in Figs. 2F and G. The COR14b frequency distribution is skewed towards the 'Tremois' parent ($P \leq 0.001$), and both plus and minus transgressive segregants were observed. A large number of positive phenotypic transgressive segregants, with TMC-Ap3 protein accumulation percentages higher than 'Nure', are apparent in the platykurtic frequency distribution ($P \leq 0.05$) shown in Fig. 2G.

The CIM analysis of COR14b accumulation revealed two QTLs, one on 5H and one on 6H. The larger effect of the QTL is on 5H, and coincident with the proximal QTL peak observed for low-temperature tolerance traits (Table 1 and Fig. 3). The second QTL is on chromosome 6H and is adjacent to the QTL for heading date (Table 1). At both QTLs, 'Nure' contributes the higher protein accumulation alleles. The CIM analysis of TMC-Ap3 revealed only one locus, and this QTL peak is in the same interval on 5H as the COR14b accumulation QTL and the proximal low-temperature tolerance QTL (Table 1 and, Fig. 3). 'Nure' contributed the higher protein accumulation allele.

Discussion

The linkage map of the NT mapping population underscores the advantages of having openly available, abundant, and informative markers for linkage map construction. Marker order and distance are conserved relative to published maps, facilitating comparative analysis. We have established the linkage map positions of two *CBF* loci: *HvCBF4* and *HvCBF8*. Several gaps remain in the map. These are due, in part, to the paucity of markers in these regions on all linkage maps, and in part to a lack of polymorphism between 'Nure' and 'Tremois' for the markers that are available. This lack of polymorphism is noteworthy in that 'Nure' and 'Tremois' are of Italian and French origin, respectively, represent different end uses ('feed' vs 'malting'), display different growth habit ('winter' vs 'spring') and have no known ancestors in common. As reported in other anther-culture-derived barley mapping populations (Heun et al. 1991) we observed segregation distortion on 2H and 6H, and it is worth noting that the same regions of segregation distortion by Costa et al. (2001) and Qi et al. (1998) occur respectively. Anyway, segregation distortion was not observed on chromosome 5H, where some of the most important and interesting QTLs were detected.

Chromosome 5H is well-resolved in this population, with 29/128 markers mapped to this chromosome. Of particular interest is the mapping of the *CBF* transcription factor genes to this chromosome. The role of these genes in the Arabidopsis low-temperature regulon has been an area of intensive research (Stockinger et al. 1997; Gilmour et al. 1998; Medina et al. 1999): their role in the *Triticeae* is an area of active investigation. We have confirmed the report of Choi et al. (2002) that positioned *HvCBF3* on 5H of barley. Von Zitzewitz et al. (2003) also confirmed this linkage map location for *HvCBF3* and they also provided evidence for the existence of a multi-locus cluster—like Arabidopsis (Gilmour et al. 1998)—of the *HvCBF* loci in the barley genome. *HvCBF8* had been assigned a tentative map position on 5H by mapping on the wheat-barley addition lines (Skinner and Hayes, unpublished results) and we have now mapped it proximal to the cluster of *HvCBF* genes that includes *HvCBF3* and *HvCBF4*.

We have also directly mapped the positions of eight *COR* genes, and inferred the map positions of 11 others. It is of interest that, out of 19 mapped and/or inferred, the only genes known to be cold responsive, or to be hypothesized to be related to low-temperature tolerance, map to the same region as the low-temperature tolerance QTLs in this population, i.e. *dhn1*, *dhn2* and *aba2*. These loci form a cluster on 5H that has been consistently implicated in low-temperature tolerance genes and/or QTLs in multiple reports (Cattivelli et al. 2002). For

example, these genes are tightly linked to the *Fr-A1* locus in wheat (Sutka et al. 1999), to an ABA accumulation QTL in barley (Quarrie et al. 1994) and to a low-temperature tolerance QTL in barley (Pan et al. 1994). In cowpea there is evidence that allelic variation at a dehydrin locus is a determinant of stress tolerance (Ismail et al. 1999), but in the *Triticeae* causal effects of these structural genes on low-temperature tolerance have yet to be demonstrated. This has, accordingly, led to the suggestion that some QTLs may represent the effects of regulatory rather than structural genes. However, no experimental evidence has been available to test this hypothesis in barley. Our QTL data may provide an important first step in this direction.

We detected QTLs for heading date under fall-sown conditions on three chromosomes. Both parents contributed early heading alleles. The multiple QTLs and alternative alleles may explain the phenotypic transgressive segregants for heading date (Fig. 2A). The largest of these QTLs is coincident with the inferred position of the *eps2* locus (Laurie et al. 1995), whereas the QTL on 6H is adjacent to the inferred positions of the *eam7* locus (Stracke and Borner 1998).

We found only one QTL for vernalization requirement, and it was on 5H in a region corresponding with the inferred position of *Vrn-H1*. We did not, however, find any vernalization-related QTL on 1H that could be attributed without doubts to *Vrn-H3* or to *Vrn-H2*, respectively. According to the epistatic genetic model for vernalization requirement proposed by Takahashi and Yasuda (1971), this would mean that 'Nure' and 'Tremois' haplotypes, respectively, are *vrn-h1Vrn-H2vrn-H3* and *Vrn-H1Vrn-H2vrn-hH3*. Although this relatively simple genetic model may be applicable, it does not account for the phenotypic transgressive segregants for vernalization requirement (Fig. 2B) and for the remaining part of variance not explained by the QTL (Table 1). Additional genes with small and undetected effects may be involved in the vernalization response. The *Hv635P2.4* SSR coincided with the vernalization requirement QTL peak. This SSR was discovered in the 635P2.4 'Morex' BAC clone (GenBank accession AY013246) which was reported to be located in our target region on 5H (J. Dubcovsky, personal communication), and which can serve as a source of sequences for designing markers tightly linked to vernalization genes (and low-temperature tolerance) or as a starting point for physically mapping this region.

QTLs for three measures of low-temperature tolerance all map to the long arm of chromosome 5H and are coincident (Fig. 3). This finding also demonstrates the utility of the physiological F_v/F_m test as an indirect measure of low-temperature tolerance in barley—as already shown in oats by Rizza et al. (2001)—even though it is measured at a different stage of plant development than field or phytotron survival. The measurement of F_v/F_m after 24 h of recovery was more informative than when measured immediately after stress, because F_v/F_m was affected to a greater extent, allowing us to observe a larger variability in the response of the genotypes.

We report for the first time the segregation of two low-temperature tolerance loci QTL on a group-5 chromosome of the *Triticeae*. Although SIM results are not able to resolve a single wide QTL of frost tolerance, the presence of two QTLs rather than effects of a single locus is clearly supported by the phenotypic values for map lines falling into the four allele classes (Table 2). The high LOD values associated with *HvCBF4* and *Hv635P2.4* should make these excellent markers for low-temperature tolerance, and they should be useful starting points for isolating the determinants of low-temperature tolerance. *Hv635P2.4* was described in the preceding section on vernalization requirement: none of the genes on this BAC clone are obvious candidates for low-temperature tolerance. We have preliminary data supporting a functional role for *CBF* genes in determining low-temperature tolerance in barley: *HvCBF4* (or one of the other *CBF* gene family members with which it co-segregates) is a potential positional and functional candidate gene for the QTL. In Arabidposis, the small family of *CBF* transcriptional activators recognize the cold and drought responsive DNA regulatory element designated by CRT (C-repeat)/DRE (dehydration responsive element), and are also associated with a low-temperature tolerance QTL (Alabadi et al. 2002; Salinas 2002). We hypothesize that the *CBF* orthologs in barley have a similar functional role, and preliminary evidence in support of this hypothesis is provided by the coincident QTLs for low-temperature tolerance and COR protein accumulation in conjunction with *HvCBF4*. The accumulation of COR14b and TMC-Ap3 proteins has been implicated in low-temperature tolerance (Baldi et al. 1999; Vagujfalvi et al 2000). The genes encoding these proteins are on chromosomes 2H and 1H (Fig. 1). Moreover, it has recently been demonstrated that a barley CBF-like sequence activates the expression of *COR* genes through the interaction with a GCCGAC motif (Xue 2002). Such a site is present in the promoter of the *cor14b* gene. The *cor14b* promoter was shown by deletion analysis to contain a 28-bp fragment (GTCACCCAAAG-GTACGTGAG *GTCGGC* AA) that conferred low-temperature responsiveness (Dal Bosco et al. 2003). This fragment contains a basic Leu zipper protein binding motif, an ABRE (opposite orientation) and a C-repeat/DRE (opposite orientation, italic), with which a CBF protein could interact. No other C-repeat/DRE motifs are present in the sequenced portion of the promoter. Only the coding region sequence is available for *tmc-ap3*, so additional work is needed before we can hypothesize a regulatory role of *CBF* loci for this *COR* gene.

The map location of *HvCBF4* coincides with the inferred position of the *Rcg1* locus that Vagujfalvi et al. (2000) hypothesized was responsible for regulating the *cor14b* gene. On the other hand, our finding that only one QTL on 5H is involved in regulating the accumulation of the COR14b and TMC-Ap3 proteins, contrasts with the two loci reported by Vagujfalvi et al. (2000) in wheat. The existence and the nature of the second regulatory locus (*Rcg2*) in the *Triticeae* genome remains a matter of

debate. Additional research is also needed to determine the nature of the second minor COR14b protein accumulation QTL on barley 6H. We have determined that two *HvCBF* loci are on chromosome 6H via the wheat-barley addition lines (Skinner and Hayes, unpublished). If one or both of these loci coincide with the COR14b accumulation QTL, this would be additional evidence for the role of the *HvCBF* loci as regulators of this *COR* gene.

In conclusion, we have generated data that support the importance of two genes on chromosome 5H in determining low-temperature tolerance and vernalization response. Previous studies have shown that the association of vernalization requirement and low-temperature tolerance is due to linkage rather than pleiotropy (Karsai et al. 2001). At the level of resolution afforded by the NT mapping population, it is noteworthy that the more distal QTL of frost tolerance, which mapped to the *Fr-H1/Vrn-H1* region of chromosome 5H, is largely overlapping and cannot be clearly separated from the QTL vernalization requirement (Fig. 3). This is interesting if we consider the exsisting debate about the role of *Vrn* genes on frost tolerance (Limin and Fowler 2002). The development of QTL-NILs for each of the two frost tolerance QTLs by means of the flanking markers would help to clarify this issue. Moreover, the assignment of a BAC clone to this region of the genome provides a starting point for physical mapping that will ultimately resolve the issue. The employment of these results presented in this paper will be of immediate utility to researchers interested in the genetic dissection of low-temperature regulons.

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