**Joint analysis for heading date QTL in small interconnected barley
populations**

Alfonso Cuesta-Marcos, Ana M. Casas, Samia Yahiaoui, M. Pilar Gracia,
José M. Lasa, Ernesto Igartua

Department of Genetics and Plant Production, Aula Dei Experimental Station, CSIC,
P.O. Box 202, E-50080 Zaragoza, Spain

Abstract

The purpose of the present work is to validate the effect of the main QTL determining heading date in a set of 281 doubled haploid lines of barley, derived from 17 small interconnected populations, whose parents are cultivars commonly used in the Spanish barley breeding program. We used seventy two molecular markers distributed across the 7 chromosomes, particularly in regions where the presence of flowering time genes or QTL is known. A combined linkage map over the 17 populations was constructed. The lines were evaluated in four field trials: two autumn sowings and two winter sowings, and in one greenhouse trial, under controlled conditions of photoperiod and temperature. We have found that it is possible to carry out QTL detection in a complex germplasm set, representative of the materials used in an active breeding programme. In most cases two alleles per QTL were detected, though polymorphism of flanking markers was notably higher. The results revealed that there is a set of QTL that accounts for an important percentage of the phenotypic variation, amenable for use in marker assisted selection. Also, the roles of the photoperiod response genes *Ppd-H1* and *Ppd-H2*, the vernalization response genes *Vrn-H1* and *Vrn-H2*, and the earliness per se locus *Eam6*, of which allele-specific or closely linked markers are available, were confirmed. These results support the use of this kind of approach for the validation of QTL found in biparental studies, or to survey allelic diversity in plant breeding sets of materials.

Keywords: *barley, consensus map, heading date, QTL validation*

1 Studies on QTL detection in plants have been usually carried out in populations of
2 progenies derived from single crosses. In small grain cereals, these parents are
3 commonly inbred lines. The advantage of this approach is the simplicity of studying
4 only two alleles per polymorphic locus. Although these studies have led to significant
5 advances on the genetic control of key traits for many crops, when researchers try to use
6 these QTL in breeding programmes, some drawbacks of the approach become evident.
7 In most cases, parents for QTL studies are selected according to the prospects of finding
8 polymorphism in the cross and, therefore, are chosen among the most extreme ones for
9 the trait of interest. This strategy often led to crosses with little relevance from the plant
10 breeding point of view (Swanston et al. 2006), and/or not adequately representative of
11 germplasm involved in breeding programmes (Varshney et al. 2005). Besides, the
12 results of biparental studies cannot be directly extrapolated to other populations in
13 which polymorphisms may be different (Flint-Garcia et al. 2003), and the resolution
14 obtained in QTL detection is low (Melchinger et al. 2004).

15 Researchers have sought other approaches to detect QTL over wider arrays of
16 more representative germplasm. Several authors proposed methods of QTL analysis in
17 sets of multiple families, either using some kind of mating design (Muranty 1996; Rebaï
18 and Goffinet 2000; Janninck and Jansen 2001; Jansen et al. 2003), or in multiple
19 families of inbred lines crosses (Xu 1998; Liu and Zeng 2000; Crepieux et al. 2004),
20 such as the ones usually found in breeding programmes. These approaches were put into
21 practice by Crepieux et al. (2005) in wheat, and by Rae et al. (2006) in barley. In this
22 last case, the authors used a set of small populations of doubled haploid lines of barley,
23 derived from crosses between elite varieties currently used in the United Kingdom
24 (*small cross mapping*). The most extreme approach is the search of QTL in sets of

1 individuals with different kinship, even if this kinship is unknown, through association
2 mapping (approach reviewed by Breseghello and Sorrells 2006).

3 The main advantages of these new approaches are the higher number of alleles
4 explored, and their direct study in target breeding populations (Breseghello and Sorrells
5 2006). They are also expected to have a deeper impact on marker assisted breeding
6 programmes, detecting marker associations robust enough for successful marker-
7 assisted selection (MAS) beyond its current use for a few genes with outstanding
8 effects.

9 This study aims at finding useful loci for MAS related to time to flowering in
10 barley. Flowering time is one of the main factors for cultivar adaptation in the dryland
11 Mediterranean agrosystems (van Oosterom and Acevedo 1992). QTL for this trait under
12 Northern Spanish dry land conditions were previously detected in the Beka x Mogador
13 population (Cuesta-Marcos et al. submitted). Before using these QTL for MAS, it is
14 necessary to validate their effect at the germplasm-pool level, and to survey the
15 variability present for each QTL in the breeding pool. For these purposes, we studied a
16 set of small populations of doubled haploid lines, derived from interconnected crosses
17 made with a set of parental lines frequently used in the Spanish National Breeding
18 Programme, and representative of the genetic diversity used by the breeders.

Materials and Methods

Plant Material

Heading date was evaluated in a set of 281 doubled haploid (DH) lines of barley, derived from the F₁s of 17 small interconnected populations, consisting of biparental crosses among 14 heterogeneous cultivars commonly used in the Spanish barley breeding programme (several parents used more than once). The number of lines was initially set to 20 for each population, to keep a manageable total population size. During the process of doubled haploid production several lines did not survive, and finally the population size ranged from a minimum of 7 to a maximum of 20 DH lines (Table 1).

Phenotyping

Four field trials were carried out at two provinces in North-Western Spain in 2003 and 2004: Vedado estate in Zuera, province of Zaragoza, and Lupiñén and Alerre, nearby locations of the province of Huesca, all latitudes around 41.5°N (Table 2). Two of them were sown in autumn in 2002, and harvested in 2003 (coded as AUTVE03 and AUTHU03) and the other two were sown in winter 2004 (coded as WINVE04 and WINHU04). Experimental design followed an alpha lattice with three replicates at each location. Plots consisted of two rows 1.2 m long and 20 cm apart. Days to heading were calculated as the number of days between the 1st of January and the day when approximately 2 cm of awns were visible in 50% of stems (Decimal growth stage (DGS) 49, Zadocs et al., 1974). Crop husbandry followed local practice at each location. Nine DH lines were not sown in 2002 because of lack of seed.

Heading date of each line in the field trials was estimated according to the alpha-lattice design, and also according to several spatial analysis models in order to minimize error due to autocorrelation among adjacent plots. These included bidimensional autoregressive models (AR1 x AR1), either alone or including tiers and columns of the field trial as covariates. The calculation procedure was Restricted Maximum Likelihood (REML), and the Bayesian Information Criterion (BIC, Schwarz 1978) was used to select the best model.

Lines were also tested under controlled conditions in two greenhouse experiments that combined presence or absence of vernalization with long photoperiod. In the vernalization followed by long photoperiod treatment (V_LP), four plants per genotype were vernalized for 8 weeks in a growth chamber at 4°C during the light time (17 hours), and 7°C in the dark. When the vernalization period was completed, one plant per DH genotype, and three for the parents, were transferred to a glasshouse with day length set to 17 h, and temperature set to 20/10°C (day/night).

In the non-vernalized plants and long photoperiod treatment (NV_LP), four seeds per genotype were sown in pots, directly in an adjacent glasshouse with similar conditions of photoperiod and temperature. After 10 days, only one plant per DH genotype and 3 for the parents were retained.

Sowing dates of both treatments were set so that, by the end of the vernalization period, vernalized and unvernallized plants reached approximately the same developmental stage. Plants in the greenhouses were randomly distributed and their positions were rotated weekly.

For each plant and experiment, heading date and number of leaves were recorded. Response to vernalization (VER) was estimated for each line as the difference in the number of main stem leaves between the NV_LP and the V_LP treatments.

1 No adjustment was made for the number of leaves recorded in the greenhouse
2 experiments since plants were regularly rotated, and hence no spatial effects were
3 expected.

Genotyping

Genomic DNA was extracted from young leaf tissue of greenhouse-grown plants as described by Casas et al. (1998). Eighty markers were used in this study: 44 SSRs, 23 EST derived SSRs, 2 EST InDel, 6 STS and 5 RFLP. Markers were distributed across the 7 chromosomes, particularly on regions where the presence of flowering time genes or QTL are known. Seventy two markers were used for all DH lines and, for those populations that were not segregating for the major vernalization and photoperiod related regions, eight additional markers were placed on chromosome 6H (scssr09398, Bmac316, Bmag500, scssr02093, Bmag009, Bmac018, scssr05599 y scssr00103). All markers were selected according to the literature and previous studies carried out with the population Beka/Mogador, tested under similar environmental conditions.

Allele specific markers for the two main vernalization response genes *Vrn-H1*, and *Vrn-H2* were used in this study. *Vrn-H1* was characterized with different combinations of primers: HvBM5.42F, HvBM5.43R, HvBM5.55F and HvBM5.56R (von Zitzewitz et al. 2005); HvBM5.85R, HvBM5.88F, HvBM5.66F and HvBM5.67R (P. Szűcs, pers. comm). These combinations of primers amplify different segments on the first intron of the candidate gene (supplementary Table 1). *VrnH2* was studied analyzing the presence of the candidate genes ZCCT-Ha and ZCCT-Hb with primers HvZCCT 01F/02R (von Zitzewitz et al. 2005). HvT SNP22, which is the allele-specific marker for the long-photoperiod response gene *Ppd-H1* was tested as reported by Turner et al. (2005). HvVRT2 was analyzed as published by Szűcs et al. (2006).

We also used the CAPS marker aMWG518/Nhe I (Primer F 5'-AAAGCTGTC ATACGTCAGC-3' and primer R 5'-CTTGTATCTTTGCTGCACG-3'), derived from the RFLP MWG518 since it is tightly linked to the photoperiod response gene *Ppd-H2* (Laurie et al. 1995).

1 For the other markers, whose polymorphism is based on amplification product
2 length, alleles of the same size coming from different parents, i.e. identical by state,
3 were also considered as identical by descent.

4 5 **Consensus map**

6 A consensus map was constructed using the 17 populations. The approximate
7 chromosome location for each marker was previously known. Recombination frequency
8 and LOD score was calculated for each combination of two markers within each
9 chromosome for the entire set of populations, using Joinmap 3.0 (van Ooijen and
10 Voorrips 2001). The number of individuals considered in each combination of two
11 markers was different, as it depended on the number of populations polymorphic for the
12 pair of markers at each comparison. The consensus map was constructed taking into
13 account the recombination frequency of all possible pairs of markers, and weighing
14 them according to their LOD scores (the higher the number of individuals, the higher
15 the LOD score for a similar recombination frequency), using Joinmap 3.0.

16 17 **QTL detection**

18 QTL analyses, and calculation of allele effects and marker interactions estimates,
19 were performed using a maximum likelihood method (mixed procedure of SAS v9, SAS
20 Institute Inc., Cary, NC, USA), independently for each field and greenhouse
21 experiment. Analyses were performed for one marker at a time, using cofactors to
22 account for variation caused at other regions. Each marker was described by a single
23 variable coded as 1, 2,...,n for its n different alleles. Marker main effects were considered
24 as fixed terms in the model. Inclusion of other markers (cofactors) for QTL analysis has
25 been reported by other authors such as Jansen (1993), Zeng (1994), or Jansen and Stam

(1994). Alleles present in less than 5% of the individuals were excluded from the analysis.

Cofactor selection was carried out independently for each experiment following a forward selection and backward elimination stepwise procedure. This method proceeds by performing analyses of variance of each marker on the studied trait. At each step, the marker with the lowest *P*-value of its F statistic is added to the model. Markers with lowest *P*-value of the partial F statistic are sequentially added to the model until no marker had a *P*-value below the 0.05 threshold. Then, we checked whether all cofactors (markers) included in the model were still significant. Markers with *P*-values above the 0.05 threshold were sequentially removed until all markers left were significant. A likelihood ratio test (LRT) was performed between the maximum likelihood of the model including markers and cofactors and the model including only the cofactors, for each marker. The maximum number of parameters in the model was kept below two times the squared root of the number of individuals (Sakamoto et al. 1986). Cofactors that mapped within a 10 cM window at each side of the marker being tested, were excluded from the model. The significance of the LRT was tested using a χ^2 with a number of degrees of freedom equal to the difference in the number of parameters between the two models (in this case $k-1$, being k the number of alleles of the marker being tested).

A False Discovery Rate multi-test adjustment (Benjamini and Hochberg, 1995) of the Likelihood Ratio Test *P*-values was performed to declare the significant markers with a genome- wide threshold of 0.05.

The proportion of variance explained by a QTL at a marker position (R^2), was calculated as:

$$R^2 = (s_0^2 - s_1^2) / s_0^2$$

1 where s_0 is the standard deviation of the error of the model including only the
2 cofactors and s_1 the standard deviation of the error of the model including the cofactors
3 and the marker being tested, (s_1)

4 **Allele effects and interactions**

5 Allele effects of each significant marker were calculated as the adjusted means of
6 days to heading or leaf number, according to the model that included the significant
7 marker and the cofactors for each experiment.

8 A multilocus model that included the significant markers was tested against the
9 same model plus all possible interactions, added sequentially, and using the BIC to
10 declare significant interactions. In case of significant interactions being present, these
11 were also included in the definitive model for allele effect estimation, and in the LRT in
12 the same way as cofactors.

14 **Classification of detected QTL**

15 We classified the significant markers as primary or secondary, according to the
16 comparison of the BIC between the multilocus model of all significant markers and the
17 multilocus model of all significant markers but the one being tested. The LRT P -value
18 of markers with higher number of alleles tended to be more significant, which seems
19 unfair. The use of BIC prevents this flaw to some extent, as it penalizes the number of
20 parameters in the model and, to a much lesser extent, the number of individuals. If the
21 removal of a marker from the multilocus model implies a smaller BIC, it means that the
22 improvement of the LRT caused by that marker was small and/or due to a large number
23 of alleles, i.e number of parameters. Markers presenting this condition were considered
24 as representative of secondary QTL. Conversely, QTL were declared primary when

- 1 their removal from the multilocus model with all significant markers caused an
- 2 increment of the BIC value.

Results

Heading date and leaf number

Ranges of variation for days to heading were similar in the winter-sown and autumn-sown field trials, around 20 days for the DH lines and 6 days for the population means (Fig. 1). In all the experiments, most of the populations showed transgressive segregation.

The parents were classified as *winter*, *facultative* or *spring*, according to previous knowledge and cultivar recommendations. Overall, there were minimal differences between parents' heading dates in the autumn-sown trials (averages for the three types of parents differed by less than 1 day). *Spring* parents flowered late in the winter-sown trials, probably as a consequence of the effect of long photoperiod in late sowings. *Spring* cultivars are usually photoperiod insensitive, and thus do not show an advancement of heading date as opposed to most of the sensitive *facultative* and *winter* cultivars. On the other hand, it seems that temperature conditions at winter-sown trials were sufficient to provide enough vernalization for most *winter* cultivars, as most were not delayed in heading date (Fig. 1). The exceptions were cultivars Angora and Tipper, rather late in these trials, suggesting a higher vernalization requirement for them. In the greenhouse, *winter* cultivars were clearly separated from the rest at the NV_LP treatment, where the lack of vernalization made them extremely late. They were also slightly later than *spring* and *facultative* parents at the V_LP treatment. *Spring* parents showed quite homogeneous behaviours across all trials, as expected. *Facultative* parents presented diverse responses, as they comprise a variety of combinations of vernalization requirement and photoperiod sensitivity. Among them, cultivar Monlon was the latest parent at the autumn-sown trials, and the earliest one at the winter-sown ones.

At the V_LP treatment (the most inductive conditions), all populations presented a rather similar response, with a narrow range of variation for all populations. At the NV_LP treatment, the distribution of the populations was influenced by the growth type of the parents, being *winter* x *winter* (ANG/CLA, BAR/PLA, BAR/TIP, CLA/PLA) populations the latest and *spring* x *spring* (NEV/BEK, SEI/ALE) the earliest populations.

Field trials showed a high correlation coefficient between experiments with similar sowing date. The correlation between the two greenhouse trials was also highly significant (Table 3).

Consensus linkage map

A consensus map was constructed with the molecular data of 80 markers analyzed in the 17 DH populations (Fig. 2). Seventy five markers were distributed across 14 linkage groups. We set a LOD grouping of 3.0. For instance, we got two linkage groups within chromosome 5H (Fig. 2), which means that there were not at least two linkages between markers of the different linkage groups with a LOD over 3.0.

Five markers were not grouped since they did not present a significant linkage with at least two other markers. Marker distances within each linkage group agree with other published barley maps. Linkage groups and unlinked markers, as well as their relative positions, were assigned to barley chromosomes according to other previously published barley linkage maps (Pillen et al. 2000; Ramsay et al. 2000; Moralejo et al. 2004; Rostoks et al. 2005).

Quantitative Trait Loci

Some markers were significantly associated with heading date under all conditions, as Bmac132 and HvBM5, whereas most of the other significant markers had

an effect in at least two experiments (Figs. 3 and 4, Table 4). The amount of phenotypic variation explained by the significant QTL and their interactions ranged from 44% to 67% in the field, and was, 93% (NV_LP), 70% (V_LP) and 90% (VER) in the greenhouse. Individual R^2 of the primary QTL detected in this study are shown in table 5. The effect of diagnostic markers for loci *Vrn-H1*, *Vrn-H2* and *Ppd-H1* reached very high values (over 40%) at some treatments. The number of alleles per locus ranged from 2 to 10. Although there were more than two alleles in most of the markers, in most cases the evidence suggested the presence of a biallelic QTL (Table 4). This will be discussed further in the next sections, where QTL will be presented according to their phenotypic effect.

Vernalization genes and their interaction. Using different primer combinations, five different products, associated with the length of the first intron of *Vrn-H1* were found (supplementary Table 1).

Vrn-H1 showed an important effect in all field trials, with a larger effect in the autumn-sown experiments. *Vrn-H2* was significant only in one of the winter-sown experiments. No interaction between both vernalization genes was detected under field conditions. Under greenhouse conditions, however, the vernalization genes presented a strong interaction. The main effects and the interaction among these two loci accounted for most of the genotypic variation: 88% of NV_LP, 77% of V_LP and 91% of the vernalization effect VER.

All possible combinations among *Vrn-H1* and *Vrn-H2* alleles were present, although the number of lines in the different categories was unbalanced (Table 6). Vernalization requirement was maximum in genotypes carrying the repressor allele at *Vrn-H2* (1400 bp) and the 5150 bp allele in *Vrn-H1*, under long photoperiod. This

allelic combination is present in the *winter* cultivars (Table 1). When the repressor *Vrn-H2* was present, the response to vernalization decreased as the size of the intron in *Vrn-H1* diminishes (1200, 1190 and 150bp), and was reduced to a minimum in genotypes lacking any amplification product. When *Vrn-H2* was absent, the effect of vernalization was rather similar on all *Vrn-H1* alleles (Table 6).

The 1200 bp allele comes from the Spanish *facultative* cultivars Albacete and Pané (both also have *Vrn-H2*). The 1190 bp allele was present only in the cultivar Orria, selected in Spain from a multicross line of CIMMYT materials. The 0 bp and 150 bp come from *spring* cultivars. Allelic combinations for each of the tested cultivars are shown in Supplementary Table 1.

Photoperiod response QTL. Markers aMWG518 and Bmag382 (chromosome 1H, bin 10-11) showed a significant effect on heading date in both autumn-sown experiments. aMWG518 also showed a significant effect in the V_LP treatment (Table 4, Figs. 3 and 4). The allele that caused delay in heading time is usually present in the *winter* cultivars (coded as 2, Table 4).

HvT SNP22, on the short arm of chromosome 2H, was the most important marker for heading date variation in winter-sown experiments. This marker showed an important effect also in the greenhouse trials (Table 4, Figs. 3 and 4). *Spring* cultivars usually carried the photoperiod insensitive allele (coded as 1, Table 4).

Earliness per se QTL. The centromeric region of chromosome 2H showed a significant effect in all field and greenhouse trials. Several markers were above the threshold in this region but, in all cases, the peak was located on the SSR marker

1 Bmac132. Lines that presented the 189 bp allele flowered significantly later with
2 respect to the other two alleles (Table 4).

3 Another notable QTL was found in the centromeric region of chromosome 6H,
4 with a peak on the SSR marker Bmag173. This marker was significant in all the field
5 trials. Bmag173 presents 5 alleles in this study. Clearly, the band of 148 bp (from
6 parents Beka and Orria) was associated with earlier heading. The other four bands also
7 presented some differences. Although means separations were not clear cut, plants
8 carrying bands of 158 and 152 bp were, on average, between 0.8 to 1.9 days later than
9 those with bands of 124 and 150 bp, suggesting the possible presence of a total of three
10 alleles for the linked QTL.

11 Other markers (Bmag120, WMC1E8, and MWG699) had a significant effect in at
12 least two experiments. For Bmag120, there were at least two alleles at the linked QTL,
13 represented by bands of 230, 236, 260 and 262 bp (always latest) versus the rest, though
14 a third allele was suggested if this last group was split into band 232 (always
15 intermediate) and bands 240 and 254 (always earliest). Lines carrying the A allele in
16 MWG699, or 238 bp on WMC1E8 (Table 4) flowered later than lines carrying any
17 other allele.

18 Several other QTL were detected in only one experiment, and they were usually
19 classified as secondary according to the criterion described in the M&M section. The
20 effects of these markers are not reported here.

21 Besides, when analyzing the effect of those markers on chromosome 6H that were
22 used to characterize only *spring x spring* and *winter x winter* populations, only
23 scssr05599 showed a significant effect, in the WINHU04 and NV_LP experiments and
24 in the VER effect (data not shown).

Discussion

The aim of the study was to validate the effect of markers detected in biparental populations, so the estimates are reduced to the positions where the markers are located, and thus no interpolation between markers was made. QTL detection may be hampered in this kind of study by the possible presence of homoplasy (bands of similar size but with different sequence). Different studies have revealed its presence and relevance at the intra- or inter-specific level (reviewed by Estoup et al. 2002). Size homoplasy is a main concern when dealing with phylogenetic relationships among species (Estoup et al. 2002), but for studies involving mapping or gene discovery in biparental crosses, looking at the diversity of accessions within a species (as in this study), or among closely related species, variation at the electromorph level (bands distinguished by electrophoretic mobility) provides sufficient resolution to be both efficient and useful (Chen et al. 2002).

Some theoretical studies have pointed out the possibility of occurrence of false QTL when analyzing complex mating designs (Verhoeven et al. 2006). The presence of genetic variance not explained by real QTL, and the presence of linkage disequilibrium generated by the mating design, may lead to the occurrence of spurious associations. Verhoeven et al. (2006) recommended accounting for family structure in these analyses, to prevent the occurrence of false QTL. This was not necessary in this study, as we could account for a great proportion of phenotypic variance (44 to 93 %), thanks to the use of markers for all major regions described as relevant for the trait, and also to the use of a cofactor approach to reduce residual variance over the genome. It is important to get a good consensus linkage map of the markers, in order to obtain accurate results, and especially to determine which cofactors are located in the 10 cM vicinity window.

1 The method used for the consensus map construction, with a maximum of only 20
2 individuals per population, was different to other methods used for this purpose, usually
3 based on the construction of individual maps for each population (Karakousis et al.
4 2003; Wenzl et al. 2006). We can conclude that this is an appropriate method, since the
5 outcome agrees well with other barley linkage maps based on single cross populations,
6 and it uses all the information on recombination in the whole set of 281 lines. Another
7 evidence of the accuracy of the map comes from the shape of the LRT score profiles
8 around the QTL. For most cases, the peaks of significance showed a decreasing trend
9 for tightly linked markers at both sides. An exception is the case of marker Hv2F4, in
10 the *Ppd-H1* region (2H), which presented a remarkable reduction in significance
11 between two much more significant flanking markers. The reason for this is not a
12 misplacement of the marker (its position fully agrees with other maps), but probably a
13 lack of power in the test at this point caused by the unbalanced number of individuals
14 present in each allele class, as most of the DH lines carried the same allele for marker
15 Hv2F4.

16 All markers significantly associated with heading date or number of leaves were
17 previously reported as allele-specific or tightly linked to flowering time determining
18 genes. This remarkable coherence with previous knowledge is a strong point supporting
19 the consistency of the analysis done. The most significant markers in this study were:

20
21 *HvT SNP22 - Ppd-H1*

22 Marker HvT SNP22, on the short arm of chromosome 2H, showed the largest
23 effect on heading date in the winter-sown experiments (between 5 and 6 days of heading
24 time difference between the two alleles, Table 4). This is the allele-specific marker for
25 the long photoperiod response gene *Ppd-H1* (Turner et al. 2005). *Ppd-H1* is a pseudo-
26 response regulator, a class of genes involved in circadian clock function and causes an

increased expression of *HvFT* with photoperiods over 12 hours (Turner et al. 2005). *HvFT* is the barley orthologue to the key flowering promoters *FT* in Arabidopsis, and *Hd3a* in rice (Turner et al. 2005; Yan et al. 2006). In plants grown under 16 hours photoperiod, under similar photoperiod as for the greenhouse trials of this experiment, peak expression of *HvFT* occurred at the end of the light period in plants carrying the photoperiod response allele *Ppd-H1*, whereas reduced expression of *HvFT* remained constant over the light period for plants carrying the opposite *ppd-H1* allele (Turner et al. 2005). As the greenhouse treatments were carried out under constant long photoperiod, *Ppd-H1* showed a significant effect, also reported in other studies (Laurie et al. 1994, 1995).

aMWG518 - Ppd-H2

The RFLP and the STS-derived marker aMWG518, and its linked SSR marker Bmag382, on the long arm of chromosome 1H, showed a significant effect on autumn-sown field experiments (Table 4). Both are linked to the photoperiod response gene *Ppd-H2*, which causes differences on heading date under short photoperiod conditions (Pan et al. 1994; Laurie et al. 1995; Boyd et al. 2003; Francia et al. 2004). Lines with the allele 2 at aMWG518, or the 105 bp allele at Bmag382, had a later heading date. This QTL is a major determinant of heading date under Northern Spanish autumn sowing conditions, in which most of the vegetative phase elapses with photoperiods around 10-11 hours, decreasing in the first developmental stages (Cuesta-Marcos et. al. submitted). However, a significant effect of these markers was also detected at the V_LP treatment, where plants were grown in the greenhouse under long photoperiods and we have found no previous report of an effect of *Ppd-H2* under long photoperiod.

Bmac132 - Eam6

The only significant marker in all field and greenhouse experiments was the SSR marker Bmac132, in bin 8 of chromosome 2H (Figs. 3 and 4, Table 4). This marker co-locates in bin 8 with *Eam6* (Franckowiak and Konishi 2002), which confers early heading under both long and short photoperiod conditions (Horsley et al. 2006). In a similar position, Laurie et al. (1995) identified *eps2*, also with an effect independent of day length. Three Bmac132 alleles were detected in this study. In all experiments, the 189 bp allele showed a marked delay with respect to alleles 183 and 191 bp, which presented similar heading dates. Thus, we clearly found two alleles in the linked QTL in this germplasm pool. The *Eam6* region has also been identified as a major determinant of heading date in the population Beka x Logan (Moralejo et al. 2004; Cuesta-Marcos et al. submitted) in autumn sowings under Northern Spanish conditions, and also in other materials under Australian Mediterranean conditions (Boyd et al. 2003). This study confirms the importance of the *Eam6* gene under Mediterranean conditions, with an overall effect between three and four days. The peak was consistently present on Bmac132, which has not been widely used thus far, and is not present in the barley genotyping set proposed by Macaulay et al. (2001). Other tightly linked markers, such as Bmac093 and EBmac640, have been used in other studies. Because of the importance of the linked QTL, we propose the use of Bmac132 as a diagnostic marker for *Eam6* in breeding programmes, until this gene is cloned.

Bmag173

The SSR marker Bmag173, in the centromeric region of chromosome 6H, was also significant in all field trials. It is located in the same region of the gene *eam7*, which confers earliness under short-photoperiod conditions (Franckowiak and

1 Gallagher 1997; Stracke and Börner 1998). Although the studies in which this QTL was
2 identified used mutants, in this set of DH lines there is, at least, a consistent effect
3 associated with the allele of 148 bp, which is the earliest in all cases. Canci et al. (2004)
4 described a QTL for heading date, in a similar position, in different mapping
5 populations (Chevron/M69, Stander/MN93 or MS92-299/M81.

6 7 *MWG699*

8 The STS marker MWG699, on the long arm of chromosome 2H, is tightly linked
9 to the *vrs1* gene, which controls the development and fertility of the lateral spikelets
10 (Komatsuda et al. 1999). This marker showed a significant effect in one of the winter-
11 sown trials and in both autumn-sown ones. Kjaer et al. (1995) found a similar effect in a
12 2-row by 6-row population, but they did not have data to prove whether this effect was
13 due to pleiotropy of the *vrs1* gene, or linkage with the QTL now known as *Eam6*. Some
14 populations of this study were 2-row by 6-row crosses, which were the origin of the
15 polymorphism at MWG699. Thus, population structure might influence heading date
16 QTL detection in a set of populations including 2-row by 6-row crosses, as in our study.
17 But, as the model we have used places cofactors at all other significant loci, any carry
18 over effect from an association between MWG699 and *Vrn-H1* (HvBM5), or *Eam6*
19 (Bmac132), has been likely removed, and the effect of MWG699 seems a true one.
20 However, it can not be ruled out that *vrs1* is associated with a gene segregating between
21 winter and spring barley and having an effect on flowering time which has not been
22 tested in the populations (since the genome coverage is not complete).

1 The SSR Bmag125 was also significant in all field trials, but with the present
2 QTL analysis method, we could not discern whether it has a direct effect on heading
3 date *per se*, or because of its relative proximity to *Eam6* and *vrs1*.
4

5 *Vernalization genes and their interaction*

6 The system of the vernalization response genes *VRN2* and *VRN1*, and their
7 epistatic interaction under long photoperiod conditions, is the main factor controlling
8 heading date in temperate grasses (Yan et al. 2003, von Zitzewitz et al. 2005). The
9 epistatic model of the interaction was suggested by Yan et al. (2004), for the *Triticeae*
10 family and was validated with the corresponding barley genes *Vrn-H1* and *Vrn-H2* by
11 von Zitzewitz et al. (2005). A central role for photoperiod in this model has also been
12 proposed (Dubcovsky et al. 2006; Trevaskis et al. 2006). According to this last model,
13 *Vrn-H2* encodes for a dominant flowering repressor that inhibits the expression of *Vrn-*
14 *H1*, which is a central control point for the transition from vegetative to reproductive
15 growth. Vernalization down-regulates the expression of *Vrn-H2*, allowing *Vrn-H1*
16 expression in the winter cultivars (genotype *vrn-H1*). If the repressor is absent
17 (genotype *vrn-H2*), there would not exist any vernalization requirement. This model has
18 been validated in the populations Dicktoo x Kompolti korai (Karsai et al. 2005), Hardy
19 x Jubilant (Kóti et al. 2006) and also in Dicktoo x Oregon Wolfe Barley Dominant,
20 Dicktoo x Calicuchima-sib and Calicuchima-sib x Oregon Wolfe Barley Dominant
21 (Szűcs et al. 2007). von Zitzewitz et al (2005) identified a 436 bp region in the first
22 intron of *Vrn-H1*, critical for the expression of the vernalization requirement.

23 Lines with all combinations of several distinct alleles at *Vrn-H1*, combined with
24 presence/absence of the *Vrn-H2* repressor were included in this study, though the
25 number of lines in each class was unbalanced (Table 6).

1 We identified 5 different alleles in *Vrn-H1*, whose differences were based on
2 different length of deletions in a region within the first intron. The 5150 bp allele, is
3 usually considered as the *winter* allele (von Zitzewitz et al. 2005) and, in this study,
4 presented the strongest vernalization response when the *Vrn-H2* repressor was present.
5 The 1200 bp allele of Albacete and Pané has a large deletion in the first intron, but
6 conserves the critical region (von Zitzewitz et al. 2005). Its effect on vernalization
7 requirement, as long as the *Vrn-H2* repressor was present (1400 bp allele), was
8 intermediate between the typical winter allele (5150 bp), and the alleles carried by
9 spring cultivars (0 and 150 bp), as shown in Table 6. These results confirm the findings
10 by Szűcs et al. (2007), who found that the 436 bp critical region is necessary but not
11 sufficient to allow the presence of full vernalization requirement. Therefore, there could
12 be other regulatory regions in *Vrn-H1*. The vernalization effect of lines carrying the
13 1190 bp allele was similar to (Table 4) or intermediate between the 1200 and 150 bp
14 alleles (Table 6), though data were not conclusive enough to declare whether it
15 corresponds to a functionally distinct allele, or it is the same as the 1200 bp allele.
16 Finally, 0 bp and 150 bp alleles, that correspond with larger deletions including the
17 critical region, showed the lowest vernalization requirements (either with or without the
18 *Vrn-H2* repressor). Thus, an allelic series at *Vrn-H1* is suggested, with strength of VER
19 effect related to size of the intron on *Vrn-H1*, when the repressor *Vrn-H2* was present,
20 as in Szűcs et al. (2007).

21
22 We have determined that it is possible to carry out QTL detection in a complex
23 germplasm set, representative of the materials used in an active breeding programme.
24 QTL position and allelic effects were consistent with estimates found in the literature.
25 In most cases, apparently the markers presented more diversity than linked QTL.

Correspondence between marker and QTL alleles was straightforward when marker allele number was low. Most QTL detected had presumably 2 alleles, with a few exceptions including HvBM5 (*Vrn-H1*). The set of markers used in this study was previously selected to represent regions with heading date QTL. The coverage of the genome achieved, though not complete, was good enough to find QTL that explained a very large proportion of the phenotypic variance. These results support the use of this kind of approach for the validation of QTL found in single cross studies, or to survey allelic diversity in plant breeding sets of materials.

Acknowledgements

This work was supported by the Spanish Ministry of Education and Research (Projects AGL2001-2289, including a scholarship granted to Mr. Alfonso Cuesta-Marcos, and AGL2004-05311) and by the European Regional Development Fund. The authors appreciate the critical reading of the manuscript by Prof. Steve Ullrich.

References

- Basten CJ, BS Weir, ZB Zeng. (1995–1996). QTL CARTOGRAPHER: A Reference Manual and Tutorial for QTL Mapping. Department of Statistics, North Carolina State University, Raleigh, NC.
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc 57:289-300
- Boyd WJR, CD Li, CR Grime, M Cakir, S Potipibool, L Kaveeta, S Men, MRJ Kamali, AR Barr, DB Moody, RCM Lance, SJ Logue, H Raman, BJ Rea (2003) Conventional and molecular genetic analysis of factors contributing to variation in the timing of heading among spring barley (*Hordeum vulgare* L) genotypes grown over a mild winter growing season. Aust J Agric Res 54:1277-1301
- Breseghello F, Sorrells ME (2006) Association analysis as a strategy for improvement of quantitative traits in plants. Crop Sci 46:1323-1330
- Canci PC, Nduulu LM, Muehlbauer GJ, Dill-Macky R, Rasmusson DC, Smith KP (2004) Validation of quantitative trait loci for Fusarium head blight and kernel discoloration in barley. Mol Breed 14:91-104
- Casas AM, Igartua E, Vallés MP, Molina-Cano JL (1998) Genetic diversity of barley cultivars grown in Spain, estimated by RFLP, similarity and coancestry coefficients. Plant Breeding 117:429-435
- Chen X, Cho YG, McCouch SR (2002) Sequence divergence of rice microsatellites in Oryza and other plant species. Mol Genet Genomics 268:331-343
- Christiansen MJ, Feenstra B, Skovgaard IM, Andersen SB (2006) Genetic analysis of resistance to yellow rust in hexaploid wheat using a mixture model for multiple crosses. Theor Appl Genet 112:581-591.

1 Crepieux S, Lebreton C, Servin B, Charmet G (2004) Quantitative trait loci (QTL)
2 detection in multicross inbred designs: Recovering QTL identical-by-descent
3 status information from marker data. *Genetics* 168:1737-1749

4 Crepieux S, Lebreton C, Flament P, Charmet G (2005) Application of a new IBD-based
5 QTL mapping method to common wheat breeding population: analysis of kernel
6 hardness and dough strength. *Theor Appl Genet* 111:1409-1419

7 Dubcovsky J, Loukoianov A, Fu D, Valarik M, Sanchez A, Yan L (2006) Effect of
8 photoperiod on the regulation of wheat vernalization genes *VRN1* and *VRN2*.
9 *Plant Mol Biol* 60:469-480

10 Estoup A, Jarne P, Cornuet JM (2002) Homoplasy and mutation model at microsatellite
11 loci and their consequences for population genetics analysis. *Molec Ecol*,
12 11:1591–1604

13 Flint-Garcia SA, Thornsberry JM, Buckler IV ES (2003) Structure of linkage
14 disequilibrium in plants. *Annu Rev Plant Biol* 54:357-374

15 Francia E, Rizza F, Cattivelli L, Stanca AM, Galiba G, Tóth B, Hayes PM, Skinner JS,
16 Pecchioni N (2004) Two loci on chromosome 5H determine low-temperature
17 tolerance in a ‘Nure’ (winter) x ‘Tremois’ (spring) barley map. *Theor Appl Genet*
18 108:670-680

19 Franckowiak JD, Gallagher LW (1997) Early maturity Locus symbol: *eam7*. *Barley*
20 *Genet Newsl* 26: 233

21 Franckowiak JD, Konishi T (2002) Early maturity 6, *Eam6*. *Barley Genet Newsl* 32: 86-
22 87

23 Horsley RD, Schmierer D, Maier C, Kudrna D, Urrea CA, Steffenson BJ, Schwarz PB,
24 Franckowiak JD, Green MJ, Zhang B, Kleinhofs A (2006) Identification of QTLs

1 associated with Fusarium head blight resistance in barley accession CIho 4196.
2 Crop Sci 46:145-156

3 Jannink JL, Jansen RC (2001) Mapping epistatic quantitative trait loci with one-
4 dimensional genome searches. Genetics 157:445–454

5 Jansen RC (1993) Interval mapping of multiple quantitative trait loci. Genetics 135:
6 205-211

7 Jansen RC, Stam P (1994) High resolution of quantitative traits into multiple loci via
8 interval mapping. Genetics 136:1447-1455

9 Jansen RC, Jannink JL, Beavis WD (2003) Mapping quantitative trait loci in plant
10 breeding populations: Use of parental haplotype sharing. Crop Sci. 43:829–834

11 Karakousis A, Gustafson JP, Chalmers KJ, Chalmers KJ, Barr AR, Langridge P (2003)
12 A Barley Consensus Map Integrating SSR RFLP, and AFLP Markers. Aust J
13 Agric Res 54:1173-1185

14 Karsai I, Szűcs P, Mészáros, Filichkina T, Hayes PM, Skinner JS, Láng L, Bedő Z
15 (2005) The *Vrn-H2* locus is a major determinant of flowering time in a facultative
16 × winter growth habit barley (*Hordeum vulgare* L) mapping population. Theor
17 Appl Genet 110:1458-1466

18 Kjaer B, Jensen J, Giese H (1995) Quantitative trait loci for heading date and straw
19 characters in barley. Genome 38:1098-1104

20 Komatsuda T, Li W, Takaiwa F, Oka S (1999) High resolution map around the *vrs1*
21 locus controlling two-rowed and sixrowed spike in barley, *Hordeum vulgare*.
22 Genome 42 248-253

23 Kóti K, Karsai I, Szűcs P, Horváth Cs, Mészáros K, Kiss GB, Bedő Z, Hayes PM
24 (2006) Validation of the two-gene epistatic model for vernalization response in a
25 winter x spring barley cross. Euphytica 152:17-24

- 1 Laurie DA, Pratchett N, Bezant JH, Snape JW (1994) Genetic analysis of a photoperiod
2 response gene on the short arm of chromosome 2 (2H) of *Hordeum vulgare*.
3 Heredity 72:619-627
- 4 Laurie DA, Pratchett N, Bezant JH, Snape JW (1995) RFLP mapping of five major
5 genes and eight quantitative trait loci controlling flowering time in a winter x
6 spring barley (*Hordeum vulgare* L) cross. Genome 38:575-585
- 7 Liu Y, Zeng ZB (2000) A general mixture model approach for mapping quantitative
8 trait loci from diverse cross designs involving multiple inbred lines. Genet Res
9 75:345-355.
- 10 Macaulay M, Ramsay L, Powell W, Waugh R (2001) A representative, highly
11 informative 'genotyping set' of barley SSRs. Theor Appl Genet 102:801-809
- 12 Melchinger AE, Utz HF, Schön CC (2004) QTL analyses of complex traits with cross
13 validation, bootstrapping and other biometric methods. Euphytica 137:1-11
- 14 Moralejo M, Swanston JS, Muñoz P, Prada D, Elia M, Russell JR, Ramsay L, Cistue L,
15 Codesal P, Casas AM, Romagosa I, Powell W, Molina-Cano JL (2004) Use of
16 new EST markers to elucidate the genetic differences in grain protein content
17 between European and North American two-rowed malting barleys. Theor Appl
18 Genet 110:116-125
- 19 Muranty H (1996) Power of tests for quantitative trait loci detection using full-sib
20 families in different schemes. Heredity 76:156-165
- 21 van Ooijen JW, Voorrips RE (2001) JoinMap® 3.0, Software for the calculation of
22 genetic linkage maps. Plant Research International, Wageningen, Netherlands
- 23 van Oosterom EJ, Acevedo E (1992) Adaptation of barley (*Hordeum vulgare* L) to
24 harsh Mediterranean environments III. Plant ideotype and grain yield. Euphytica
25 62:29-38

1 Pan A, Hayes PM, Chen F, Chen THH, Blake T, Wright S, Karsai I, Bedö Z (1994)
2 Genetic analysis of the components of winterhardiness in barley (*Hordeum*
3 *vulgare* L). Theor Appl Genet 89:900-910

4 Pillen K, Binder A, Kreuzkam B, Ramsay L, Waugh R, Förster J, Léon J (2000)
5 Mapping new EMBL-derived barley microsatellites and their use in
6 differentiating German barley cultivars. Theor Appl Genet 101:652-660

7 Rae SJ, Keith R, Mackie A, Matthew D, Felix G, Morris PC, O'Sullivan D, Donini P,
8 Thomas WTB (2006) Small cross mapping of barley quality characters. Eucarpia
9 meeting, Cereals Section, Lleida, Spain, November 13-17

10 Ramsay L, Macaulay M, degli Ivanissevich S, MacLean K, Cardle L, Fuller J, Edwards
11 KJ, Tuvešson S, Morgante M, Massari A, Maestri E, Marmiroli N, Sjakste T,
12 Ganai M, Powell W, Waugh R (2000) A simple sequence repeat-based linkage
13 map of barley. Genetics 156:1997-2005

14 Rebaï A, Goffinet B (2000) More about quantitative trait locus mapping with diallel
15 designs. Genet Res 75:243–247

16 Rostoks N, Mudie S, Cardle L, Russell J, Ramsay L, Booth A, Svensson JT,
17 Wanamaker SI, Walia H, Rodriguez EM, Hedley PE, Liu H, Morris J, Close TJ,
18 Marshall DF, Waugh R (2005) Genome-wide SNP discovery and linkage analysis
19 in barley based on genes responsive to abiotic stress. Mol Gen Genomics
20 274:515-527

21 Sakamoto Y, Ishiguro M, Kitagawa G (1986) Akaike information criterion statistics.
22 KTK Scientific Publishers, Tokyo, pp 83-85

23 Schwarz G (1978). Estimating the dimension of a model. Ann Statistic 6:461-464

24 Stracke S, Börner A (1998) Molecular mapping of the photoperiod response gene *ea7* in
25 barley. Theor Appl Genet 97:797-800

Swanston JS, Russell JR, Pérez-Vendrell AM, Elia M, Moralejo M, Molina-Cano JL (2006) Efficient selection of β -glucan content enhances wider utilisation of barley grain. Eucarpia meeting, Cereals Section, Lleida, Spain, November 13-17

Szűcs P, Karsai I, von Zitzewitz, Cooper L, Gu Y, Chen T, Hayes P, Skinner J (2006) Positional relationships between photoperiod response QTL and photoreceptor and vernalization genes in barley. *Theor Appl Genet* 112:1277-1285

Szűcs P, Skinner JS, Karsai I, Cuesta-Marcos A, Haggard KG, Corey AE, Chen THH, Hayes PM (2007) Validation of the *VRN-H2/VRN-H1* epistatic model in barley reveals that intron length variation in *VRN-H1* may account for a continuum of vernalization sensitivity. *Mol Genet Genom*, 10.1007/s00438-006-0195-8

Trevaskis B, Hemming MN, Peacock WJ, Dennis ES (2006) *HvVRN2* Responds to daylength, whereas *HvVRN1* is regulated by vernalization and developmental status. *Plant Physiol* 140:1397-1405

Turner A, Beales J, Faure S, Dunford RP, Laurie DA (2005) The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. *Science* 310:1031–1034

Varshney RK, Graner A, Sorrells ME (2005) Genomics-assisted breeding for crop improvement. *Trends in Plant Science* 10:621-630

Verhoeven KJ, Jannink JL, McIntyre LM (2006) Using mating designs to uncover QTL and the genetic architecture of complex traits. *Heredity*. 96:139-149

Wenzl P, Li H, Carling J, Zhou M, Raman H, Paul E, Hearnden P, Maier C, Xia L, Caig V, Ovesna J, Cakir M, Poulsen D, Wang J, Raman R, Smith KP, Muehlbauer GJ, Chalmers KJ, Kleinohs A, Huttner E, Kilian A (2006) A high-density consensus map of barley linking DArT markers to SSR, RFLP and STS loci and agricultural traits. *BMC Genomics* 7:206-215

- 1 Xu SZ (1998) Mapping quantitative trait loci using multiple families of line crosses.
2 Genetics 148:517-524.
- 3 Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003)
4 Positional cloning of the wheat vernalization gene *VRN1*. Proc Natl Acad Sci,
5 USA 100:6263-6268
- 6 Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P,
7 Bennetzen JL, Echenique V, Dubcovsky J (2004) The wheat VRN2 gene is a
8 flowering repressor down-regulated by vernalization. Science 303:1640-1644
- 9 Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Yasuda
10 S, Dubcovsky J (2006) The wheat and barley vernalization gene *VRN3* is an
11 orthologue of FT. Proc Natl Acad Sci, USA 103:19581-19586
- 12 Zeng ZB (1994) Precision mapping of quantitative trait loci. Genetics 136:1457-1468
- 13 von Zitzewitz J, Szűcs P, Dubcovsky J, Yan L, Pecchioni N, Francia E, Casas A, Chen
14 THH, Hayes PM, Skinner JS (2005) Molecular and structural characterization of
15 barley vernalization genes. Plant Mol Biol 59:449–467

Table 1. Description of the 17 small populations of doubled haploid (DH) lines of barley used in this study. Parent name abbreviations in brackets. Growth type is expressed as follows: S (Spring); F (Facultative); W (Winter).

Population	Parents	Growth type	Row type	DH number	No. markers analyzed	No. polymorphic markers
1	Seira (SEI) Orria (ORR)	S-F	2-6	20	71	52
2	Seira (SEI) Alexis (ALE)	S-S	2-2	18	79	29
3	Seira (SEI) Tipper (TIP)	S-W	2-2	20	71	44
4	Albacete (ALB) Monlon (MON)	F-F	6-6	7	71	42
5	Albacete (ALB) Plaisant (PLA)	F-W	6-6	19	71	46
6	Alexis (ALE) Pané (PAN)	S-F	2-6	20	71	52
7	Angora (ANG) Clarine (CLA)	W-W	2-2	20	80	29
8	Barberousse (BAR) Albacete (ALB)	W-F	6-6	10	71	50
9	Barberousse (BAR) Monlon (MON)	W-F	6-6	12	79	36
10	Barberousse (BAR) Plaisant (PLA)	W-W	6-6	20	71	22
11	Barberousse (BAR) Tipper (TIP)	W-W	2-6	8	71	27
12	Beka (BEK) Monlon (MON)	S-F	2-6	20	71	55
13	Clarine (CLA) Plaisant (PLA)	W-W	2-6	20	80	39
14	Gaelic (GAE) Tipper (TIP)	F-W	2-2	8	71	29
15	Nevada (NEV) Beka (BEK)	S-S	2-2	20	79	31
16	Pané (PAN) Plaisant (PLA)	F-W	6-6	19	71	46
17	Plaisant (PLA) Orria (ORR)	W-F	6-6	20	71	38

Table 2. Environmental conditions for every field and greenhouse trial. Average daily temperatures for field experiments were gathered from nearby meteorological stations. For growth chambers and greenhouses, thermohygrographes were used.

Trial/treatment	Location	Year	SD	CDD	TT	HL-S ⁽¹⁾	HL-H ⁽¹⁾	RF
AUTHU03	Lupiñén	2002-2003	11-08-2002	687	1291	10.8	14.5	316
AUTVE03	Zuera	2002-2003	11-15-2002	623	1283	10.6	14.5	209
WINHU04	Alerre	2004	01-28-2004	468	932	10.6	15.6	196
WINVE04	Zuera	2004	01-22-2004	427	1055	10.5	15.6	152
NV_LP	greenhouse	2003-2004	-	-	1510	17.0	17.0	-
V_LP	greenhouse	2003-2004	-	392	1147	17.0	17.0	-

(1) Natural day length includes civil twilight

SD: Sowing date

CDD: Cooling degree-days (from sowing to average heading date of the trial)

TT: Thermal time (°C) (from sowing to average heading date of the trial)

HL-S: Hours of light (in sowing date)

HL-H: Hours of light (in the average heading date of the trial)

RF: Rain fall (mm) from sowing to the average heading date of the trial

Table 3. Correlation coefficients of heading dates and/or leaf number among the experiments carried out in this study (codes for experiments in the text).

	AUTHU03	WINVE04	WINHU04	NV_LP	V_LP
AUTVE03	0,88***	0,67***	0,65***	0,15*	0.18***
AUTHU03		0,55***	0,57***	0,23***	0.23***
WINVE04			0,88***	0,03	0.19***
WINHU04				-0,03	0.11*
NV_LP					0.86***

*, **, *** Pearson correlation coefficients significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively

Table 4. Significant markers detected at each experiment. The table shows the number of lines and average days to heading, or leaf number, for the different alleles of the significant markers for each experiment. Means are adjusted for other significant markers in each experiment. Letters indicate means separation. We used a False Discovery Rate multi-test adjustment for the *P*-values and confidence limits for the differences of means with alpha = 0.05. In *italics*, markers considered as secondary QTL according to the methodology described in the M&M section.

Chr.	Marker	AUTVE03			AUTHU03			WINVE04		WINHU04		NV_LP		V_LP		VER	
	(putative gene)	Allele	No.	Mean	No.	Mean	No.	Mean	No.	Mean	No.	Mean	No.	Mean	No.	Mean	
1H	aMWG518 (<i>Ppd-H2</i>)	1	99	112.2 b	87	110.0 b	-	-	-	-	-	-	101	6.6 b	-	-	
		2	164	114.5 a	142	113.4 a	-	-	-	-	-	-	168	7.5 a	-	-	
1H	WMC1E8	191	66	112.7 b	-	-	62	138.5 b	-	-	-	-	-	-	-	-	
		238	197	114.0 a	-	-	194	139.4 a	-	-	-	-	-	-	-	-	
2H	HvTSNP22 (<i>Ppd-H1</i>)	1	-	-	-	-	114	142.1 a	115	143.1 a	110	11.0 a	119	7.8 a	-	-	
		2	-	-	-	-	142	135.9 b	142	138.1 b	145	9.1 b	152	6.6 b	-	-	
2H	Bmac132 (<i>Eam6</i>)	183	27	112.7 b	28	110.4 b	28	138.5 b	28	139.5 b	27	9.8 b	28	6.9 b	-	-	
		189	82	115.5 a	72	114.3 a	80	141.3 a	81	142.3 a	81	10.6 a	85	7.6 a	-	-	
		191	154	111.8 b	129	110.4 b	148	138.2 b	148	139.5 b	147	9.7 b	156	6.6 b	-	-	
2H	MWG699 (<i>vrs1</i>)	A	97	114.7 a	89	112.9 a	95	139.8 a	-	-	-	-	-	-	-	-	
		D	41	112.8 b	37	110.2 b	38	137.8 b	-	-	-	-	-	-	-	-	
		K	125	112.6 b	103	119.9 b	124	138.1 b	-	-	-	-	-	-	-	-	
4H	HvZCCT (<i>Vrn-H2</i>)	0	-	-	-	-	-	-	95	138.6 b	98	8.1 b	97	6.5 b	89	1.0 b	
		1400	-	-	-	-	-	-	162	139.9 a	157	12.0 a	172	7.8 a	163	3.7 a	
5H	HvBM5 (<i>Vrn-H1</i>)	0	58	111.7 c	51	110.4 b	57	138.0 c	57	138.6 c	56	7.9 c	60	6.2 c	56	1.0 c	
		150	25	115.2 ab	28	112.5 ab	27	138.7 bc	28	140.7 b	28	9.5 b	27	7.3 b	25	1.4 bc	
		1190	17	112.3 c	18	110.8 b	18	137.3 c	18	138.8 c	19	9.1 b	18	6.4 c	16	2.4 b	
		1200	25	112.8 bc	19	111.4 ab	24	139.6 b	24	140.4 b	30	9.9 b	26	6.8 bc	24	2.3 b	
		5150	138	114.7 a	113	113.0 a	130	141.2 a	130	142.1 a	122	13.7 a	138	8.5 a	131	4.3 a	
6H	Bmag173 (<i>Eam7</i>)	124	134	113.2 ab	118	111.1 bc	135	139.1 a	135	140.4 b	-	-	-	-	-	-	
		148	35	111.8 b	38	109.6 c	39	137.4 b	39	138.6 c	-	-	-	-	-	-	
		150	23	113.5 ab	19	111.5 ab	24	138.8 a	25	140.2 b	-	-	-	-	-	-	
		152	38	114.2 a	37	112.8 a	38	139.8 a	38	141.3 ab	-	-	-	-	-	-	
		158	18	115.5 a	17	113.5 a	20	139.7 a	20	142.3 a	-	-	-	-	-	-	
7H	Bmag120	230	-	-	58	112.6 b	61	140.0 a	62	141.2 ac	-	-	-	-	-	-	
		232	-	-	35	110.8 bc	36	138.9 ab	36	140.1 bd	-	-	-	-	-	-	
		236	-	-	43	111.9 b	55	139.8 a	55	141.6 a	-	-	-	-	-	-	
		240	-	-	11	110.7 bc	13	137.1 c	13	138.5 d	-	-	-	-	-	-	
		254	-	-	15	108.7 c	15	137.4 bc	15	139.8 bcd	-	-	-	-	-	-	
		260	-	-	57	111.4 b	65	139.5 a	65	141.2 ab	-	-	-	-	-	-	
		262	-	-	10	115.7 a	11	140.2 a	11	141.6 ab	-	-	-	-	-	-	

1 **Table 5.** Individual R² of QTL declared as primary according to the methodology described in the M&M

Marker (<i>putative gene</i>)	AUTVE03	AUTHU03	WINVE04	WINHU04	NV_LP	V_LP	VER
aMWG518 (<i>Ppd-H2</i>)	0.05	0.13	-	-	-	0.06	-
WMC1E8	0.03	-	0.03	-	-	-	-
HvTSNP22 (<i>Ppd-H1</i>)	-	-	0.51	0.44	0.19	0.15	-
Bmac132 (<i>Eam6</i>)	0.09	0.14	0.17	0.18	0.04	0.06	-
MWG699 (<i>vrs1</i>)	0.05	0.10	0.07	-	-	-	-
HvZCCT (<i>Vrn-H2</i>)	-	-	0.04	-	0.48	0.29	0.25
HvBM5 (<i>Vrn-H1</i>)	0.08	0.05	0.18	0.20	0.57	0.47	0.40
Bmag173 (<i>Eam7</i>)	0.04	0.09	0.08	0.14	-	-	-
Bmag120	-	0.13	0.10	0.09	-	-	-

2 section. R² were calculated at the positions of the markers.

3

4

Table 6. Interaction between markers at the vernalization genes. Number of lines and average leaf number for all allele combinations of the vernalization genes *Vrn-H1* and *Vrn-H2*. Means separation as in Table 4.

HvBM5 (<i>Vrn-H1</i>)	HvZCCT (<i>Vrn-H2</i>)	NV_LP		V_LP		VER	
		No.	Mean	No.	Mean	No.	Mean
allele size (bp)							
5150	1400	100	18.4 a	115	10.2 a	112	7.2 a
1200	1400	27	12.5 b	24	7.2 bc	22	4.6 b
1190	1400	15	9.8 cd	14	6.9 bcd	14	2.8 c
150	1400	4	11.0 bc	4	8.0 b	2	2.3 cde
0	1400	11	8.4 ef	15	6.6 cd	13	1.2 de
5150	0	22	9.0 de	23	6.8 bcd	19	1.5 d
1200	0	3	7.3 f	2	6.4 bcde	2	0.1 de
1190	0	4	8.4 ef	4	5.9 de	2	2.0 cde
150	0	24	8.0 f	23	6.5 cd	23	0.5 e
0	0	45	7.5 f	45	5.7 e	43	0.8 de

4

5

Figure 1. Range of days to heading in the field experiments, and leaf number until heading under greenhouse conditions, for the 17 DH populations evaluated in this study. Days to heading of the DH lines are averaged by sowing season. P1 and P2 are the abbreviations of the parents of each line (see Table 1 for description)

Figure 2. Consensus map for the 17 DH populations. Linkage groups are set at a LOD score of 3.0. Distances are in Kosambi cM. Dotted lines bind linkage groups and unlinked markers within each chromosome. Major heading time loci are indicated in bold types, positions according to literature. (*) Markers analyzed only in populations 2, 7, 10, 11, 13, 14 and 15 and excluded from the QTL analysis

Figure 3. P values of the Likelihood Ratio Test (LRT) for all the markers in the field experiments, expressed as $-\log P$. Significance threshold for individual QTL detection is based on an experiment-wise error of $\alpha=0.05$ ($-\log \alpha=1.3$). A False Discovery Rate multi-test adjustment (Benjamini and Hochberg, 1995) of the LRT P -values was performed. In brackets are written the values of markers whose value was over 15. Tests were made only at the positions of the markers. Full lines join markers only to facilitate visualization, but they are not indicating test values in the intervals. Dotted lines join linkage groups within the same chromosome.

Figure 4. P values of the Likelihood Ratio Test (LRT) for all the markers in the greenhouse experiments.