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ORIGINAL PAPER

# The flowering locus *Hr* colocalizes with a major QTL affecting winter frost tolerance in *Pisum sativum* L.

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**Abstract** An understanding of the genetic determinism of frost tolerance is a prerequisite for the development of frost tolerant cultivars for cold northern areas. In legumes, it is not known to which extent vernalization requirement or photoperiod responsiveness are necessary for the development of frost tolerance. In pea (*Pisum sativum* L.) however, the flowering locus *Hr* is suspected to influence winter frost tolerance by delaying floral initiation until after the main winter freezing periods have passed. The objective of this study was to dissect the genetic determinism of frost tolerance in pea by QTL analysis and to assess the genetic linkage between winter frost tolerance and the *Hr* locus. A population of 164 recombinant inbred lines (RILs), derived from the cross Champagne x Terese was evaluated both in the greenhouse and in field conditions to characterize the photoperiod response from which the allele at the *Hr* locus was inferred. In addition, the population was also assessed for winter frost tolerance in 11 field conditions. Six QTL were detected, among which three were consistent among the different experimental conditions, confirming an oligogenic determinism of frost tolerance in pea. The *Hr* locus was found to be the peak marker for the highest explanatory QTL of this study. This result supports the hypothesis of the prominent part played by the photoperiod responsiveness in the determinism of frost tolerance for this species. The consistency of three QTL makes these positions interesting targets for marker-assisted selection.

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## Introduction

Fall sowings allow for enhanced plant productivity in a number of important crop species. They are however

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limited by low temperature together with other stresses associated with winter climatic conditions. Overwintering plants have developed adaptative responses to the seasonal weather changes. First, they sense the upcoming winter through the perception of environmental cues, which are principally temperature and daylength. Then, they adapt both at the developmental and physiological levels, using complementary strategies: freezing escape and cold acclimation. Freezing escape consists in delaying the transition from the vegetative to the reproductive phase, given that frost sensitivity increases after floral initiation (Fowler et al. 2001). Associated with this postponed floral initiation a progressive chilling and freezing tolerance is acquired under low temperature. This cold acclimation response also drives improved tolerance to other winter stresses such as frost heaving, freeze–thaw cycles, water logging, wind dehydration, photoinhibition and diseases such as *Mycosphaerella pinodes* one of the most injurious pathogen in wet and cold conditions. Winter hardiness integrates the interactive responses to the whole set of winter stresses and can be assessed in field conditions only while frost tolerance sensu stricto is generally evaluated in controlled conditions.

The development of molecular tools has allowed significant progress toward the understanding of winter hardiness, freezing escape and freezing tolerance. The identification of regions controlling winter hardiness has been completed for major cultivated species through the assessment of mapping populations in field conditions and QTL mapping (in cereals: Pan et al. 1994; Börner et al. 2002; Francia et al. 2004; rape: Teutonico et al. 1995; and legumes: Kahraman et al. 2004). In parallel, the molecular basis of freezing tolerance has been extensively studied, particularly in the model species *Arabidopsis thaliana*, allowing sustained progress in understanding the cold acclimation signalling pathways and the identification of numerous cold responsive genes. QTL mapping also permitted tests of the genetic relationship between the plant phenology controlled by developmental genes and the expression of winter hardiness or freezing tolerance. In barley, the coincidence between a QTL of winter hardiness and the vernalization gene *Vrn*1 was reported (Francia et al. 2004). In wheat, a genetic linkage was found between the *Vrn*1 locus and a major gene (Galiba et al. 1995) or QTL (Toth et al. 2003) governing freezing tolerance. Beyond these mapping results, it has also been shown, for cereals, that the vernalization requirement and photoperiod response genes regulate the duration of the expression of cold responsive genes by controlling the date of the switch from the vegetative to the reproductive phase (Fowler et al. 1996, 2001; Mahfoozi et al. 2001).

In legumes, the nature of the relationship between winter hardiness, frost tolerance and developmental genes has still to be elucidated. Pea (*Pisum sativum* L.) is particularly adapted to this goal. Genetic variation for winter hardiness has been reported for pea (Auld et al. 1983; Cousin et al. 1985; Liesenfeld et al. 1986). Pea is a diploid species highly polymorphic for a number of morphological traits and molecular markers (Baranger et al. 2004; Ellis and Poyser 2002) and for which consensus maps are available (Aubert et al. 2006; Ellis and Poyser 2002; Loridon et al. 2005; Weeden et al. 1998). Moreover, major loci that control the pea transition to the reproductive stage have been identified (Weller et al. 1997). Among these loci, we have paid a particular attention to the *Hr* locus, responsible for a high qualitative response of floral initiation to the photoperiod (Murfet 1973). Initially identified in controlled conditions of photoperiod and associated to the Murfet's type line 63, the *Hr* dominant allele was also found in a set of forage cultivars (Lejeune-Hénaut et al. 1999; Murfet 1981). These lines remain vegetative until a threshold daylength of 13h30 is reached, which is completed in mid-April in northern latitudes (49°52′N in Lejeune-Hénaut et al., 1999) and they are also known to be frost tolerant. The present study was undertaken to check the genetic linkage between the winter frost tolerance and the *Hr* locus in pea.

## Materials and methods

### Plant material

One hundred and sixty-four $F_8$ recombinant inbred lines (RILs), a set of plant material already identified in Loridon et al. (2005) and Aubert et al. (2006) as Pop2, were obtained by single seed descent from a $F_2$ population descending from the cross between Champagne and Terese. The parental lines were chosen for their polymorphism for the targeted traits, according to the observations reported by Lejeune-Henaut et al. (1999). Champagne is a forage line derived from a local French population. It exhibits the *Hr* flowering phenotype, i.e., delayed floral initiation under short days (SD) and is also a freezing tolerant and winter hardy line, formerly used as a parent in winter dry pea breeding in France. In contrast, Terese is only slightly reactive to the daylength, which reveals its *hr* phenotype. It is a spring French dry pea variety, sensitive to frost and thus generally not able to survive winter in northern latitudes. Champagne and Terese are also contrasted for morphological traits, such as the foliage formation type (conventional and afila, resp.), the flower color (purple and white, resp.), the seed coat color (with and without marbling, resp.), the hilum color (black and clear, resp.) and finally internode length (long and dwarf, resp.), these traits being useful classical makers for anchoring the genetic map to already published pea linkage

maps (Aubert et al. 2006; Ellis and Poyser 2002; Loridon et al. 2005; Weeden et al. 1998).

## Evaluation of sensitivity to the daylength

The occurrence of floral initiation is not easy to evaluate for a large population, because it relies on destructive sampling and time-consuming observations of plant apices under magnifying glasses. Then, according to the phenotypic classification proposed by Murfet (1973), we characterized the late high response (LHR) phenotype associated to the dominant *Hr* allele by recording the date of beginning of flowering under appropriate environmental conditions. Two complementary experiments allowed us to differentiate *Hr* from *hr* lines within Pop2: the first one was conducted in the greenhouse and the second one in the field.

### *Greenhouse experiment*

A random set of 80 RILs and the parental lines were grown in the greenhouse under a 8h-photoperiod (short days, SD), artificially provided by black plastic curtains pulled over the plants from 16 p.m. to 8 a.m. Due to a limited area covered by the curtains, the experiment was conducted successively with two different subsets of 40 RILs. In order to avoid high temperatures, both subsets were grown during the winter periods of 1999–2000 and 2000–2001. The sowing date was 29th September for both repetitions. The parental lines and four control RILs (i.e., the lines 43, 73, 118 and 183) were studied in both subsets. The plants were grown in cans of 30 cm diameter distributed over two contiguous replicates, each line being represented by a can of three plants in each replicate. The substrate was a peat mixture. The plants were watered regularly. Sodium vapor lamps provided additional light each time the natural radiation felt under 80 $W/m^2$ during the day period. Temperature was maintained at 13°C during the night and never exceeded 20°C during the day period. A cylindric netting wire was adjusted vertically to the top diameter of each can to ensure an erected growth of the plants. Basal and upper branches were regularly excised and the flowering behavior was recorded on main shoots. The experiment was stopped after the end of the flowering period of the latest flowered lines, which occurred in late February 2000 or 2001. The lines were then attributed a flowering phenotype, i.e., able to flower under short days (A) or not able to flower under short days (NA).

### *Field experiment*

Pop2 was sown twice at a monthly interval (Date 1 = D1: 30 September and Date 2 = D2: 29 October 2001) at the INRA experimental station of Mons (49°52′N, 3°00′E). The experimental design was a split-plot with two replicates, the sowing dates being the main plots and the replicates being the subplots. Each individual plot consisted of a 2 m row of 25 seeds, with a 1 m spacing between two rows. Plants were grown against wire in order to prevent them from lodging. Insects, diseases and weeds were controlled chemically and plants grew in well-watered conditions. The date of the beginning of flowering (DBF) was recorded for each plot as the date at which half of the plants exhibited at least one fully opened flower. No significant difference was observed between the replicates within each sowing date condition and the lines mean values will thus be presented in the results. The choice of D1 and D2 to reveal the reaction to photoperiod proceeded from the field characterization proposed by Lejeune-Hénaut et al. (1999). In this paper, a 1-month delay in autumn sowing dates had little effect on the date of floral initiation of *Hr* lines because they need at least a 13h30 photoperiod to trigger off floral initiation, which happens only in mid-April of the following spring. In contrast, *hr* lines, which are not strictly dependent from long days, will initiate flowers earlier in spring if they have been sown earlier in autumn. In other words, we expected *Hr* lines to have similar DBF for D1 and D2 and *hr* lines to have an earlier DBF in D1 than in D2.

## Evaluation of winter frost damages

The winter frost tolerance of Pop2 has been evaluated in the field in 11 location x year conditions (Table 1). In 2000/2001, 2001/2002 and 2002/2003, experiments were conducted at the INRA locations of Mons, Clermont-Ferrand-Theix and Dijon. In addition, experiments were also conducted in 2001/2002 at the INRA locations of Colmar and Lusignan. In each location, plots were organized following a complete block design with two replicates in 2000/2001 and three replicates in the other conditions. Individual plots consisted in two rows in 2000/2001 (2 m long, 20 seeds per row, 20 cm between rows) and six rows (1.6 m long, 15 seeds per row, 20 cm between rows) in the other conditions. Weeds and diseases were controlled chemically. Although winter survival usually depends on the response to different abiotic stresses, the climatic (Table 1) and biological observations supported the idea that frost was the major stress for the 11 conditions studied here. Frost damages were evaluated after the winter freezing periods had passed. A score was attributed to a plot as a whole based on the aspect of the aerial parts of the plants according to the following scale: 0 for no damages, 1 for frost burn limited to the leaf edges, 2 for frost burn on the majority

**Table 1** Main characteristics of the experimental locations in which Pop2 was assessed for winter survival

| Location | Latitude and longitude | Altitude (m) | Type of climate | Date of sowing | Date of emergence | Date of the record of freezing damages | Abbreviation | Characteristics of the winter period (*) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Number of days with minimum air temperature ≤0°C | Minimum air temperature (°C) |
| Mons | 49°88′N, 5°09E | 211 | Maritime cold | 04-10-2000 | 20-10-00 | 19-02-01 | mon0001 | 24 | −4.5 |
| | | | | 27-09-2001 | 08-10-01 | 18-01-02 | mon0102 | 37 | −8.7 |
| | | | | 02-10-2002 | 11-10-02 | 06-02-03 | mon0203 | 26 | −11.4 |
| Clermont-Ferrand Theix | 45°70′N, 3°02′E | 890 | Mid mountain | 06-10-2000 | 22-10-00 | 11-03-01 | cle0001 | 44 | −10 |
| | | | | 27-09-2001 | 08-10-01 | 30-01-02 | cle0102 | 58 | −15.3 |
| | | | | 26-09-2002 | 28-10-02 | 19-03-02 | cle0203 | 69 | −14.3 |
| Dijon | 47°25′N, 5°09′E | 211 | Continental | 04-10-2000 | 14-11-00 | 20-02-01 | dij0001 | 28 | −5.3 |
| | | | | 26-09-2001 | 06-10-01 | 05-03-02 | dij0102 | 66 | −12.9 |
| | | | | 01-10-2002 | 23-10-02 | 11-02-03 | dij0203 | 32 | −12.9 |
| Colmar | 48°05′N, 7°03′E | 200 | Continental | 02-10-2001 | 12-10-01 | 04-03-02 | col0102 | 61 | −18.2 |
| Lusignan | 46°44′N, 0°14E | 150 | Martime mild | 27-09-2001 | 04-10-01 | 17-01-02 | lus0102 | 38 | −8.6 |

* Values have been calculated or recorded from the date of emergence to the date of the record of freezing damages

of the leaf surface, 3 for frost burn on upper third of the stem, 4 for frost burn on upper three-quarters of the stem, and 5 for frost dead plants. Intermediate scores were given to record irregular damages within a plot.

Analyses of variance were performed with the SAS package (SAS Institute Inc., 1999) using the 'general linear model' procedure with the following model for each condition:

$$Y_{ij} = \mu + \text{geno}_i + \text{rep}_j + \text{e}_{ij},$$

where $Y_{ij}$ is the value of frost damages recorded for the recombinant inbred line $i$ and the replicate $j$, where $\mu$ is the population mean, and where geno, rep and e are, respectively, the genotypic, replication and residual effects of the model. The normality of residual distributions was tested using skewness, kurtosis and Shapiro–Wilk statistics (Shapiro and Wilk 1965) displayed by the 'univariate' procedure. This procedure was also used to detect abnormal residues and the corresponding records were either corrected or deleted. For each of the 11 conditions, the RILs genotypic means adjusted from the replication effect were calculated with the 'lsmeans' statement of the 'general linear model' procedure. This resulted in a set of 11 winter frost damages (WFD) values per genotype further used for QTL detection.

## Map construction and QTL analysis

The Pop2 genotyping data are already included in the composite maps proposed by Aubert et al. (2006) and Loridon et al. (2005), the first one comprising gene-anchored markers. Using both sources, we built a map for Pop2 using the 'map' command of MAPMAKER/EXP version 3.0b (Lander et al. 1987; Lincoln et al. 1992). Additional markers only available for Pop2 (Loridon et al. 2005) were also placed using the 'try' and 'map' commands. Finally, the 'assign' and 'try' commands were run to map the *Hr* locus, phenotyped as described above and the resulting order was verified with the 'ripple' command. The Haldane function was used to calculate distances in centiMorgan (cM).

The QTL analyses were performed using the package Windows QTL Cartographer Version 2.5 (Wang et al. 2005). Composite interval mapping (CIM) was run using model 6. The LOD significance threshold was determined after running 1,000 permutations tests ($\alpha = 0.05$) for each of the 11 traits. The higher resulting threshold, LOD = 3.59, was chosen to perform CIM for all traits. Cofactors were selected with the forward and backward regression method (probability in and probability out were each set to a value of 0.05). The window size in which the cofactors are not considered was set up to 10 cM on either side of the markers flanking the test site.

## Results

### Phenotyping of the photoperiod reaction within Pop2

#### *Greenhouse experiment*

Among the 80 RILs studied in the greenhouse to assess their responsiveness to the photoperiod, 38 were completely unable to flower after 5 months of the SD treatment, like the parent Champagne; 36 RILs showed a flowering time continuum between 29 November 1999 and 16 February 2000 or 4 December 2000 and 3 February 2001, according to the sowing date.

The parental line Terese flowered a little later in the second period of experimentation, i.e., 23 December 2000 to 8 January 2001, than in the first one, i.e., 12 to 21 December 1999. The same delay was observed for the control RILs that were studied twice and able to flower under SD (lines 43 and 73). Thus the qualitative flowering behavior, A (able to flower under SD) versus NA (not able to flower under SD), was reproducible between the two periods of experimentation: Terese, the line 43 and the line 73 being A, and Champagne the line 118 and the line 183 being NA.

Four lines (14, 16, 70 and 138) did not show a clear phenotype with only one or two plants among six being able to flower under SD before the date defined as the end of the experiment, which prevented us from assigning them to one of the two flowering phenotypic classes A or NA. Besides, the lines 74 and 192 did not open flowers before the end of the experiment which should have placed them in the NA class, but traces of aborted flowers were detected on upper nodes of the line 74 and flower primordia were observed in the apex of the line 192 under magnifying glasses, so they were also excluded from the greenhouse classification.

#### *Field experiment*

The reaction to the photoperiod could also be deduced from the flowering behavior in the field, as shown in Fig. 1. On this figure, each line was represented by its date of beginning of flowering (DBF) in each of the two sowing dates (D1 and D2) of the field experiment. The lines that were previously studied in the greenhouse were plotted with black squares and dots. Among them, the parental lines and the 4 control RILs were splitted over

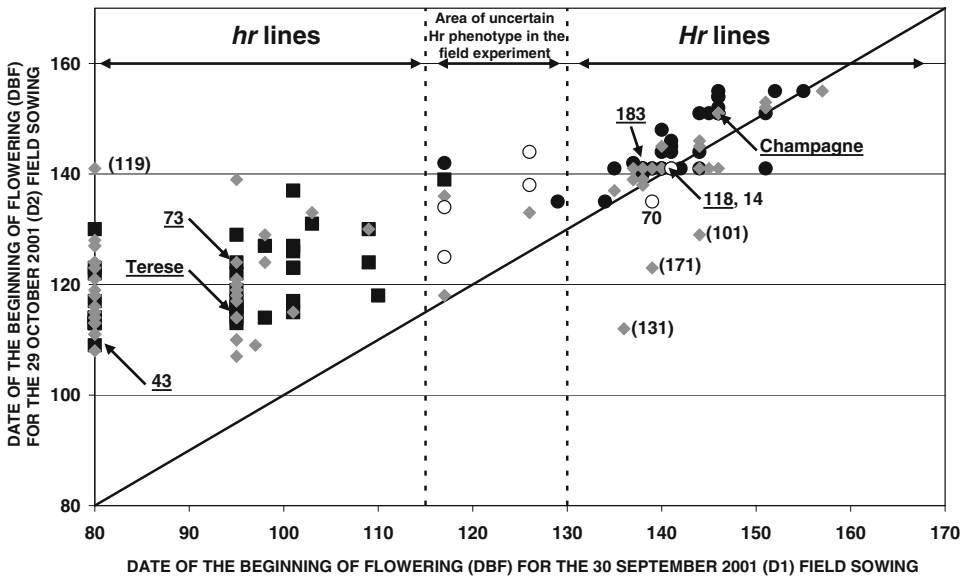


**Fig. 1** Dates of the beginning of flowering (*DBF*) for Pop2 lines at the two field sowing dates, D1 = 30 September 2001 and D2 = 29 October 2001. DBF at D1 and DBF at D2 are represented respectively on the x and y-axis. DBF is expressed as the number of days from 1st January 2002, e.g., 100 corresponds to the 10 April and 150 corresponds to the 30 May. The filled symbols are a reminder of the flowering phenotype observed in the greenhouse short days (SD) experiment, *filled square*: lines that were able to flower under SD; *filled circle*: lines that were unable to flower under SD. The other symbols are attributed as follows, *open circle*: lines with an unclear SD reaction phenotype in the greenhouse experiment; *shaded diamond*: lines that were only studied in the field. The lines Terese, 43, 73 as well as Champagne, 118 and 183 (*labels underlined*) were studied twice in the greenhouse during the winter periods of 1999–2000 and 2000–2001. They showed each time the same SD reaction, i.e., Terese, 43 and 73 were able (*A*) to flower under SD, while Champagne, 118 and 183 were not (*NA*). In the field experiment, some early lines freezed and died when sown at D1; they were arbitrarily plotted on the *y*-axis; their early DBF at D2 allows to allocate them to the *hr* group, except for the line 119, represented into brackets, which flowered too late in D2 to be considered as equivalent to the rest of the group. Three other lines of the field experiment, i.e., the lines 101, 131 and 171 also represented into brackets, were excluded from the graphical classification although they plotted in the *Hr* part of the graph because they were far away from the first bissector

two distinct groups for DBF in the field. Terese and the lines 43 and 73 had an early DBF and they began to flower much earlier in D1 than in D2, which placed the points on the left part of the figure and quite far above the first bissector. At the opposite, Champagne and the lines 118 and 183 flowered late but began to flower approximately at the same date for D1 and D2, which brought the corresponding points on the right part of Fig. 1 and close to the first bissector. Even when sown earlier, these lines flowered at the same date (mid May for Champagne) behaving as if they were waiting for an appropriate daylength to initiate flowers. This behavior appeared to be consistent with the obligate photoperiod requirement observed in the greenhouse experiment, where they were not able to flower under unvariable SD. The results of the greenhouse and field experiments were globally consistent, except for a middle group of seven lines for which the different experiments did not allow to assure the *Hr* phenotype. We thus decided to exclude this zone, corresponding to $115 < D1 < 130$, for the following. This graphical method was extended to the RILs that had not been evaluated in the greenhouse, represented by grey diamonds on Fig. 1, which allowed allocation of most of the lines to either the *Hr* or *hr* group. Three of the lines only studied in the field fell in the area of uncertain phenotypes and were excluded. So were the lines 101, 131 and 171 which plotted in the right part of the figure because although they were late at flowering at D1, they were far away under the first bissector and thus too different from Champagne to be affected to the *Hr* group. In the field experiment, some early lines freezed and died when sown at D1; they were arbitrarily plotted on the *y*-axis; their early DBF at D2 allows to allocate them to the *hr* group, except for the line 119 which flowered too late in D2 to be considered as equivalent to the rest of the group. The 164 lines were genotyped as follows for the *Hr* locus: 67 *Hr*, 82 *hr*, 15 missing data. The 67:82 ratio of the genotyped RILs fitted the hypothesis of a single locus segregation (Chi square = 1.51).

## Morphological characteristics of the *Hr* versus *hr* lines in the field

Additionally to their strong reaction to the photoperiod, the *Hr* lines of Pop2 were characterized by a rosette-type growth habit during the winter period (Fig. S1). They developed more branches and the aerial organs remained dwarf, i.e., short internodes and small leaflets, during the whole winter period, whatever the allele at the major internode-length locus Le (Ingram et al. 1984; Mendel 1866). In the following spring, when the daylength reached approximately 13h30 (value observed for Champagne, Lejeune-Hénaut et al. 1999), and allowed the switch to the reproductive stage, the *Hr* lines recovered an erected growth and larger leaflets. In the *Hr* background, a significant difference of internode length between the dwarf (*le*) and normal (*Le*) lines was visible only from that time.

## Mapping the *Hr* locus and QTL of WFD

A genetic map was developed for Pop2, which comprises 213 markers, including *Hr*, and covers 1,491 cM Haldane (Aubert et al. 2006; Loridon et al. 2005). The *Hr* locus, for which the genotype was deduced from the photoperiod reaction as explained above mapped on the linkage group (LG) 3, between the microsatellite marker AA175 and the seed marbling locus, M (Fig. 2). An analysis of the WFD recorded for Pop2 in 11 field conditions was performed with Windows QTL Cartographer (LOD threshold = 3.59, determined after 1,000 permutations tests for each condition). Composite interval mapping (CIM) allowed us to detect six distinct QTL positions, referred to as WFD 1.1 to WFD 6.1, as shown in Fig. 2 and Table 2. Two genomic regions, WFD 3.1 and WFD 6.1 were very consistently detected in the different conditions: WFD 3.1 on LG3 near *Hr* and AA175 was detected in all environments and WFD 6.1 on LG6 near markers AD141, AD59, AD159 and AA200 was detected in 10 environments out of 11. The WFD 3.2 position corresponded to 5 significant QTL mapped at the bottom of LG3, close to the dwarfism locus *Le*. Another position, WFD 5.1, was consistent among 11 conditions (Table 2). Finally, the positions WFD 1.1 and WFD 5.2 were characterized by QTL detected in only one condition, the corresponding closest markers being R13.1800 for WFD 1.1, and Y02.900 for WFD 5.2. Besides the positions presented in Fig. 2, Table 2 also gives the coefficient of determination ($R^2$) of each QTL peak as well as the allelic value of Terese. These values highlight the prominent part played by WFD 3.1 in the genetic control of winter freezing damages, with $R^2$ ranging from 0.19 to 0.52. The five other positions are comparatively less explanatory with individual $R^2$ ranging from 0.03 to 0.12. Another important observation is that the favorable WFD alleles, i.e., those giving a smaller WFD score equivalent to a higher frost tolerance, are all from Champagne except for the WFD 3.2 position, where the favorable allele at the peak marker, *le*, corresponds to the dwarf type Terese. Table 3 presents the allele sizes of the microsatellite markers close to the QTL peaks for the three most consistent QTL, WFD 3.1, WFD 5.1 and WFD 6.1. We also used the AA175 genotyping data to try to infer the Hr genotype of the 11 lines located in the area of uncertain Hr phenotype in the field experiment (Fig. 1). Among these lines, 3 presented the Terese allele for AA175 and 8

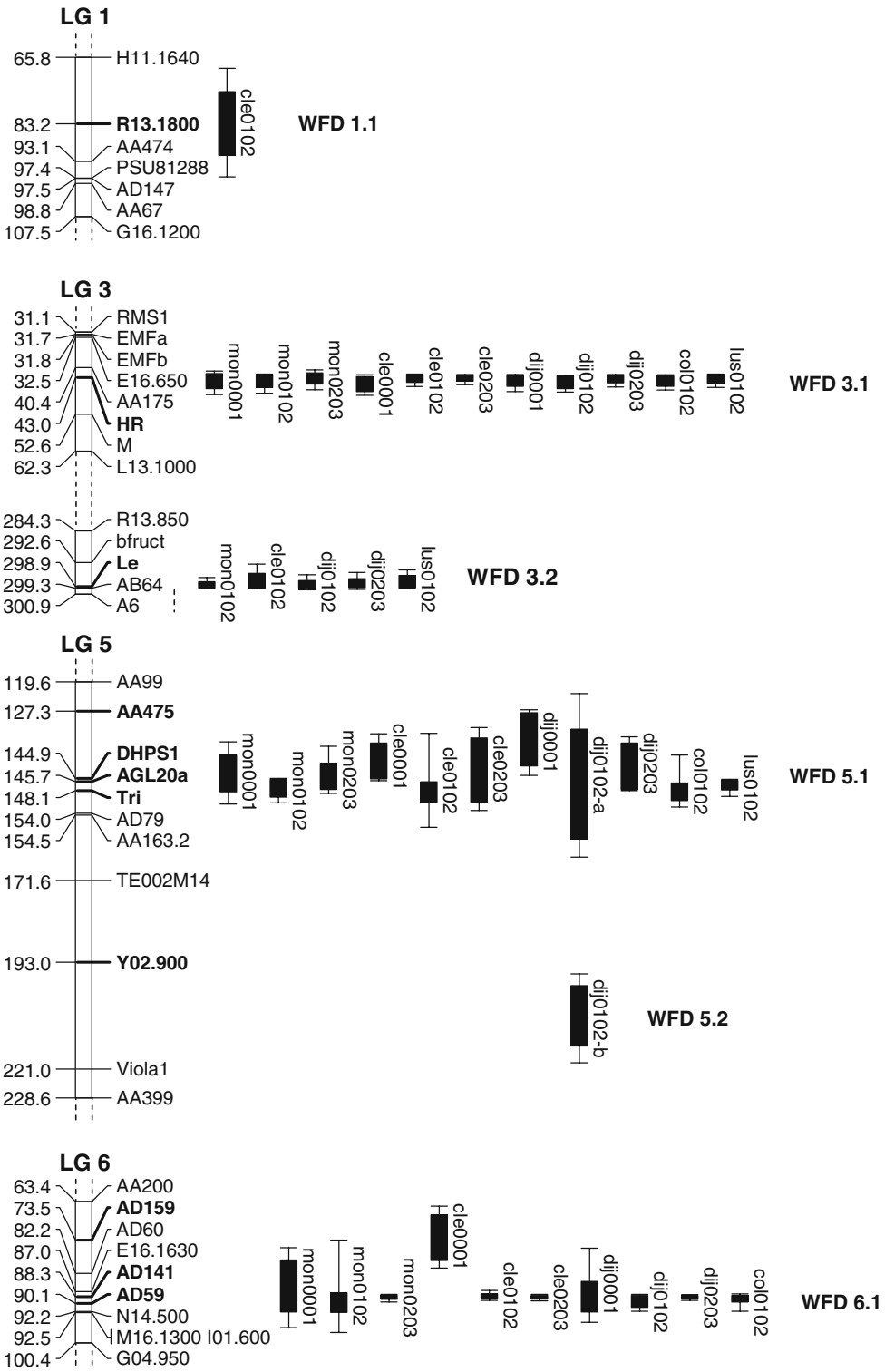


**Fig. 2** Genetic maps of the linkage group segments containing QTL for winter freezing damages. Each QTL is identified by the corresponding environmental condition abbreviated as mentionned in Table 1. Two intervals are specified for each QTL: the inner interval corresponds to a 1-LOD drop-down and the outer interval corresponds to a 2-LOD drop-down. Markers in bold are either the closest marker to a QTL peak either the QTL peak itself. Consensual positions of the QTL are revealed by overlapping confidence intervals and are identified by the symbols WFD 1.1 to WFD 6.1

the Champagne allele. The mean DBF of these 3 and 8 lines were similar at D1, 120 and 121 days respectively but different at D2, 127 and 137 days, respectively, this last result reflecting globally the expected difference between hr and Hr lines. Thus, even if the photoperiod response would have to be tested again for the 11 uncertain lines, the microsatellite marker AA175 seems to be a satisfying indicator of the flowering phenotype.

**Table 2** Characteristics of the QTL of winter freezing damages in the F8 RILs population derived from Champagne x Terese

| Linkage group | QTL identification | Condition | Markers flanking the QTL peak, **Marker at the QTL peak** | LOD | $R^2$, for loci where LOD > 3.59 | Allelic value of Terese |
|---|---|---|---|---|---|---|
| 1 | WFD 1.1 | cle0102 | R13.1800, AA474 | 4.04 | 0.03 | +0.21 |
| 3 | WFD 3.1 | mon0001 | AA175, ***Hr***, M | 13.80 | 0.19 | +0.36 |
| | | mon0102 | AA175, ***Hr***, M | 29.21 | 0.35 | +0.63 |
| | | mon0203 | AA175, ***Hr***, M | 15.38 | 0.24 | +0.40 |
| | | cle0001 | *Hr*, M | 29.69 | 0.49 | +0.86 |
| | | cle0102 | AA175, ***Hr***, M | 37.46 | 0.41 | +0.79 |
| | | cle0203 | AA175, ***Hr***, M | 35.07 | 0.41 | +0.83 |
| | | dij0001 | AA175, ***Hr***, M | 26.62 | 0.40 | +0.75 |
| | | dij0102 | AA175, ***Hr***, M | 43.21 | 0.52 | +0.81 |
| | | dij0203 | AA175, ***Hr***, M | 32.25 | 0.35 | +0.70 |
| | | col0102 | AA175, ***Hr***, M | 32.40 | 0.47 | +0.78 |
| | | lus0102 | AA175, ***Hr***, M | 38.39 | 0.49 | +0.63 |
| | WFD 3.2 | mon0102 | *bfruct*, ***Le***, AB64 | 10.08 | 0.09 | −0.32 |
| | | cle0102 | *bfruct*, ***Le***, AB64 | 4.41 | 0.03 | −0.20 |
| | | dij0102 | *bfruct*, ***Le***, AB64 | 5.72 | 0.04 | −0.22 |
| | | dij0203 | *bfruct*, ***Le***, AB64 | 5.66 | 0.04 | −0.23 |
| | | lus0102 | *bfruct*, *Le* | 7.53 | 0.06 | −0.25 |
| 5 | WFD 5.1 | mon0001 | AA475, ***DHPS1***, *AGL20a* | 7.12 | 0.09 | +0.24 |
| | | mon0102 | *AGL20a*, *Tri* | 8.74 | 0.08 | +0.30 |
| | | mon0203 | *DHPS1*, ***AGL20a***, *Tri* | 8.28 | 0.12 | +0.28 |
| | | cle0001 | AA475, *DHPS1* | 8.32 | 0.12 | +0.43 |
| | | cle0102 | *AGL20a*, *Tri* | 12.33 | 0.09 | +0.37 |
| | | cle0203 | *DHPS1*, ***AGL20a***, *Tri* | 8.02 | 0.06 | +0.31 |
| | | dij0001 | AA475, *DHPS1* | 6.37 | 0.09 | +0.36 |
| | | dij0102 | AA475, *DHPS1* | 7.11 | 0.06 | +0.29 |
| | | dij0203 | *DHPS1*, ***AGL20a***, *Tri* | 11.89 | 0.09 | +0.35 |
| | | col0102 | *AGL20a*, *Tri* | 8.04 | 0.08 | +0.32 |
| | | lus0102 | *DHPS1*, ***AGL20a***, *Tri* | 6.54 | 0.05 | +0.20 |
| | WFD 5.2 | dij0102 | Y02.900, *Viola1* | 6.56 | 0.08 | +0.32 |
| 6 | WFD 6.1 | mon0001 | E16.1630, **AD141**, AD59 | 10.70 | 0.14 | +0.29 |
| | | mon0102 | E16.1630, **AD141**, AD59 | 3.89 | 0.03 | +0.18 |
| | | mon0203 | E16.1630, **AD141**, AD59 | 8.92 | 0.13 | +0.28 |
| | | cle0001 | AA200, AD159 | 6.69 | 0.08 | +0.35 |
| | | cle0102 | E16.1630, **AD141**, AD59 | 12.43 | 0.09 | +0.35 |
| | | cle0203 | E16.1630, **AD141**, AD59 | 11.64 | 0.09 | +0.37 |
| | | dij0001 | E16.1630, **AD141**, AD59 | 7.32 | 0.08 | +0.33 |
| | | dij0102 | AD141, **AD59**, N14.500 | 11.53 | 0.08 | +0.32 |
| | | dij0203 | E16.1630, **AD141**, AD59 | 12.86 | 0.10 | +0.36 |
| | | col0102 | E16.1630, **AD141**, AD59 | 10.14 | 0.07 | +0.30 |

The markers in bold are at the QTL peak

## Discussion

### The *Hr* locus: map position and putative orthologous sequences

The molecular identity of *Hr* has yet to be determined. To map the *Hr* locus in our population, we carried out a short day experiment proposed by Murfet (1971, 1973) to reveal the segregation between *Hr* and *hr* plants. In our greenhouse experiment, the *Hr* phenotype of the parental line Champagne was clearly distinct from that of the *hr* line Terese: flowering was not obtained for Champagne after 5 months of SD treatment although it was for Terese. Moreover, a significant 1:1 segregation was revealed for

**Table 3** Allele sizes of the microsatellite markers close to the QTL peaks for WFD 3.1, WFD 5.1 and WFD 6.1

The primers and optimal conditions of amplification are described in Loridon et al. (2005, electronic supplemental material), – indicates the absence of the band

| Linkage group | QTL identification | Microsatellite markers close to the QTL peaks | Allele size (base pairs) for Terese/Champagne |
|---|---|---|---|
| 3 | WFD 3.1 | AA175 | 270/260 |
| 5 | WFD 5.1 | AA475 | –/180 |
| 6 | WFD 6.1 | AA200 | –/220 |
| | | AD159 | 160/150 |
| | | AD141 | 330/– |
| | | AD59 | 330/320 |

the 74 Pop2 lines showing a clear phenotype in the greenhouse experiment, confirming a genetic control by a single locus. The field experiment allowed us to extend the *Hr* classification to 150 of the 164 Pop2 lines and to assign unambiguously the *Hr* locus to LG3 on the Pop2 genetic map. This position of *Hr* close to the seed marbling gene (Fig. 2) is in agreement with the tight linkage between *Hr* and M reported by Murfet (1973), which calculated a recombination value of 3% between both loci and deduced therefore the position of *Hr* on LG3. It is also consistent with the approximate position of *Hr* reported on the consensus map proposed by Weeden et al. (1998).

It is of course a challenging goal to identify the DNA sequence of *Hr*. *Arabidopsis* flowering genes are an interesting source of candidate sequences. Some of them (pea orthologous sequences for *Arabidopsis* AGL20 = SOC1, COL, CRY2, EMF, FCA, FPA, LD, PHYA, TFL1) have already been mapped in Pop2 (Aubert et al. 2006). None are positional candidates for *Hr*, although we formerly thought that it could be the case for the COL sequences (PsCOLa and PcCOLb), which belong to the photoperiod pathway in *Arabidopsis*. Hecht et al. (2005) suggested however that some *Arabidopsis* flowering genes could be candidates for this locus. They searched legume (*Medicago truncatula*, *Glycine max* and *Lotus japonicus*) databases for orthologous sequences of a number of *Arabidopsis* flowering genes. They located nine *Arabidopsis* ortholog pairs to corresponding map positions in *Pisum sativum* and *Medicago truncatula* by using the syntenic relationships described for pea and Medicago by Kaló et al. (2004) and Choi et al. (2004). This result supports a map-based strategy to identify pea flowering genes. Concerning *Hr*, Hecht et al. (2005) have given a particular interest to the flowering locus C (FLC) and to Frigida (FRI), because they share with the dominant *Hr* allele a strong delay in flowering particularly under SD. They did not find any FRI or FLC orthologs in pea, but proposed to pay attention to the *Medicago truncatula* FRLa gene, which is similar to the *Arabidopsis* FRI-like genes, and whose mapping position in *Medicago truncatula* corresponds to the approximate position of *Hr*. Although it may appear relatively hazardous to screen *Arabidopsis* flowering genes for a precise target orthologous sequence in *Medicago* or pea, because crucifers and legumes may have evolved independently, this strategy remains promising provided that *Arabidopsis* candidates are chosen accordingly to similar phenotypes in both species.

## Winter frost tolerance is determined by a few but consistent QTL positions in Pop2

The quantitative trait mapping study presented here relies on the assessment of the pea segregating population Pop2 using eleven field environments. This experimental effort was considered necessary for a cultivated species to ensure that the results would be reliable for further winter pea breeding programs. It is difficult to repeatably evaluate frost tolerance in field conditions. We overcame this difficulty by constituting a field experimental network among INRA locations representing various cold winters with more or less maritime influence. Three sites, Mons, Dijon and Clermont-Ferrand-Theix, are particularly relevant to screen for frost resistance because frost events occur there quite regularly. With this strong experimental basis, we found consistent positions for 4 of the 6 detected QTL.

The availability of numerous molecular markers allows geneticists to develop linkage maps with an appropriate density of markers for QTL mapping. Compared with the previous studies of the transmittance of winterhardiness in pea (Auld et al. 1983; Cousin et al. 1985; Liesenfeld et al. 1986), it is now possible to discover new information about the genome location of the areas responsible for winter frost tolerance. It is interesting to note that, whatever the statistical method previously employed, i.e., diallel analyses (Auld et al. 1983; Cousin et al. 1985) or dissection of the frequency distributions among segregating populations (Liesenfeld et al. 1986), the authors generally reported a quantitative inheritance of winter hardiness with additive effects of few genes. Liesenfeld et al. (1986) estimated that as few as three additive genes may condition winter hardiness in the lines evaluated. Among other legume species, Kahraman et al. (2004) detected five QTL positions for winter survival in a RIL population of lentil, with only one QTL being consistent

across three location × year conditions. Our QTL detection in Pop2, showing six significant QTL, is in agreement with an oligogenic determinism of winter frost tolerance. Moreover, the consistency of three QTL, WFD 3.1, WFD 5.1 and WFD 6.1 among almost all the experimental conditions makes these loci interesting targets for marker assisted selection.

## A genetic linkage between winter frost tolerance and the response to photoperiod in pea

The position of the *Hr* locus as the peak marker for the highest explanatory QTL of this study supports our hypothesis of the prominent part played by this gene in the variability of freezing resistance, at least in Pop2. It is the first report in a legume species for understanding the nature of the genetic relationship between frost resistance and developmental, i.e., photoperiod or vernalization, genes. Whether this relationship is only due to a genetic linkage or reflects an epistatic effect of developmental genes upon frost tolerance genes has been extensively sought in other cultivated species, particularly cereals. In wheat (*Triticum aestivum*), QTL mapping has revealed genetic links between presumed homologous QTL for frost resistance and the vernalization locus *Vrn*1 (Galiba et al. 1995; Toth et al. 2003). A colocalization between a QTL for photoperiod sensitivity and a QTL for winterhardiness has also been pointed out on chromosome 6A by a QTL meta-analysis (Hanocq et al. 2007). In barley (*Hordeum vulgare*), highly significant QTL for winter hardiness and related measures of low-temperature tolerance were found on chromosome 5H (Francia et al. 2004; Pan et al. 1994) and the coincidence with the *Vrn*1 region of the *Triticeae* was also evidenced (Francia et al. 2004). In oilseed rape (*Brassica napus* L.), QTL controlling vernalization requirement and freezing tolerance were also mapped to the same genomic region (Teutonico and Osborn 1995; Teutonico et al. 1995). In annual legumes, QTL mapping of winterhardiness was realized for lentil but did not point out any genetic linkage with a developmental gene (Kahraman et al. 2004). However, in the perennial legume *Medicago sativa*, coincident QTL were found for winterhardiness and fall dormancy which is a developmental factor triggered by short days and cool temperatures (Brouwer et al. 2000).

The observation of a winter-adapted plant morphology within each species can also be related to the expression of developmental genes. It is a common observation that winter hardy herbaceous plants exhibit a prostrate growth, numerous branches and reduced aerial organs when submitted to acclimating temperatures and this supports the pleiotropic effects on both growth habit and low-temperature tolerance attributed to developmental genes. In wheat, Roberts (1990) reported that *Vrn*1 controls or is tightly linked to a locus partly controlling the rosette growth habit. In pea, it has already been observed that the fall rosette formation is closely associated with winter hardiness (Andersen and Markarian 1968; Liesenfeld et al. 1986; Markarian and Andersen 1966). Markarian and Andersen (1966), studying a cross including the forage pea Austrian Winter, have proposed a genetic control with two genes for this trait. They concluded that the formation of a compact rosette is essential for winter survival. Our results also support this conclusion considering that most of the *Hr* lines of Pop2 are characterized by a marked rosette in autumn and winter field conditions, and that the *Hr* locus colocalizes with the highest explanatory WFD 3.1 QTL of our study. Also in support, is the statement (Murfet and Reid 1993) that the flowering allele *Hr* enhances the capacity of pea photoperiodic lines to produce basal laterals. Murfet and Reid (1993) brought together the behavior of *Hr* lines with that of many primitive accessions of *Pisum sativum* sp. *humile*; *P. sativum* sp. *elatius* and *P. fulvum*, which display an enhanced branching response to short photoperiods when compared with the domestic cultivars. They suggested that such a profuse basal branching is of evolutionary interest for wild ecotypes that are adapted to grow vegetatively through the cool and moist conditions of winter and that flower with the advent of long days in the spring.

Our present findings and previous results (Lejeune-Hénaut et al. 1999) highlight the prominent relationship between winter frost tolerance and the photoperiod response in *P. sativum*. For legumes, it is tempting to suggest that low-temperature tolerance is primarily regulated by the photoperiod response. This is in contrast to cereals where the vernalization response appears to have this role. Further assessment of this hypothesis in pea requires determination of the molecular sequence of *Hr*, and in this prospect, the knowledge of the *Arabidopsis* flowering pathways will be beneficial. The recent considerations upon the epigenetic transmission of the vernalization response in Arabidopsis constitute an attractive model to explain the coincidence between the repression of the flowering transition and the up-regulation of low-temperature-responsive genes (Amasino 2004a, b).

## Genetic linkages between winter frost tolerance and agronomical traits

Besides the large effect of WFD 3.1/*Hr* which deserved attention, other colocations have to be considered. It is the case for the WFD 3.2 QTL associated to the *Le* locus on LG3. This position is the only one, in the genetic background of the studied population, for which the favorable allele for winter frost tolerance is brought by the

susceptible parent Terese. We have, however, reported above that the field-autumn-sown *Hr* lines remain dwarf until a longer spring daylength has triggered off the switch from the prostrate to the erected growth habit, which suggests an epistatic effect of *Hr* upon the expression of the dwarfism. Finally, even if the independant effect of WFD 3.2 and the candicacy of the colocated *Le* locus needs to be checked. Agronomically, the recessive *le* allele is required in the dry pea cultivars for northern Europe, where all the dry peas are dwarf to minimize crop lodging. The position of the *Tri* locus under the WFD 5.1 QTL will have to be carefully considered in breeding programs, as the favorable allele for WFD 5.1 could bring together the dominant *Tri* allele which is unfavorable for a seed use for pig and poultry feeding, because it contains two structural genes encoding the major pea seed trypsin inhibitors (Domoney et al. 1994, 1995). Page et al. (2002) developed a set of PCR primers suitable to distinguish a number of pea genotypes for the *Tri* locus and which was applied to genotype Pop2 (Page et al. 2003). This marker could be used to breed for low trypsin inhibitor activity, although a favorable effect of the dominant *Tri* allele on winter frost tolerance cannot be excluded.

## Conclusion: perspectives for marker assisted selection (MAS) of winter frost tolerant peas

Considering the high explanatory level of the WFD 3.1 QTL in the genetic variation of winter frost tolerance in pea, but the difficulty to assess the *Hr* phenotype, efficient breeding progress could be made by using the closest molecular markers to follow the introgression of the favorable *Hr* dominant allele in diverse genetic backgrounds using the microsatellite marker AA175. A second flanking marker would be necessary for a better efficiency, as the marbling locus (M) falls 9.6 cM apart from *Hr*. Thus, unless a rapid identification of the *Hr* molecular sequence among *Arabidopsis* flowering candidates, a denser genetic map around *Hr* is necessary and additional markers will be searched in the orthologous region of *Medicago truncatula*. Following the same strategy, the microsatellite markers AD59, AD141, AA200 and AD159 can help a marker-assisted introgression of WFD 6.1. Considering the largest confidence interval of WFD 5.1, four markers AA475, DHPS1, AGL20a and *Tri*, should be used to follow the introgression, with the restrictions related to the *Tri* locus mentioned above.

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## References

Amasino R (2004a) Take a cold flower. Nat Genet 36:111–112

Amasino R (2004b) Vernalization, competence, and the epigenetic memory of winter. Plant Cell 16:2553–2559

Andersen RL, Markarian D (1968) The inheritance of winterhardiness in *Pisum* III. Stem branching in Autumn growth. Euphytica 17:473–478

Aubert G, Morin J, Jacquin F, Loridon K, Quillet M, Petit A, Rameau C, Lejeune-Hénaut I, Huguet T, Burstin J (2006) Functional mapping in pea, as an aid to the candidate gene selection and for investigating synteny with the model legume *Medicago truncatula*. Theor Appl Genet 112:1024–1041

Auld DL, Adams KJ, Swensen JB, Murray GA (1983) Diallel analyses of winterhardiness in peas. Crop Sci 23:763–766

Baranger A, Aubert G, Arnau G, Laine AL, Deniot G, Potier J, Weinachter C, Lejeune-Henaut I, Lallemand J, Burstin J (2004) Genetic diversity within *Pisum sativum* using protein- and PCR-based markers. Theor Appl Genet 108:1309–1321

Börner A, Schumann E, Fürste A, Cöster H, Leithold B, Röder M, Weber W (2002) Mapping of quantitative trait loci determining agronomic important characters in hexaploid wheat (*Triticum aestivum* L.). Theor Appl Genet 105:921–936

Brouwer DJ, Duke SH, Osborn TC (2000) Mapping genetic factors associated with winter hardiness, fall growth, and freezing injury in autotetraploid alfalfa. Crop Sci 40:1387–1396

Choi H-K, Mun J-H, Kim D-J, Zhu H, Baek J-M, Mudge J, Roe B, Ellis N, Doyle J, Kiss GB, Young ND, Cook DR (2004) Estimating genome conservation between crop and model legume species. PNAS 101:15289–15294

Cousin R, Messager A, Vingere A (1985) Breeding for yield in combining peas. In: Hebblethwaite PD, Heath MC, Dawkins TCK (eds) The pea crop. A basis for improvement. Butterworths, London, pp 115–136

Domoney C, Welham T, Ellis N, Hellens R (1994) Inheritance of qualitative and quantitative trypsin-inhibitor variants in *Pisum*. Theor Appl Genet 89:387–391

Domoney C, Welham T, Sidebottom C, Firmin JL (1995) Multiple isoforms of *Pisum* trypsin inhibitors result from modification of two primary gene products. FEBS Lett 360:15–20

Ellis THN, Poyser SJ (2002) An integrated and comparative view of pea genetic and cytogenetic maps. New Phytol 153:17–25

Fowler DB, Breton G, Limin AE, Mahfoozi S, Sarhan F (2001) Photoperiod and temperature interactions regulate low-temperature-induced gene expression in barley. Plant Physiol 127:1676–1681

Fowler DB, Chauvin LP, Limin AE, Sarhan F (1996) The regulatory role of vernalization in the expression of low-temperature-induced genes in wheat and rye. Theor Appl Genet 93:554–559

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

Galiba G, Quarrie SA, Sutka J, Morgounov A, Snape JW (1995) Rflp Mapping of the Vernalization (Vrn1) and Frost-Resistance (Fr1) Genes on Chromosome 5a of Wheat. Theor Appl Genet 90:1174–1179

Hanocq E, Laperche A, Jaminon O, Lainé AL, Le Gouis J (2007) Most significant genome regions involved in the control of earliness traits in bread wheat, as revealed by QTL meta-analysis. Theor Appl Genet 114:569–584

Hecht V, Foucher F, Ferrandiz C, Macknight R, Navarro C, Morin J, Vardy ME, Ellis N, Beltran JP, Rameau C, Weller JL (2005) Conservation of *Arabidopsis* flowering genes in model legumes. Plant Physiol 137:1420–1434

Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, MacMillan J (1984) Internode length in *Pisum*. The *Le* gene controls the 3ß-hydroxylation of gibberellin $A_{20}$ to gibberellin $A_1$. Planta 160:454–463

Kahraman A, Kusmenoglu I, Aydin N, Aydogan A, Erskine W, Muehlbauer FJ (2004) QTL mapping of winter hardiness genes in lentil. Crop Sci 44:13–22

Kaló P, Seres A, Taylor SA, Jakab J, Kevei Z, Kereszt A, Endre G, Ellis THN, Kiss GB (2004) Comparative mapping between *Medicago sativa* and *Pisum sativum*. Mol Genet Genomics 272:235–246

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Lejeune-Hénaut I, Bourion V, Eteve G, Cunot E, Delhaye K, Desmyter C (1999) Floral initiation in field-grown forage peas is delayed to a greater extent by short photoperiods, than in other types of European varieties. Euphytica 109:201–211

Liesenfeld DR, Auld DL, Murray GA, Swensen JB (1986) Transmittance of winterhardiness in segregated populations of peas. Crop Sci 26:49–54

Lincoln M, Daly M, Lander E (1992) Constructing genetic maps with MAPMAKER/EXP version 3.0. Technical report, 3rd edn. Whitehouse Institute, Mass, Cambridge

Loridon K, McPhee K, Morin J, Dubreuil P, Pilet-Nayel M, Aubert G, Rameau C, Baranger A, Coyne C, Lejeune-Hénaut I, Burstin J (2005) Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). Theor Appl Genet 111:1022–1031

Mahfoozi S, Limin AE, Fowler DB (2001) Developmental regulation of low-temperature tolerance in winter wheat. Ann Bot 87:751–757

Markarian D, Andersen RL (1966) The inheritance of winterhardiness in *Pisum* I. Euphytica 15:102–110

Mendel G (1866) Versuche über Pflanzenhybriden. Verhandlungen des Naturforschenden Vereines in Brünn 4:3–47

Murfet IC (1971) Flowering in *Pisum*. Three distinct phenotypic classes determined by the interaction of a dominant early and a dominant late gene. Heredity 26:243–247

Murfet IC (1973) Flowering in *Pisum*. *Hr*, a gene for high response to photoperiod. Heredity 26:243–257

Murfet IC (1981) The likely flowering genotype for several cultivars and mutants. Pisum Newslett 13:40–41

Murfet IC, Reid JB (1993) Developmental mutants. In: Casey R, Davies DR (eds) Peas: genetics, molecular biology and biotechnology. CAB International, Wallingford, pp 165–216

Page D, Aubert G, Duc G, Welham T, Domoney C (2002) Combinatorial variation in coding and promoter sequences of genes at the Tri locus in *Pisum sativum* accounts for variation in trypsin inhibitor activity in seeds. Mol Genet Genomics 267:359–369

Page D, Duc G, Lejeune-Henaut I, Domoney C (2003) Marker-assisted selection of genetic variants for seed trypsin inhibitor contents in peas. Pisum Genetics 35:19–21

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

Roberts DWA (1990) Identification of loci on chromosome 5A of wheat involved in control of cold hardiness, vernalization, leaf length, rosette growth habit, and height of hardened plants. Genome 33:247–259

SAS (1999) SAS/STAT® User's Guide, version 8. SAS Institute Inc., Cary, NC, 3884 p

Shapiro SS, Wilk MB (1965) An analysis of variance for normality (complete samples). Biometrika 52:591–611

Teutonico RA, Osborn TC (1995) Mapping loci controlling vernalization requirement in *Brassica rapa*. Theor Appl Genet 91:1279–1283

Teutonico RA, Yandell B, Satagopan JM, Ferreira ME, Palta JP, Osborn TC (1995) Genetic analysis and mapping of genes controlling freezing tolerance in oilseed *Brassica*. Mol Breed 1:329–339

Toth B, Galiba G, Feher E, Sutka J, Snape JW (2003) Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat. Theor Appl Genet 107:509–514

Wang S, Basten CJ, Zeng Z-B (2005) Windows QTL Cartographer Version 2.5. Department of Statistics, North Carolina State University, Raleigh, NC. (http://www.statgen.ncsu.edu/qtlcart/WQTLCart.htm)

Weeden NF, Ellis THN, Timmerman-Vaughan GM, Swiecicki WK, Rozov SM, Berdnikov VA (1998) A consensus linkage map for *Pisum sativum*. Pisum Genetics 30:1–4

Weller JL, Reid JB, Taylor SA, Murfet IC (1997) The genetic control of flowering in pea. Trends Plant Sci 2:412–418