Theor Appl Genet (2009) 118:1561–1571
DOI 10.1007/s00122-009-1004-7

ORIGINAL PAPER

# Association of sugar content QTL and PQL with physiological traits relevant to frost damage resistance in pea under field and controlled conditions

**Estelle Dumont · Véronique Fontaine · Christophe Vuylsteker · Hélène Sellier · Sylvie Bodèle · Najia Voedts · Rosemonde Devaux · Marlène Frise · Komlan Avia · Jean-Louis Hilbert · Nasser Bahrman · Eric Hanocq · Isabelle Lejeune-Hénaut · Bruno Delbreil**

Received: 17 June 2008 / Accepted: 8 March 2009 / Published online: 26 March 2009


**Abstract** To increase yield in pea (*Pisum sativum* L.), autumn sowing would be preferable. Hence, frost tolerance of pea became a major trait of interest for breeders. In order to better understand the cold acclimation in pea, Champagne a frost tolerant line and Terese, a frost sensitive line, and their recombinant inbred lines (RIL) were studied. RIL frost tolerance was evaluated by a frost damage scale under field as well as controlled conditions. A quantitative trait loci (QTL) approach was used to identify chromosomal regions linked to frost tolerance. The detected QTL explained from 6.5 to 46.5% of the phenotypic variance. Amongst them, those located on linkage groups 5 and 6 were consistent with over all experiments, in field as well as in controlled environments. In order to improve the understanding of the frost tolerance mechanisms, several cold acclimation key characters such as concentration of sugars, electrolyte leakage, osmotic pressure, and activity of RuBisCO were assessed. Some of these physiological QTL colocalised with QTL for frost damage, in particular two raffinose QTL on LG5 and LG6 and one RuBisCO activity QTL on LG6, explaining 8.8 to 27.0% of the phenotypic variance. In addition, protein quantitative loci were mapped; some of them colocalised with frost damage and physiological QTL on LG5 and LG6, explaining 16.0–43.6% of the phenotypic variance. Raffinose metabolism and RuBisCO activity and its effect on photosynthesis might play a major role in cold acclimation of pea.

Communicated by D. Hoisington.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00122-009-1004-7) contains supplementary material, which is available to authorized users.

E. Dumont · C. Vuylsteker · N. Voedts · J.-L. Hilbert · N. Bahrman · B. Delbreil
SADV Laboratoire des Stress Abiotiques et Différenciation des Végétaux cultivés bât SN2, 3ème étage, UMR USTL-INRA 1281, Université des Sciences et Technologies de Lille1, 59655 Villeneuve d'Ascq, France

V. Fontaine · H. Sellier · S. Bodèle · R. Devaux · M. Frise · K. Avia · N. Bahrman · E. Hanocq (✉) · I. Lejeune-Hénaut
SADV Laboratoire des Stress Abiotiques et Différenciation des Végétaux cultivés, UMR USTL-INRA 1281, Estrées-Mons, BP 50136, 80203 Péronne Cedex, France
e-mail: hanocq@mons.inra.fr

## Introduction

A stress is a condition which inhibits the normal functioning of living beings (Mahajan and Tuteja 2005). Higher plants, representing about 99% of the eukaryotic biomass of the planet (Trewavas 2003), might be better adapted to changes than animals, because their immobility resulted in a stronger selection for a greater phenotypic plasticity (Bradshaw 1972). Cold stress is an important factor in agriculture because yield and biomass often largely vary with temperature conditions. Facing frost stress, plants may develop two strategies: frost escape and/or frost tolerance achieved by a preceding cold acclimation. In some plants such as cereals, vernalization corresponds to a frost escape strategy, because it delays the transition from the vegetative to the reproductive phase, the latter being the most sensitive to frost (Fowler et al. 2001). Some species can tolerate negative temperatures if they were previously exposed to a few days of low, but still positive, temperatures. This ability is known as cold acclimation (Levitt 1980). It depends on metabolic changes that lead to plant adaptation. For winter wheat, Baga et al. (2007) have described a quantitative trait loci (QTL) for low-temperature tolerance, located close to

the *vrn-A1* locus (vernalization) and to two CBF (C-repeat binding factor) genes within the confidence intervals. These genes are expressed during cold acclimation (Thomashow 1999). Some of the characters often cited for a relationship with the process of cold acclimation, are the accumulation of sugars (Castonguay et al. 1995), the modification of the plasma membrane composition (Uemura et al. 1995) preventing electrolyte leakage (Dexter et al. 1932), and an increase of the RuBisCO activity (Holaday et al. 1992).

Pea (*Pisum sativum* L.), a crop used as protein source in animal feeding because of its relatively high protein seed content (21–26%) is distinguished from cereals by a high concentration of the essential amino acid lysine. Thus, breeders are interested in an increase of yield via an increase of the production of biomass and/or an extension of the production area. Both of these goals could be realised by the availability of frost resistant peas. Higher biomasses, as well as increased seed numbers, can be naturally achieved by autumn sowing (peas are normally sown in spring) and the resulting longer growth period until the flowering state. But to achieve this goal, they have to resist different winter stresses amongst which frost is particularly important.

The pea forage line Champagne possesses the *Hr* allele (high response to the photoperiod on linkage group 3) which delays flowering initiation (Murfet 1973) under short days. Plants remain in a vegetative stage until a minimum threshold day length of 13.5 h is reached, normally corresponding to the beginning of April. In this way, plants escape frost because the vegetative state is less sensitive to frost (Lejeune-Hénaut et al. 1999). In fact, in a recombinant inbred line population derived from Champagne (*Hr*) × Terese (*hr*), a field winter frost damage (WFD) QTL previously described colocalizes with the *Hr* locus (Lejeune-Hénaut et al. 2008). In addition to this escape ability (*Hr*), other QTL have been also detected. Bourion et al. (2003) have already shown that, in a controlled environment chamber, Champagne also exhibits a better frost tolerance than Terese.

Amongst known cold acclimation characters, measurements were performed for sugar contents, RuBisCO activity, electrolyte leakage, and osmotic pressure in field and controlled environment experiments. De Vienne et al. (1999) have shown that proteins for which PQL (protein quantitative loci, Damerval et al. 1994) colocalize with QTL of interest could be candidate proteins. Our objective was to detect QTL as well as PQL for physiological and phenotypical parameters and to verify a possible linkage with frost damage QTL related to cold acclimation.

In order to eliminate the major effect of the *Hr* locus, a homogeneous subpopulation was used for which all lines possessed the dominant *Hr* allele from Champagne. Two regions on the pea linkage groups 5 and 6 were correlated to the frost damage QTL in the whole population or the *Hr* subpopulation, in the field as well as under controlled conditions. Validations of the subpopulation choice and the controlled environment study were established. Some PQL and physiological and morphological QTL colocalised with the frost damage QTL; the involvement of these characters in cold acclimation was examined.

## Materials and methods

### Plant material

Two pea lines were chosen for their contrasted behaviour against frost: Champagne, a winter forage frost tolerant variety and, Terese, a spring dry frost sensitive variety. Champagne and Terese differ for leaf structure: Champagne is a wild-type pea and Terese an *afila*-type. A population of 164 recombinant inbred lines (RIL) has been derived by SSD (single seed descent) from the cross between Champagne and Terese until the F8 generation. This population named Pop2 has been described by Loridon et al. (2005). It segregates for the *Hr* locus: 78 lines are *Hr and* 86 lines are *hr*.

By delaying the floral initiation, a critical stage, the *Hr* allele confers a better frost tolerance to peas. An earlier study (Lejeune-Hénaut et al. 2008) has described a major QTL of frost damage colocalizing with the *Hr* locus. The strong influence of this gene might limit or even prevent the detection of other QTL having lower effects. We, therefore, only used the subpopulation carrying the *Hr* allele issued from the frost tolerant parent Champagne in order to eliminate its influence. In any case, a recovery of *hr* lines is normally excluded, as they are generally destroyed by frost.

### Growth conditions and samplings

#### *In field experiments*

For the *Hr* subpopulation, plants were sown in two different INRA experimental stations: Clermont-Ferrand Theix (45°70′N, 3°02′E) in the middle of France (mountainous area, 890 m) and Mons (49°53′N, 3°00′E) in northern France. Sowing dates were 27 September 2004 in Clermont-Ferrand and 30 September 2004 in Mons. Each plot was sown in three replicates contained 12 rows of 1.5 m, 20 cm apart, with a density of 20 seeds per row in Clermont-Ferrand and 25 seeds per row in Mons. For biochemical measurements, four samplings (S1–S4) were carried out: three of them were performed during the acclimation period (S1: 26 October 2004, S2: 16 November 2004 and S3: 07 December 2004 in Clermont-Ferrand or S1: 19 October 2004, S2: 22 November 2004 and S3: 13 December 2004 in

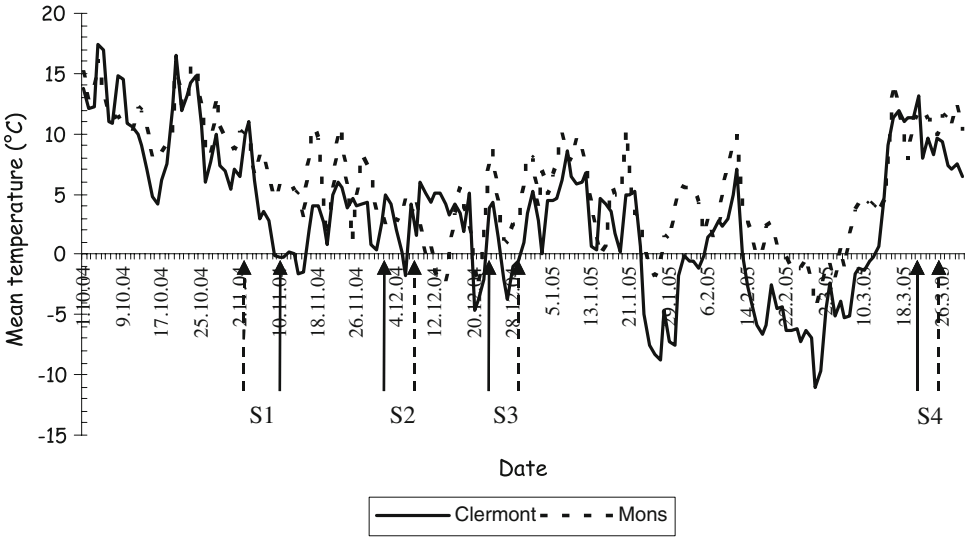


**Fig. 1** Temperature conditions in field experiment. Mean daily temperature during field experiments in Clermont-Ferrand (*continuous line*) and in Mons (*dotted line*). Standard screen air temperature was registered 2 m above the ground. Four samplings were conducted (S1–S4) at each location. *S1* 10.26.2004, *S2* 11.16.2004 and *S3* 12.07.2004 in Clermont-Ferrand and *S1* 10.19.2004, *S2* 11.22.2004 and *S3* 12.13.2004 in Mons, and the fourth at the end of winter *S4* 03.20.2005 in Clermont-Ferrand and *S4* 03.24.2005 in Mons

Mons) and the fourth at the end of winter (S4: 20 March 2005 in Clermont-Ferrand or S4: 24 March 2005 in Mons) as shown on Fig. 1.

*In the controlled environment chamber*

In the controlled environment chamber, plants were grown in 12 cm high isolating trays, each having one hundred holes containing a Jiffy block (Ets Puteaux, France) with one seed. The chamber allows 800 plants to be grown at a time. Plants were grown according to the temperature and day length conditions described in Table 1 with a photosynthetically active radiation varying from 250 µmol $m^{-2}$ $s^{-1}$ (for cold and frost periods) to 300 µmol $m^{-2}$ $s^{-1}$ (for nursery and recovery).

After a 11-day-nursery period at 19°C/12°C (day/night) which allowed the seeds to emerge and to produce up to two fully expanded leaves, the plants were submitted to an 11-day-cold acclimation period at 10°C/2°C. Frost (6°C/−8°C) was then applied during 4 days and plants were finally allowed to recover at 16°C/5°C during 8 days. A first set of experiments was carried out with the whole Pop2 population (78 *Hr* and 86 *hr* lines). Three successive repetitions were realised with four plants per line. Owing to this small number only the frost damage could be scored at the end of these experiments. A second set of experiments was undertaken in order to evaluate the cold acclimation traits on the *Hr* subpopulation. Four experiments were performed successively in the same chamber using the resistant parental line Champagne as a control. Each experiment followed a 3-block design in which each line was represented by 15 plants. At each sampling date, five plants were harvested for each of the three biological repetitions. Before harvesting, the developmental stage of each plant was evaluated. For the *afila* lines, the score 0.25 was attributed at the emergence of the tendrils; 0.50 to the entire spreading of the tendrils; 0.75 to the beginning of the opening of the stipules and 1 to the complete opening of both tendrils and stipules. For the other lines, leaflets were considered instead of the tendrils. Samples were furthermore separated in aerial part and roots. Three samplings (T1–T3) were realised during each experiment: the first one after 10 days of the nursery period (T1), and the second one after 10 days of the cold acclimation period (T2). Five plants remained in the controlled room for an evaluation of their frost damage before being sampled for the other measurements at the end of the recovery period (T3).

**Table 1** Growth conditions for the frost experiment with cold acclimation in the controlled environment chamber

| Period | Nursery | Cold acclimation | Frost | Recovery |
|---|---|---|---|---|
| Number of days | 11 | 11 | 4 | 8 |
| Daylength (h) day/night | 10/14 | 10/14 | 8/16 | 10/14 |
| Temperature (°C) day/night | 19/12 | 10/2 | 6/−8 | 16/5 |

Total length of the experiment was 34 days

Evaluation of frost damage

In the controlled chamber, at the end of the recovery period (T3), seedlings were at the four leaf stage and frost damage (FD) were evaluated through the extent of yellowing and necrotic areas on leaves. Injury were ranged from 0 to 5, 0 corresponded to uninjured plants, one to plants with their first leaf injured at the margin, two for their first and second leaves injured, three for three leaves injured, four for three leaves totally injured and five for the dead plants. The

number of total branches (NTB) and green branches (NGB) at T3 were assessed. The number of green branches is another means to evaluate frost damage. In the field experiment, adult plants were submitted to winter frost and WFD was evaluated at S4 with scores varying from 0 to 5 (Lejeune-Hénaut et al. 2008).

### Electrolyte leakage assay

The electrolyte leakage assay was used to estimate cell damage following frost. Measurements were realised after 8 days of recovery period (T3) in the controlled environment chamber. In the field, the electrolyte leakage assay was performed at S1, S2, and S3, both in Clermont-Ferrand and Mons.

Two stipules per plant were taken on five plants at each repetition. They were placed in 15 mL of deionised water for 24 h and the first measurement was done ($C_0$) with an Ion Check 30 conductivity metre (Radiometer Analytical, France). $C_0$ corresponds to the quantity of ions that diffuse freely through the membranes. The samples were then placed at −80°C for 24 h in order to make the cells burst and to measure the total quantity of ions in the cells ($C_1$). The ratio $(C_0/C_1) \times 100$ corresponded to the percentage of electrolyte leakage (Campos et al. 2003).

### RuBisCO activity

In the field experiment in Clermont-Ferrand (S2), the carboxylase activity of RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) was determined via the oxidation rate of NADH at 340 nm. Plants were ground in liquid nitrogen to a fine powder and 500 µL of an extraction buffer [100 mM bicine pH8.2, 1 mM EDTA, 20 mM $MgCl_2$, 5 mM DTT, 10% (v/v) glycerol and inhibitors cocktail (Sigma)] were added to 500 mg of fresh material to extract soluble proteins. Samples were centrifuged (21,000 g, 4°C, 10 min), the supernatant was transferred to a new tube and centrifuged a second time. The assay was performed in the presence of two enzymes: 3-phosphoglycerate kinase (PGK, EC2.7.2.3) and glyceraldehyde-3-phosphate dehydrogenase (Gal3PDH, EC1.2.1.12). Phosphocreatine and creatine phosphokinase (EC2.7.3.2) were used to regenerate ATP during the assay. Ten microlitres of the supernatant (soluble proteins) were placed in 1 mL of a solution composed of 100 mM bicine KOH pH8, 25 mM $NaHCO_3$, 20 mM $MgCl_2$, 0.25 mM NADH, 3.5 mM ATP, 5 U $mL^{-1}$ 3PGK, 5 U $mL^{-1}$ Gal3PDH, 5 U $mL^{-1}$ creatine phosphokinase and 5 mM phosphocreatine. The extract was incubated 15 min at 30°C. The reaction was initiated by the addition of 0.5 mM RuBP (ribulose-1,5-bisphosphate) (Di Marco and Tricoli 1983; Ward and Keys 1989). The assay was performed at 30°C and the absorbance at 340 nm was measured in a spectrophotometer (CARY50, Varian). Enzyme activities were expressed as nanomoles $CO_2$ (RuBisCO) degraded per second (nanokatal) in relation to total protein unit. Total soluble proteins were determined by the method of Bradford (1976) with BSA (bovine serum albumin) as a standard.

### Osmotic pressure

For the samples from the field experiment in Clermont-Ferrand (S2), the osmotic pressure was estimated by measuring the vapour pressure deficit, which is a linear function of the osmolarity. After grinding the plants, deionised water was added to the powder in order to recover all the soluble substances (ions, soluble sugars…) of the cells. The samples were then centrifuged and 10 µL of the supernatant were put on a disc of blotting paper which was inserted in the chamber of the micro-osmometer (Vapro® 5520, Wescor, UT, USA) (Ball and Oosterhuis 2005).

### Analysis of soluble sugars

#### *Sample preparation*

Analysis of soluble sugars was realised for the samples T1 and T2 in the controlled environment chamber (both aerial parts and roots) and for the sample S2 in Clermont-Ferrand, for the aerial parts only.

Plant material was ground in liquid nitrogen in a mortar to a fine powder. For carbohydrate extraction, 200 mg of this powder were lyophilised and mixed with 1 mL 80% ethanol, vortexed and placed at 60°C for 30 min. After a 15 min centrifugation at 15,000 *g* and 4°C, the supernatant was transferred to a new tube. The pellet was resuspended in 500 µL 80% ethanol for 15 min at 60°C, followed by a second centrifugation under the same conditions. Supernatants were combined. The same extraction procedure was repeated with 250 µL 80% ethanol and the supernatants were gathered and dried in a speed vacuum. The dried residue was resuspended in 150 µL of distilled water for aerial parts and 100 µL for roots, and the aerial extracts were filtered and diluted (1/3) before the HPLC assay (high-performance liquid chromatography).

#### *High-performance liquid chromatography*

A Prominence system (Shimadzu) was used. Separations were performed using an Alltech OA-1000 organic acids column (300 × 6.5 mm) with 0.01 N $H_2SO_4$ mobile phase. The flow was 0.4 mL $min^{-1}$ and the column was heated to 40°C. Runs lasted for 20 min. Peaks corresponding to raffinose, glucose and sucrose were detected with a refractive index detector (RID-10A, Shimadzu). The use of standards

allowed us to determine the concentration of the different metabolites with the LCsolution software (Shimadzu). For each sample, the sugar content was expressed as mg $g^{-1}$ of dry matter.

### Two-dimensional electrophoresis

Protein gels were realised with leaves harvested at T2 in the controlled environment. Stipules, leaflets and tendrils were harvested for wild-type leaf pea and stipules and tendrils for *afila*-type leaf pea. Plant material was ground in liquid nitrogen in a mortar to a fine powder and the extraction protocol was performed according to Dupire et al. (1999). Immobilized pH gradient strips (17 cm ReadyStrip IPG Strips, BioRad) with a linear pH gradient from 4 to 7 were used with 80 µg of proteins. After active rehydration at 20°C during 16 h at 50 V, the IEF was run until 35,000 V h were reached. The second dimension was realised in a 12.5% polyacrylamide gel at 350 V using an Ettan DALTtwelve (GE Healthcare). Protein spots were silver-stained according to Blum et al. (1987). Gels were compared using ImageMaster 2D Platinum (GE Healthcare) and spots were quantified in each gel. Seventy spots were identified by nanoLC-MS/MS.

### Statistical and QTL and PQL analyses

Statistical analyses were done with the SAS package version 8 (SAS Institute Inc. 1999). The GLM (general linear model) procedure was used for the analysis of variance, the GLM procedure with manova for the genetic correlations and the univariate procedure to test the normality and to detect abnormal residues. The data being out of the Henry's line in the normal probability plot were verified and measured once again if possible. If necessary, they were eliminated from the data-set. For each variable, the adjusted means were calculated and used in the QTL and PQL analyses.

Quantitative trait loci and PQL analyses were performed with Windows QTL Cartographer version 2.5 (Wang et al. 2007). The genetic map has already been used for QTL mapping in Lejeune-Hénaut et al. (2008), and the genetic data are detailed in Aubert et al. (2006) and Loridon et al. (2005). The genetic map comprises 1,491 cM with 258 markers on seven linkage groups (LG). QTL mapping was realised by composite interval mapping (model 6) with the forward and backward regression method for covariate selection (method 3). The LOD threshold was determined for each variable after running five times a 1,000 permutation test ($\alpha = 0.05$) and calculating the mean LOD for the five repetitions. In addition, the part of the phenotypic variance ($R^2$) explained by the QTL and the corresponding additive effect (a) were calculated for each QTL.

## Results

### Frost damage QTL

The evaluation of the *Hr* subpopulation in the field in Mons and Clermont-Ferrand resulted in the detection of six winter frost damage (WFD) QTL: on LG1 (WFDcle.a), LG4 (WFDmon.a), LG5 (WFDcle.b and WFDmon.b) and LG6 (WFDcle.c and WFDmon.c), as shown in Fig. 2 and Table 2. Based on the scale 0–5, the WFD varied from 0.5 to 4.5 for Mons, and from 0.0 to 5.0 for Clermont-Ferrand.

The QTL mapping for frost damage on the 164 RIL in a controlled cold-acclimated experiment led to the detection of three frost damage (FD) QTL on LG3 (FD164.a), LG5 (FD164.b) and LG6 (FD164.c) (Fig. 2; Table 2). The FD values for this experiment ranged from 0.03 to 2.39. For the *Hr* subpopulation, three FD QTL were detected: on LG5 (FD.a) and LG6 (FD.b) as for the whole population and another one on LG7 (FD.c) (Fig. 2; Table 2). The FD varied from 0.06 to 3.

Linkage group 5 and LG6 QTL were very consistent between the field and the controlled chamber conditions. LOD values ranged from 3.54 (FD.c) to 17.31 (WFDcle.c) and phenotypic variance explanation ($R^2$) from 8.0 (FD164.c) to 46.5% (WFDcle.c). The favourable alleles for winter frost damage on LG5 and LG6 were those given by the Champagne parent.

### Colocalization of frost damage QTL with QTL for soluble sugars concentrations and other physiological measurements in the aerial parts

Two QTL (RafCleS2.c and GlcT2.b) were detected on LG5 between the markers AA475 and AA163.2, the first being a QTL for the concentration of raffinose at S2 in the field and the second related to the concentration of glucose at T2 in the controlled environment chamber (Fig. 3). For example, the genetic correlation between frost damage and raffinose concentration is 0.78 ($\alpha < 0.0001$) with minimum and maximum values of 0.60 and 7.89 (Table 3). On linkage group 6, six QTL were detected between the markers AD159 and G04.950. For the controlled chamber plants, one QTL for the concentration of raffinose was detected after 10 days of cold acclimation (RafT2.b). The other QTL were detected from field data during the cold acclimation period: the concentration of raffinose at S2 (RafCleS2.d), the RuBisCO activity at S2 (RuBisCOcleS2), and the electrolyte leakage at S1 and S2 (LeakCleS1 and LeakCleS2) for Clermont-Ferrand, and S3 for Mons (LeakMonS3). All these QTL colocalised with the QTL of frost damage in the LG5 and LG6 (Fig. 2). The LOD values ranged from 3.78 (GlcT2.b) to 8.31 (RafCleS2.d) and $R^2$ from 14.3 (RafT2.b) to 25.2% (RafCleS2.d).

In roots no QTL for sugar concentrations were found to colocalize with QTL for frost damage, whereas in leaves no

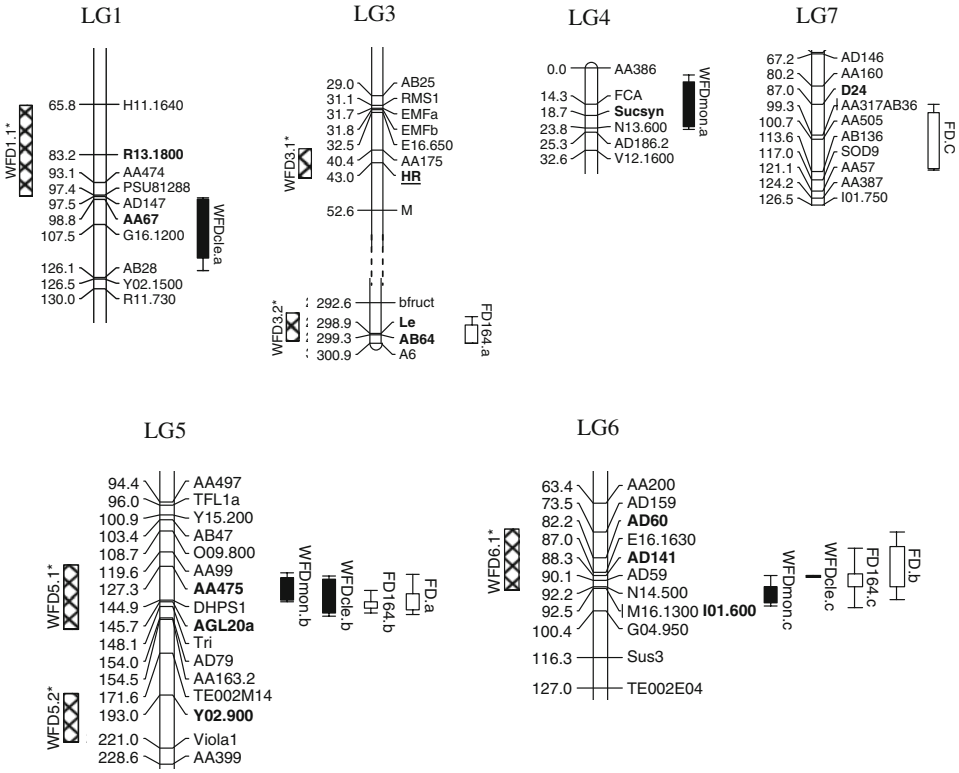


**Fig. 2** QTL of winter frost damage (WFD) and frost damage (FD). Each field QTL (*black box*) is identified by WFD and the location abbreviation (*mon* Mons, *cle* Clermont-Ferrand). For the controlled chamber experiment (*white box*), QTL were named only FD for the *Hr* subpopulation or followed by 164 for the whole population. Confidence intervals are defined as 1 (*box*) and 2 (*whisker*) units decrease in LOD. The closest markers to the peaks are in *bold*. On the *left*, the QTL detected by Lejeune-Hénaut et al. (2008) in field with the whole population (*asterisks* name used in the published article) were figured with *hatched boxes*. Only major QTL regions are shown

**Table 2** Characteristics of the QTL obtained in the entire or sub F8 RIL population derived from Champagne × Terese

| QTL identification | Population | Condition | Linkage group | Closest marker | LOD | $R^2$ (%) | *a* | Phenotypical values | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Minimal | Maximal |
| WFDcle.a | Hr | Clermont-Ferrand | 1 | AA67 | 4.74 | 6.53 | 0.33 | 0.00 | 5.00 |
| WFDcle.b | | | 5 | *AGL20a* | 7.31 | 16.22 | 0.53 | | |
| WFDcle.c | | | 6 | AD141 | 17.31 | 46.46 | 0.88 | | |
| WFDmon.a | Hr | Mons | 4 | *SucSyn* | 7.36 | 14.10 | 0.31 | 0.50 | 4.50 |
| WFDmon.b | | | 5 | AA475 | 7.71 | 17.01 | 0.33 | | |
| WFDmon.c | | | 6 | I01.600 | 10.66 | 24.71 | 0.40 | | |
| FD164.a | Whole | Controlled chamber | 3 | AB64 | 5.91 | 10.03 | 0.12 | 0.03 | 2.39 |
| FD164.b | | | 5 | *AGL20a* | 8.02 | 16.56 | 0.16 | | |
| FD164.c | | | 6 | AD141 | 4.19 | 8.06 | 0.11 | | |
| FD.a | Hr | Controlled chamber | 5 | *AGL20a* | 4.55 | 20.81 | 0.30 | 0.06 | 3.00 |
| FD.b | | | 6 | AD60 | 3.66 | 15.72 | 0.24 | | |
| FD.c | | | 7 | D24 | 3.54 | 14.32 | 0.24 | | |

*LOD* logarithm of odds, *WFD* field winter frost damage, *cle* Clermont-Ferrand, *mon* Mons, *FD* frost damage in controlled environment chamber, $R^2$ phenotypic variance, *a* additive allelic value of Terese

osmotic pressure QTL could be detected. The data of additional QTL are shown in Figs S1 and S2.

## Colocalization of frost damage QTL with QTL for plant development

In the cold-acclimated experiment, one QTL for NTB (NTB.a) and another for NGB (NGB) were detected on LG5 (Fig. 3). This was in agreement with the correlations in cold-acclimated experiment between frost damage and NTB or NGB (Table 3). In addition, two QTL for seedling stage were detected at T1 (Stage T1) and T2 (Stage T2) in the same linkage group 5 region. The location might be independent of the physiological stage of the seedlings. On LG6, only a QTL for NTB was detected (NTB.b). LOD ranged from 3.66 (NTB.b) to 6.24 (NGB) and $R^2$ from 10.8 (NTB.b) to 27.0% (Stage T2).

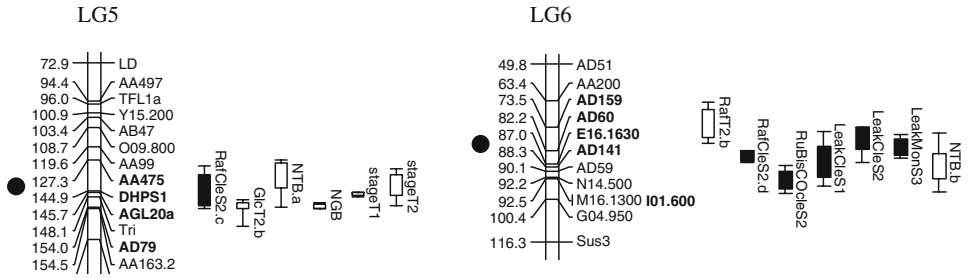


**Fig. 3** Metabolites, physiological and morphological QTL on LG5 and LG6. Field data QTL (*black box*) in Clermont-Ferrand or Mons are identified by cle or mon, respectively. Controlled chamber QTL are represented in *white boxes*. *T1* harvest after 10 days of nursery, *T2* harvest after 10 days of cold acclimation, *S2* harvest during cold acclimation in field, *Leak* electrolyte leakage, *Raf* concentration of raffinose, *Glc* concentration of glucose, *Suc* concentration of sucrose, *stage* number of leaves on the plants, *NTB* total number of branches, *NGB* number of green branches after recovery period, *RuBisCO* activity of RuBisCO. When the term root is added, the QTL was detected with root data. *black circle*, on the *left*, indicates the position of frost damage QTL represented in Fig. 2. Only QTL regions of interest are shown

**Table 3** Characteristics of metabolites, physiological and morphological QTL obtained in the sub F8 RIL population derived from Champagne × Terese

| QTL identification | Condition | Linkage group | Closest marker | LOD | $R^2$ (%) | *a* | Phenotypical values | | Genetic correlation with WFD or FD |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Minimal | Maximal | |
| RafCleS2.c | Clermont-Ferrand | 5 | *DHPS1* | 4.51 | 8.8 | −0.53 | 0.60 | 7.89 | 0.78*** |
| RafCleS2.d | | 6 | AD60 | 8.31 | 25.2 | −0.93 | | | |
| RafT2.b | Controlled chamber | 6 | AD159 | 4.71 | 14.3 | −0.04 | 0.01 | 0.54 | 0.36** |
| GlcT2.b | Controlled chamber | 5 | AD79 | 3.78 | 12.3 | −0.57 | 1.54 | 7.45 | n.s. |
| LeakCleS1 | Clermont-Ferrand | 6 | E16.1630 | 3.92 | 14.8 | 3.15 | 5.15 | 46.83 | 0.44*** |
| LeakCleS2 | | 6 | AD159 | 4.73 | 23.4 | 6.91 | 5.15 | 65.32 | 0.49*** |
| LeakMonS3 | Mons | 6 | AD159 | 5.97 | 24.6 | 11.56 | 5.95 | 97.93 | – |
| RuBisCOcleS2 | Clermont-Ferrand | 6 | I01.600 | 4.89 | 15.8 | −0.16 | 3.29 | 5.70 | 0.56*** |
| NTB.a | Controlled chamber | 5 | AA475 | 4.80 | 21.8 | −0.42 | 0.26 | 4.20 | 0.43*** |
| NTB.b | | 6 | AD141 | 3.66 | 10.8 | −0.29 | | | |
| NGB | Controlled chamber | 5 | AD79 | 6.24 | 26.3 | −0.45 | 0 | 3.90 | 0.71*** |
| StageT1 | Controlled chamber | 5 | *AGL20a* | 5.05 | 15.5 | −0.14 | 0.52 | 2.12 | |
| StageT2 | | 5 | AA475 | 4.90 | 27.0 | −0.17 | 2.29 | 3.51 | n.s. |

*LOD* logarithm of odds, $R^2$ phenotypic variance, *a* additive allelic value of Terese, *cle* Clermont-Ferrand, *mon* Mons, *Raf* concentration of raffinose, *Glc* concentration of glucose, *Suc* concentration of sucrose, *Leak* electrolyte leakage, *RuBisCO* activity of RuBisCO, *NTB* total number of branches, *NGB* number of green branches after recovery period, *stage* number of leaves on the plants, T1 and T2: harvest after 10 days of nursery and after 10 days of cold acclimation in controlled environment chamber respectively, *S1–S3* harvest during cold acclimation in field, *root* QTL detected with data of roots in the controlled environment chamber

Genetic correlation between WFD (or FD) and other parameters, level of significance (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, *n.s.* not significant)

### Colocalization of frost damage QTL with PQL

Amongst 264 spots detected in 2D gels, 191 spots allowed for the mapping of 315 PQL across seven linkage groups (Fig. S3). LOD ranged from 2.83 (L264, GL2) to 18.80 (L269, GL3) and $R^2$ from 12.1 (L173, GL3) to 63.0% (L606: OEE1, GL3) (Table 4). Twenty-two PQL were colocalised with frost damage QTL on LG5 (Fig. 4). Amongst these PQL, four spots were identified: fructose bisphosphate aldolase (L522), triose phosphate isomerase (L680), chaperonin 21 (L768), and RuBisCO small subunit (L925). On LG6 five PQL colocalized with the frost damage QTL; one of them was related to plastocyanin (L917).

## Discussion

Several QTL described by Lejeune-Hénaut et al. (2008) in a field experiment with the Pop2 lines were used to verify that our detection of QTL was relevant. In our field study, two QTL were detected on LG5 (Mons and Clermont-Ferrand) and two on LG6 (Mons and Clermont-Ferrand). These QTL were at the same position (except for LG3) as those found with the entire population by Lejeune-Hénaut et al. (2008). Therefore, the use of the *Hr* subpopulation allowed us to eliminate the influence of *Hr* (no colocalized QTL) and to observe the intrinsic frost tolerance obtained by cold acclimation.

**Table 4** Characteristics of PQL detected on linkage groups LG5 and LG6 derived from the sub F8 RIL population derived from Champagne × Terese

| PQL identification | Linkage group | Closest marker | LOD | $R^2$ (%) | *a* |
|---|---|---|---|---|---|
| L132 | 5 | *Tri* | 5.44 | 31.1 | −0.02 |
| L191 | 5 | *Tri* | 4.73 | 27.6 | −0.08 |
| L257 | 5 | *Tri* | 4.20 | 25.4 | −0.06 |
| L294 | 5 | AD79 | 4.12 | 29.0 | −0.06 |
| L305 | 5 | AA163.2 | 4.53 | 23.0 | −0.05 |
| L339 | 5 | Tri | 7.17 | 35.0 | −0.07 |
| L365 | 5 | AA99 | 5.27 | 32.1 | −0.04 |
| L664 | 5 | Tri | 4.44 | 29.0 | −0.01 |
| L680 | 5 | *Tri* | 6.19 | 26.0 | −0.01 |
| L727 | 5 | AD79 | 4.02 | 21.3 | −0.11 |
| L732 | 5 | *Tri* | 5.16 | 31.6 | −0.10 |
| L733 | 5 | *Tri* | 5.02 | 30.3 | −0.17 |
| L749 | 5 | Tri | 3.50 | 16.0 | −0.04 |
| L768 | 5 | AA475 | 5.14 | 30.9 | −0.16 |
| L779 | 5 | Tri | 6.03 | 28.9 | −0.09 |
| L873 | 5 | AA475 | 3.32 | 25.2 | 0.25 |
| L905 | 5 | DHPS1 | 6.54 | 26.5 | −0.09 |
| L925 | 5 | AGL20a | 7.61 | 38.6 | −0.49 |
| L1024 | 5 | *Tri* | 9.32 | 35.2 | −0.01 |
| L1071 | 5 | *Tri* | 4.99 | 27.4 | −0.03 |
| L1185b | 5 | AD79 | 5.68 | 28.3 | −0.08 |
| L349b | 6 | AD59 | 4.77 | 27.2 | 0.01 |
| L733 | 6 | AD141 | 7.83 | 34.7 | 0.20 |
| L779 | 6 | AD59 | 5.69 | 36.7 | 0.10 |
| L802 | 6 | AD59 | 4.72 | 23.1 | 0.05 |
| L917 | 6 | E16.1630 | 5.31 | 38.6 | −1.06 |

LOD logarithm of odds, $R^2$ phenotypic variance, *a* additive allelic value of Terese


**Fig. 4** PQL colocalised with frost damage QTL on LG5 and 6. PQL were identified by the spot name (*L* leaf). *Black circle*, on the *left*, indicates the position of frost damage QTL represented in Fig. 2. Only QTL regions of interest are shown

Both analyses in field and controlled environment showed the same QTL on LG5 and LG6. The reliability of the detection for these QTL did not depend on the number of individuals since the results were consistent between the whole population (164 lines) and the *Hr* subpopulation (78 lines). These QTL probably depend on mechanisms

independent of the vegetative developmental stage of the plants, as described for pathogenesis-related QTL (Prioul et al. 2004). Our study was focused on linkage groups 5 and 6.

## Involvement of physiological parameters and some proteins during cold acclimation

We chose to analyse soluble sugars because of their involvement in cold acclimation as osmoprotectants (Levitt 1980) and their detection in different pea lines (Bourion et al. 2003). In the field, a QTL for raffinose concentration was observed during the cold acclimation on both LG5 and LG6, in the controlled chamber only on LG6. They all colocalize with the QTL of frost damage. This triholoside certainly plays a role in cold acclimation as in other species (*Arabidopsis*: Ristic and Ashworth 1993; *Lonicera*: Imanishi et al. 1998; poplar: Renaut et al. 2004). Raffinose is the soluble sugar having been shown to have a major effect during cold acclimation for different species like poplar, with a correlation of −0.96 between raffinose and LT50 (temperature inducing 50% of injured cells) (Renaut et al. 2004) but its effect is also much debated (Taji et al. 2002; Zuther et al. 2004). Even if the role of raffinose is not yet well established, our results for pea strongly suggest its implication in frost tolerance.

A QTL for glucose at T2 in the controlled environment chamber was detected on LG5. Additional QTL for glucose, sucrose or raffinose were detected but did not colocalize with those for frost damage. In 3-week-old seedlings of *Arabidopsis*, Wanner and Junttila (1999) have observed that sucrose, glucose and fructose are accumulated within a few hours after the transfer of plants to 1°C and during the 5 days of cold acclimation under different photoperiods. The content of these sugars increased in our experiment under cold acclimation, but no correlation with frost damage could be observed for sucrose and fructose.

On LG5, a QTL for branching traits was detected. Cold-acclimated seedlings having more branches (NTB) and green branches (NGB) were more resistant to frost. This was in accordance with the fact that pea seedlings must have a minimum number of leaves (1 or 2) to resist frost (Lejeune-Hénaut et al. 2004). Hence, the number of green branches represents an indication for the degree of frost tolerance in resistant plants.

Two hundred and sixty-four protein spots were detected and 70 proteins amongst them identified. Amongst the 22 PQL having been mapped on LG5, four could be attributed to functions: triose phosphate isomerase (L680) and fructose bisphosphate aldolase (L522) are involved in glycolysis and the RuBisCO small subunit (L925) in photosynthesis. These two primary metabolisms are linked with sugars and these three PQL colocalised with glucose concentration QTL. The fourth PQL corresponded to chaperonin 21. This protein binds to cpn60 which is indispensable for the assembly of RuBisCO (Yong et al. 2006). Chaperonin 21 shows homologies with *E. coli* GroES (Baneyx et al. 1995) involved in the folding of several proteins. The chaperonin 21 might promote the protein folding in order to maintain metabolism during the cold acclimation. PQL can colocalize or not with their structural genes (de Vienne et al. 1999). The density of the actual genetic map of pea (one marker every 5.7 cM corresponding to 16,820 kb: 1 cM = 2,951 kb) and the relatively low number of identified genes does not yet allow us to draw conclusions for possible colocalizations.

For a given QTL, many genes could be found within the confidence interval focusing on markers with a known function localised on the genetic map. On LG5, QTL were located in the same region as the *Tri* locus (trypsin inhibitor activity), *AGL20a* (Agamous-like 20a = SOC1 suppressor of overexpression of CONSTANS) and *DHPS1* (dihydrodipicolinate synthase). With respect to the *Tri* locus, Page et al. (2002) have shown that trypsin inhibitor activity decreases the nutritional value of pea seeds. Therefore, caution should be exercised in the use of this QTL in a breeding programme for improvement of frost tolerance in pea. Besides, it will be difficult to consider *Tri* as a good candidate gene for our study.

The gene *agl20a* is an activator of flowering (Lee et al. 2000) and is regulated by photoperiod, vernalization, and autonomous floral induction pathways. Ohto et al. (2001) have demonstrated that sugars have a negative effect on the floral transition: sucrose, glucose, fructose and galactose, can delay flowering time. As raffinose can be hydrolysed to sucrose and galactose by α-galactosidase, it might be interesting to measure the activity of this enzyme during cold acclimation. Raffinose accumulation and its degradation in sucrose and galactose could possibly delay floral initiation which would result in an increase in resistance to cold temperatures since the reproductive state is the most sensitive to frost (Fowler et al. 2001). The observed increase in raffinose content in our seedlings during cold acclimation is consistent with this hypothesis.

*DHPS1* is a key enzyme for amino acid biosynthesis, in particular lysine biosynthesis via the DAP (diaminopimelic acid) pathway. Amongst the amino acids, proline might be an osmoprotectant in plants. According to Trischuk et al. (2006), thylakoid membranes are protected from frost inactivation by exogenous proline, arginine, threonine and lysine. Another hypothesis was that the higher amino acid concentrations are caused by the need to enhance protein synthesis.

A QTL for the activity of RuBisCO colocalised with a QTL for frost damage on LG6. RuBisCO is the key enzyme of the Calvin cycle that allows the fixation of $CO_2$ during the photosynthesis. Holaday et al. (1992) have observed

that the activity of RuBisCO in spinach increases by 20% after 10 days at 10°C. In winter rye, the photosynthetic capacity of cold hardening leaves at 5°C increases and is correlated with a threefold increase in RuBisCO activity (Hurry et al. 1994). The same phenomena have been observed for wheat and rape at 5°C (Hurry et al. 1995).

Quantitative trait loci for electrolyte leakage measured in field experiments were detected on LG6. This measurement gives information on cell damage (Dexter et al. 1932). If it is high, damage in the plasma membrane are significant so the plant is not able to adapt itself to frost. In the field, a correlation between electrolyte leakage and the score for frost damage of 0.44 ($\alpha < 1‰$) was detected. Electrolyte leakage is the physical measurement of a phenomenon (membrane damage), which could be used to screen more quantitatively and objectively for tolerance to frost damage.

Markers under the QTL on the LG6 did not provide candidate genes, but with the map from Aubert et al. (2006), different markers with known function were found: *Gbsts2* (granule-bound starch synthase II), *GA20ox* (Gibberellin20 oxidase) and *RNAhel* (RNA helicase).

Up till now, 70 out of 264 protein spots have been identified. Consequently, many of the observed PQL are not yet informative, but the ongoing identification process should enable us to further elucidate the mechanisms of cold acclimation and frost resistance.

To our knowledge, our study is the first one to focus on frost damage QTL (abiotic stress) in pea under both field and controlled conditions. Gusta et al. (2005) noticed some difficulties in this kind of comparison, namely the differences in plant age and growth conditions. Our study was conducted under field and controlled conditions on an entire population (*Hr* and *hr* lines, 164 lines) and also on the *Hr* subset of the population. QTL found on LG5 and LG6 were consistent in all experiments, hence independent of plant age (seedling or adult). Using the entire population under field conditions, Lejeune-Hénaut et al. (2008) have detected a major QTL around the *Hr* locus that we chose to eliminate by working on the homogenous *Hr* subpopulation. Our results enable us to conclude that the QTL on LG5 and LG6 are linked to cold acclimation and not to frost escape conferred by the *Hr* allele. The second new was approach involved the use of physiological data measured during cold acclimation to detect QTL. The increase in RuBisCO activity seems to have an important impact on cold acclimation in pea, as it has been shown for others species including spinach (Holaday et al. 1992) and rye (Hurry et al. 1994). Raffinose is a soluble sugar which seemed to have a major effect during the cold acclimation for different species. Although the mechanism of its action has not been well established, it seems to be linked to frost tolerance in pea. The observed electrolyte leakage might represent the major damage caused by frost. The consistency of the results for field and controlled growth conditions represents a major finding of our study. The search and validation of candidate genes will be performed by dissecting chromosomal regions of interest. Experiments will also be expanded to a higher number of segregating populations in different varieties with the aim to confirm the localised regions in the linkage groups.

**Acknowledgments** We thank Dr J. Le Gouis for pre-reviewing this paper and M. Morchen for her helpful comments on the manuscript. Thanks also to anonymous reviewers for their valuable recommendations. Thanks to F. Batifoy and B. Debote for technical assistance in the plant material at INRA experimental station of Clermont-Ferrand Theix, and to J.J. Stempniak, J.F. Hû, D. Rabier, M. Thomas, F. Depta and B. Decaux from INRA station Mons. This work is part of the PhD thesis of Estelle Dumont, financially supported by a grant from the French Ministère de la Jeunesse, de l'Education Nationale et de la Recherche. This work was also supported by Genoplante (project GOP-PEAD2) and the region Picardie (project ProgenFroid).

## References

Aubert G, Morin J, Jacquin F, Loridon K, Quillet MC, 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

Baga M, Chodaparambil SV, Limin AE, Pecar M, Fowler DB, Chibbar RN (2007) Identification of quantitative trait loci and associated candidate genes for low-temperature tolerance in cold-hardy winter wheat. Funct Integr Genomics 7(1):53–68

Ball RA, Oosterhuis DM (2005) Measurement of root and leaf osmotic potential using the vapor-pressure osmometer. Env Exp Bot 53(1):77–84

Baneyx F, Bertsch U, Kalbach CE, van der Vies SM, Soll J, Gatenby AA (1995) Spinach chloroplast cpn21 co-chaperonin possesses two functional domains fused together in a toroidal structure and exhibits nucleotide-dependent binding to plastid chaperonin 60. J Biol Chem 270(18):10695–10702

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93–99

Bourion V, Lejeune-Hénaut I, Munier-Jolain N, Salon C (2003) Cold acclimation of winter and spring peas: carbon partitioning as affected by light intensity. Eur J Agron 19:535–548

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bradshaw AD (1972) Some of the evolutionary consequences of being a plant. Evol Biol 5:25–47

Campos PS, Quartin V, Ramalho JC, Nunes MA (2003) Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. J Plant Physiol 160(3):283–292

Castonguay Y, Nadeau P, Lechasseur P, Chouinard L (1995) Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winterhardiness. Crop Sci 35:509–516

Damerval C, Maurice A, Josse JM, de Vienne D (1994) Quantitative trait loci underlying gene product variation: a novel perspective for analyzing regulation of genome expression. Genetics 137:289–301

de Vienne D, Leonardi A, Damerval C, Zivy M (1999) Genetics of proteome variation for QTL characterization: application to drought-stress responses in maize. J Exp Bot 50(332):303–309

Dexter ST, Tottingham WE, Graber LF (1932) Investigations of the hardiness of plants by measurement of electrical conductivity. Plant Physiol 7(1):63–78

Di Marco DI, Tricoli D (1983) RuBP carboxylase determination by enzymatic estimation of D-3-PGA formed. Photosynth Res 4:145–149

Dupire L, Décout E, Vasseur J, Delbreil B (1999) Histological and 2-D protein patterns comparisons between a wild type and a somatic embryogenic mutant of *Asparagus officinalis* L. Plant Sci 147:9–17

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

Gusta LV, Trischuk R, Weiser CJ (2005) Plant cold acclimation: the role of abscisic acid. J Plant Growth Regul 24:308–318

Holaday AS, Martindale W, Alred R, Brooks AL, Leegood RC (1992) Changes in activities of enzymes of carbon metabolism in leaves during exposure of plants to low temperature. Plant Physiol 98:1105–1114

Hurry V, Malmberg G, Gardeström P, Oquist G (1994) Effects of a short-term shift to low temperature and of long-term cold hardening on photosynthesis and ribulose-1, 5-bisphosphate carboxylase/oxygenase and sucrose phosphate synthase activity in leaves of winter rye (*Secale cereale* L.). Plant Physiol 106:983–990

Hurry V, Strand A, Tobiaeson M, Gardeström P, Oquist G (1995) Cold hardening of spring and winter wheat and rape results in differential effects on growth, carbon metabolism and carbohydrate content. Plant Physiol 109:697–706

Imanishi HT, Suzuki T, Masuda K, Harada T (1998) Accumulation of raffinose and stachyose in shoot apices of *Lonicera caerulea* L. during cold acclimation. Sci Horti Amsterdam 72:255–263

Lee H, Suh SS, Park E, Cho E, Ahn JH, Kim SG, Lee JS, Kwon YM, Lee I (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in *Arabidopsis*. Gen Dev 14:2366–2376

Lejeune-Hénaut I, Bourion V, Etévé 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

Lejeune-Hénaut I, Delbreil B, Devaux R, Guilioni L (2004) Températures froides et fonctionnement d'un couvert de pois. In: Munier-Jolain N, Chaillet I, Lecoeur J, Jeuffroy MH (eds) Agrophysiologie du pois protéagineux. INRA-Arvalis, Paris, pp 184–195

Lejeune-Hénaut I, Hanocq E, Béthencourt L, Fontaine V, Delbreil B, Morin J, Petit A, Devaux R, Boilleau M, Stempniak JJ, Thomas M, Lainé AL, Foucher F, Baranger A, Burstin J, Rameau C, Giauffret C (2008) The flowering locus *Hr* colocalizes with a major QTL affecting winter frost tolerance in *Pisum sativum* L. Theor Appl Genet 116(8):1105–1116

Levitt J (1980) Responses of plants to environmental stresses. Academic Press, New York

Loridon K, McPhee K, Morin J, Dubreuil P, Pilet-Nayel ML, 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

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 444:139–158

Murfet IC (1973) Flowering in *Pisum. Hr*, a gene for high response to photoperiod. Heredity 31(2):157–164

Ohto MA, Onai K, Furukawa Y, Aoki E, Araki T, Nakamura K (2001) Effects of sugar on vegetative development and floral transition in *Arabidopsis*. Plant Physiol 127:252–261

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

Prioul S, Frankewitz A, Deniot G, Morin G, Baranger A (2004) Mapping of quantitative trait loci for partial resistance to *Mycosphaerella pinodes* in pea (*Pisum sativum* L.), at the seedling and adult plant stages. Theor Appl Genet 108:1322–1334

Renaut J, Lutts S, Hoffmann L, Hausman JF (2004) Responses of poplar to chilling temperatures: proteomic and physiological aspects. Plant Biol 6:81–90

Ristic Z, Ashworth EN (1993) Changes in leaf ultrastructure and carbohydrates in *Arabidopsis thaliana* L. (Heyn) cv. Columbia during rapid cold acclimation. Protoplasma 172:111–123

SAS User's guide (1999) ver. 8.2, Cary, NC, SAS Institute

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J 29:417–426

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571–599

Trewavas A (2003) Aspects of plant intelligence. Ann Bot 92:1–20

Trischuk RG, Schilling BS, Wisniewski M, Gusta LV (2006) Freezing stress: systems biology to study cold tolerance. In: Rao KVM, Raghavendra AS, Reddy KJ (eds) Physiology and molecular biology of stress tolerance in plants. Springer, Berlin

Uemura M, Joseph RA, Steponkus PL (1995) Cold acclimation of *Arabidopsis thaliana*: effect on plasma membrane lipid composition and freeze-induced lesions. Plant Physiol 109:15–30

Wang S, Basten CJ, Zeng ZB (2007) Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC. http://statgen.ncsu.edu/qtlcart/WQTLCart.htm

Wanner LA, Junttila O (1999) Cold-induced freezing tolerance in *Arabidopsis*. Plant Physiol 120:391–399

Ward DA, Keys AJ (1989) A comparison between the coupled spectrophotometric and the uncoupled radiometric assays for RuBP carboxylase. Photosynth Res 22:167–171

Yong ZH, Chen GY, Shi JN, Xu DQ (2006) *In vitro* reassembly of tobacco ribulose-1, 5-bisphosphate carboxylase/oxygenase from fully denatured subunits. Acta Biochem Biophys Sin 38:737–745

Zuther E, Büchel K, Hundertmark M, Stitt M, Hincha DK, Heyer AG (2004) The role of raffinose in the cold acclimation response of *Arabidopsis thaliana*. FEBS Lett 576:169–173