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ORIGINAL PAPER

QTL analysis of frost damage in pea suggests different mechanisms involved in frost tolerance

Anthony Klein · Hervé Houtin · Céline Rond · Pascal Marget · Françoise Jacquin · Karen Boucherot · Myriam Huart · Nathalie Rivière · Gilles Boutet · Isabelle Lejeune-Hénaut · Judith Burstin

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Abstract
Key message **Avoidance mechanisms and intrinsic resistance are complementary strategies to improve winter frost tolerance and yield potential in field pea.**
Abstract The development of the winter pea crop represents a major challenge to expand plant protein production in temperate areas. Breeding winter cultivars requires the combination of freezing tolerance as well as high seed productivity and quality. In this context, we investigated the genetic determinism of winter frost tolerance and assessed its genetic relationship with yield and developmental traits. Using a newly identified source of frost resistance, we developed a population of recombinant inbred lines and evaluated it in six environments in Dijon and Clermont-Ferrand between 2005 and 2010. We developed a genetic map comprising 679 markers distributed over seven linkage groups and covering 947.1 cM. One hundred sixty-one quantitative trait loci (QTL) explaining 9–71 % of the phenotypic variation were detected across the six environments for all traits measured. Two clusters of QTL mapped on the linkage groups III and one cluster on LGVI reveal the genetic links between phenology, morphology, yield-related traits and frost tolerance in winter pea. QTL clusters on LGIII highlighted major developmental gene loci (*Hr* and *Le*) and the QTL cluster on LGVI explained up to 71 % of the winter frost damage variation. This suggests that a specific architecture and flowering ideotype defines frost tolerance in winter pea. However, two consistent frost tolerance QTL on LGV were independent of phenology and morphology traits, showing that different protective mechanisms are involved in frost tolerance. Finally, these results suggest that frost tolerance can be bred independently to seed productivity and quality.

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A. Klein (✉) · H. Houtin · C. Rond · P. Marget · F. Jacquin · K. Boucherot · M. Huart · J. Burstin
INRA, UMR 1347 Agroécologie, BP 86510, 21000 Dijon Cedex, France
e-mail: anthony.klein@dijon.inra.fr

N. Rivière
Biogemma, Route d'Ennezat, CS 90126, 63720 Chappes, France

G. Boutet
INRA, UMR 1349 IGEPP, BP 35327, 35653 Le Rheu Cedex, France

I. Lejeune-Hénaut
INRA, UMR 1281 SADV, Estrées-Mons, BP 50136, 80203 Péronne Cedex, France

Introduction

In Europe, insufficient plant protein production is a long-standing problem. Increasing home-grown legume seed production is needed to satisfy an increasing protein rich raw material demand and enhance agriculture sustainability locally. Breeding efforts to improve grain yield and seed protein content have proven difficult. Quantitative trait loci (QTL) for yield, yield components, seed protein content and developmental traits were identified in spring pea (Krajewski et al. 2011; Burstin et al. 2007; Timmerman-Vaughan et al. 2005; Tar'an et al. 2004). Traits are complex, integrating all the processes occurring during the plant growth cycle and being highly influenced by environmental conditions. Today, the

development of winter pea cultivars should be a way to improve yield potential and stability through a longer life cycle and hence increase crop competitiveness. Autumn sowing allows for a higher biomass production as well as the avoidance of drought and heat stresses of late spring (Stoddard et al. 2006). However, winter hardy cultivars able to survive low freezing temperatures and other associated winter stresses are still needed. Freezing temperature is a major environmental limitation to the distribution of plant wild species and to crop productivity (Levitt, 1980). In pea (Lejeune-Hénaut et al. 2008) as in other plant species (Avia et al. 2013; Dhillon et al. 2010; Mikkelsen and Thomashow 2009; Franklin and Whitelam 2007) this complex trait may involve both intrinsic antifreeze and avoidance mechanisms such as photoperiod sensitivity or vernalization, through co-regulation or pleiotropy. Quantitative trait loci (QTL) for frost tolerance have been mapped in a recombinant inbred line (RIL) population derived from a cross between a French winter tolerant fodder pea landrace, Champagne, and a spring field pea cultivar Terese (Pop2). Based on the evaluation of winter freezing damage (WFD) in field or controlled conditions, several QTL were consistently detected for this population in different environments highlighting four genomic regions related to frost tolerance on linkage groups III (two regions), V and VI (Lejeune-Hénaut et al. 2008; Dumont et al. 2009). Additionally, some other WFD QTL were detected on linkage groups I, IV and VII in one environment only. A major QTL of pea frost tolerance on LGIII was located in the vicinity of the *Hr* locus. *Hr* is a gene controlling plant response to photoperiod (Weller et al. 2012). This gene is an essential component of frost avoidance, since it delays the vegetative to reproductive stage transition until longer days, when the risk of frost occurrence is lower. Interesting co-locations between WFD QTL and QTL for physiological traits were also discovered on LGV and VI (Dumont et al. 2009). Co-location of QTL on LGVI suggested the potential role of RuBisCO activity and raffinose content in frost tolerance, possibly in relation to the ability to maintain photosynthesis and accumulate protective sugars under low but positive temperatures as described in other species (Dumont et al. 2009). In addition, WFD QTL on LGVI in pea coincides with the location of a major freezing tolerance QTL on the chromosome 6 in *M. truncatula* (Tayeh et al. 2013a).

The objective of the present study was to specify the mechanisms involved in frost tolerance in pea by (a) using a different source of frost tolerance found in a Chinese winter tolerant landrace, (b) assessing the genetic relationship between frost tolerance, plant phenology and morphology and (c) investigating the genetic interplay between frost tolerance and seed production.

Materials and methods

Plant material

A pea population of 129 F6-derived recombinant inbred lines (RIL, Pop9) was produced by Single-Seed Descent at INRA Dijon (France) from the cross between China (JI1491), a pea germplasm accession originating from China, and a spring garden pea cultivar, Caméor. China was found to be freezing tolerant in germplasm screens conducted in Dijon, while Caméor was shown to be highly sensitive to frost. China and Caméor both exhibit a conventional foliage type and white flowers but are contrasted for several other morphological traits (Supplementary Table S1), such as the internode length (long and short, respectively), the branching type (high and low basal branching, respectively), the hilum color (black and white, respectively), as well as agronomic traits such as thousand seed weight (177 and 216 g, respectively), and harvest index (0.50 and 0.64, respectively). Both genotypes exhibit high seed protein content.

Field trials

The 129 RILs and parental lines were evaluated in six environments at INRA Dijon, Domaine d'Epoisses, Bretenière, France (47°14′N, 5°05′E) in autumn sowing in 2007/2008 (Dij08), 2008/2009 (Dij09) and 2009/2010 (Dij10) and in spring sowing in 2008 (Dij08S), and at INRA Clermont Ferrand-Theix, France (45°42′N, 3°00′E) in autumn sowing in 2005/2006 (Cler06) and 2009/2010 (Cler10). INRA Clermont Ferrand-Theix is a mountainous area (altitude 820 m) used to evaluate winter frost tolerance. INRA Dijon is a continental area (altitude 210 m). Climatic data recorded in the field trials are shown in Fig. 1 and Supplementary Table S2.

Fields experiments were carried out using a randomized complete block design with two replicates. Each plot consisted of 25 seeds sown in a row of 2 m long, with 1 m spacing between two adjacent rows. Plants were grown against trellises. Weeds, insects and diseases were controlled chemically. At INRA Dijon, irrigation was provided at the beginning and end of flowering.

Measurements

In Cler06, Cler10 and Dij08, Dij09 and Dij10 experiments, we scored winter frost damage as well as plant architecture traits. Winter frost damage (WFD) was evaluated after each winter freezing period. A score was assigned to a line as a whole, based on the aspect of the aerial parts of the plants according to the scale defined in Lejeune-Hénaut et al. (2008): (0) for no damage, (1) for frost burn limited to the

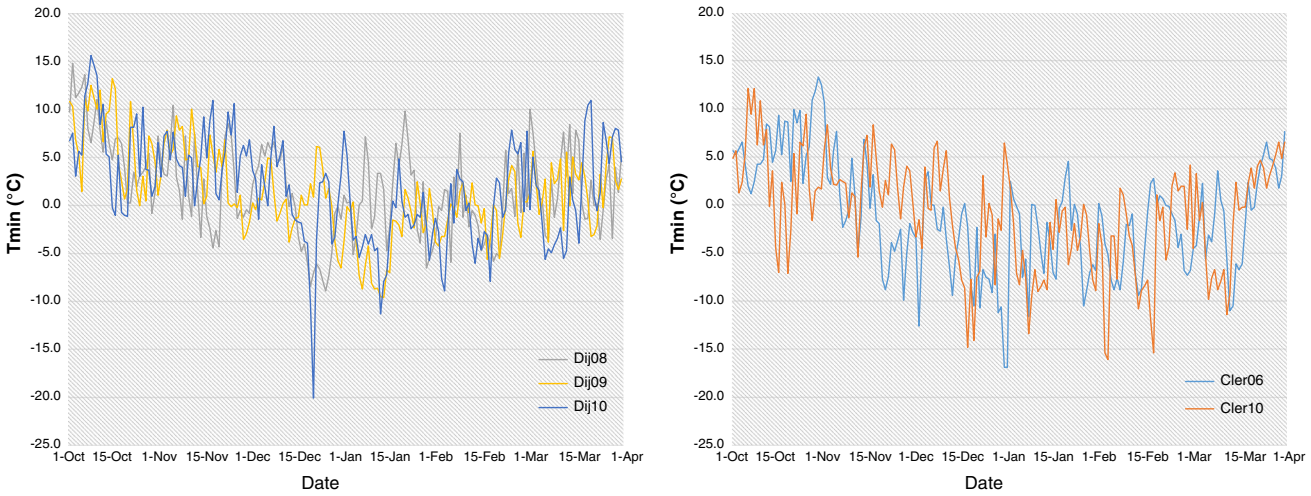

Fig. 1 Minimal temperature recorded in the winter field trials at INRA Dijon and INRA Clermont-Ferrand between 2005 and 2010 from October to March. *Tmin* (°C) minimal temperature (in degree Celcius), *Cler06* Clermont-Ferrand autumn sowing in 2005, *Cler10* Clermont-Ferrand autumn sowing in 2009, *Dij08* Dijon autumn sowing in 2007, *Dij09* Dijon autumn sowing in 2008, *Dij10* Dijon autumn sowing in 2009 (color figure online)

leaf edges, (2) for frost burn on the majority 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. The branching type (NB) was described at the end of winter with scores varying from 0 to 3: (0) corresponded to plants without primary branches and (3) to plants with primary and secondary branches, characteristics of a rosette-type habit. For Dij10, the leaf projected area (Area1 and Area2) was determined by image analysis of pictures taken before and after winter respectively, using the WinRhizo software as described in Bourion et al. (2010). In addition, leaf chlorophyll content after winter (Chloro) was estimated on the last expanded leaf using a SPAD-meter (Minolta, Japan). In Dijon experiments, stages of beginning of flowering (BegFlo), end of flowering (EndFlo), beginning of seed filling (BSF), maturity (Harvest) and the height at BSF (hBSF) were scored along the plant life cycle. All phenological traits were expressed as a function of cumulative °C/days from sowing, using a 0 °C base temperature. Flowering time (Flo), seed filling (Fill), and reproductive period (Repro) were calculated as follows: Flo = EndFlo−BegFlo, Fill = Harvest−BSF, Repro = Harvest−BegFlo. At plant maturity, a sample of ten plants per line was harvested. Yield components and growth parameters were measured: the height at harvest (hHarvest), the number of basal branches per plant (NBB), the pod number per plant (PN), the seed number per plant (SN), the seed number per pod (SNP), the seed weight per plant (SW), the thousand seed weight (TSW), the straw dry weight per plant (SDW), the biomass dry weight per plant (BDW = SDW + SW), the seed protein content (SPC) and the harvest index (HI) supplied by seed weight/biomass dry weight. SDW was weighed after 48 h at 80 °C. Seed protein content was determined by near-infrared spectroscopy (NIRS) as described in Burstin et al. (2007).

Phenotypic data analysis

For each environment, ANOVA were performed using SAS GLM procedure (SAS Institute Inc. 1999) to determine the significance levels of the genotype and replication effects. The statistical model was: $Y_{ijk} = \mu + g_i + r_j + b_{k/j} + e_{ijk}$ where Y_{ijk} is the value of the trait for genotype i in block k of the replicate j, μ the general mean, g_i the genotypic effect, r_j the replicate effect, $b_{k/j}$ the block k effect in the replicate j and e_{ijk} the residual. Broad sense heritability (h^2) was estimated from ANOVA by $h^2 = \sigma_g^2/[\sigma_g^2 + (\sigma_e^2/n)]$ with σ_g^2 the genetic variance, σ_e^2 the residual variance and n the number of replicates. Normality of residuals and homogeneity of variances were checked using Shapiro–Wilk and Bartlett's test ($P \geq 0.05$) from SAS UNIVARIATE procedure. RILs' adjusted means calculated using the LSMEANS command of SAS GLM procedure were used for QTL analysis. Pearson correlation coefficients (r^2) between traits were calculated from RILs adjusted means using SAS CORR procedure.

Principal component analysis was performed using "FactoMineR" library from R (R Development Core Team 2013). Hierarchical clustering using "ade4" library and the "hclust" function with the "ward" method. Frequency distributions from RILs adjusted means using "car" library and the "hist" function.

Genetic linkage map and QTL analysis

The Pop9 genotyping data were described in Deulvot et al. (2010) and Duarte et al. (2013, submitted). Additionally, a microsatellite marker (AA175) was genotyped according to Loridon et al. (2005), as well as the *Elf3* locus using a Single Nucleotide Polymorphism (SNP) between China and Caméor. The Pop9 map was performed using MAPMAKER/EXP, version 3.0 (Lander et al. 1987), with a minimum LOD score threshold of 3.0 and a recombination frequency <0.3. Marker order was established using the "order" command. CentiMorgan (cM) distances were calculated with the Kosambi function. QTL composite interval mapping was done using the iterative QTL mapping method (iQTLm) of the MCQTL software v5.2.4 (Jourjon et al. 2005). Cofactor selection and QTL detection *P* value thresholds were determined after 1,000 permutation tests on all traits, for a global genome-wide type I risk of 10 % for cofactor selection, and 5 % for QTL detection. Cofactors were searched by forward regression, using a threshold of $P = 2.3$. QTL were searched by iQTLm, using a threshold of $P = 3.0$. The global R^2, individual R^2, and allelic effect at each QTL were estimated for each trait. The QTL map was drawn using MapChart software (Voorrips 2002).

Results

Phenotypic variability for phenology, morphology, physiology and yield components

In winter field trials conducted between 2005 and 2010 at Dijon and Clermont-Ferrand/Theix, China confirmed its very high tolerance to cold, associated with a highly branched phenotype at the end of winter (Supplementary Table S1). To the contrary, Caméor encountered high winter frost damage (WFD) and showed fewer branches at the end of winter. Highly significant ($P < 0.0001$) genotype effects were detected for all but one trait. Heritability ranged from 0.15 to 0.99. It was high for WFD, TSW and low for SPC and SW. The only spring field trial (Dij08S) showed lower heritabilities for most traits. The effect of the trial environment was also detected for all traits: for example, WFD means of Pop9 ranged from 0.90 in Dij08 to 3.12 in Cler06 and 2.50 in Dij09. Conversely, basal branching (NB) of Pop9 was the lowest in Dij09 (0.77) and the highest in Dij08 (1.15). Yield (SW), yield components (SN, SNP, and TSW) and plant biomass (SDW, BDW) were higher for Dij09 than for Dij08, Dij08S and Dij10. This was associated with the contrasted climates encountered during winter trials, with frost episodes and cold temperatures in Cler06, Cler10 and Dij10, and milder weather in Dij08 (Fig. 1, Supplementary Table S2). Dij08S showed the lowest plant and seed biomass but the highest SPC. Frequency distributions of adjusted means (Supplementary Figure S1) showed a normal distribution for most traits (Shapiro–Wilk test, $P > 0.05$). Compared with China, some transgressive progenies with increased resistance to frost were observed in Pop9. A high genetic variability and transgressive progenies were also observed for yield, yield components and SPC: SN and SW ranged from 133 to 165 and 29.8 to 30.3 g in Caméor and China, respectively, and varied from 30 to 532 and 8 to 86 g in the Pop9. TSW ranged from 177 to 216 g in China and Caméor, respectively, and ranged from 119 to 243 g in Pop9. SPC varied from 20.1 to 30.1 % in Pop9 while China and Caméor displayed similar and high value (25.3 and 25.7 %, respectively).

Phenotypic structuring

Hierarchical clustering of phenotypic traits (Fig. 2) revealed four groups of variables: group 1 [1] combined architecture (NB, NBB, Area2) with earliness (BegFlo, BSF, EndFlo, Harvest)—group 2 [2] associated morphology (hHarvest, hBSF, Area1) with frost damage (WFD) and reproduction duration (Flo, Fill, Repro)—group 3 [3] associated seed filling components (TSW, SNP,SPC, HI) and Chloro—group 4 [4] linked yield (SW) and seed onset components (SN, PN) and growth (SDW, BDW). Group [1] was opposite to group [2]. Winter frost tolerance was associated with a high number of branches, low leaf area before winter, high chlorophyll content after winter, and late beginning of flowering (Supplementary Table S3). Group [3] was opposite to group [4] (Fig. 3a). Indeed, yield (SW) was highly positively correlated with pod and seed number (PN, SN), many pods being associated with late end of flowering and to a lesser extent, a long flowering period. Yield was positively correlated with plant height and total biomass production (hBSF, hHarvest, SDW, BDW). TSW was moderately positively correlated with BSF but not with yield. Seed protein content (SPC) was moderately negatively correlated with yield (SW) and seed number (SN). Furthermore, principal component analysis of phenotypic variation among inbred lines clustered the population into six groups (Fig. 3b). The hierarchical clustering of lines grouped lines into two groups of 59 and 60 lines, suggesting a major gene involved. The partitioning of lines was in accordance with the *Elf3* genotyping except for 12 lines that were grouped together.

QTL mapping for phenological, morphological and agronomical trait

A new genetic map was built for the China × Caméor population: it included 679 well distributed SNP markers and one SSR marker, and spanned 947.1 cM. A total of 161

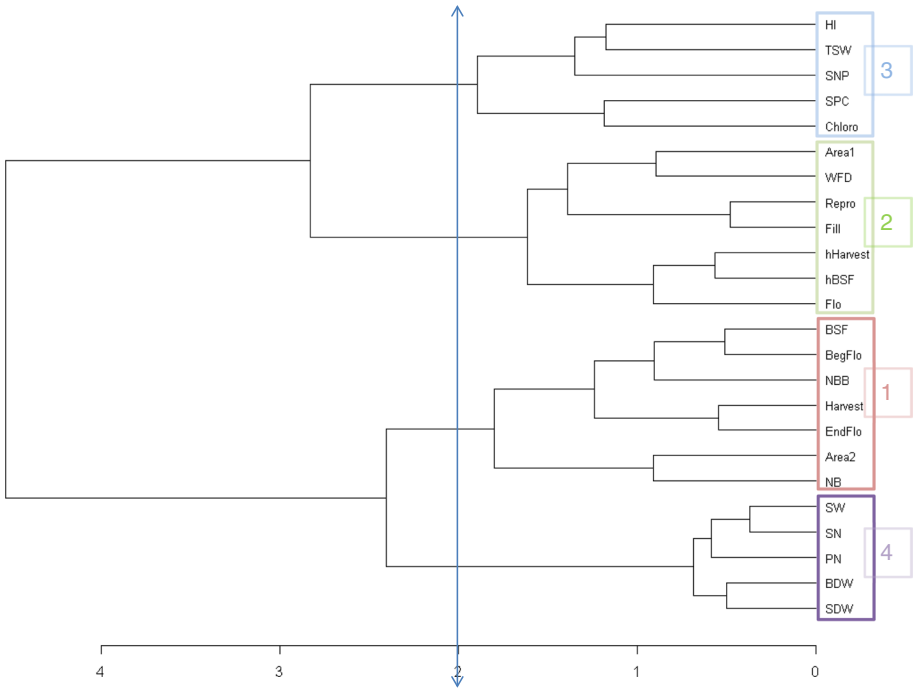

Fig. 2 Hierarchical clustering of phenotypics traits observed on one-row field trial between 2005 and 2010 at INRA Dijon and INRA Clermont-Ferrand. *BegFlo* beginning of flowering, *BSF* beginning of seed filling, *EndFlo* end of the flowering, *Harvest* maturity, *Flo* flowering period, *Fill* filling period, *Repro* reproductive period, *WFD* winter frost damage, *NB* branching type, *Area1* and *Area2* leaf projected area before and after the winter respectively, *Chloro* SPAD chlorophyll measurements, *hBSF* height at BSF, *hHarvest* height at harvest, *NBB* number of basal branches per plant, *PN* pod number per plant, *SN* seed number per plant, *SNP* seed number per pod, *SW* seed weight per plant, *TSW* thousand seed weight, *SDW* straw dry weight per plant, *BDW* biomass dry weight per plant, *HI* harvest index and *SPC* seed protein content (color figure online)

QTL distributed over the seven pea linkage groups were detected across the six environments for all traits measured (Table 1; Fig. 4, Supplementary Table S4). A total of 16 QTL corresponding to six genomic regions on the LGIII, V, VI and VII were detected for WFD over all winter environments; 56 QTL for phenological traits distributed over nine genomics regions on the LGI, II, III, V, VI and VII, 34 QTL for morphological traits over five environments, corresponding to ten genomic regions. Finally, 55 QTL were detected across four environments for agronomical traits corresponding to sixteen genomic regions.

Three major clusters of QTL were detected (Fig. 4): (a) The cluster on LGVI (VI.1) from Ps001502 to FVE corresponded to 16 QTL of phenological traits detected over four environments (R^2 = 14–42 %), 8 QTL of morphological traits (R^2 = 20–37 %), 5 QTL of WFD observed across all environments (R^2 = 38–71 %) and 3 QTL controlling SDW, SPC and TSW (R^2 = 15–17 %). This cluster included QTL of WFD and NB for all the environments measured and explained up to 71 % of the WFD variation at Cler10. The positive additive effect for winter frost tolerance brought by the China allele was coupled with a rosette type combining a low leaf area before the winter, a late flowering with a quickly reproductive period and associated with higher SDW, TSW and SPC; (ii) Another cluster on LGIII (III.1) from Ps001727 to Ps000439 associated 15 QTL of phenology (R^2 = 12–44 %), across all environments, with 15 QTL controlling yield and yield components (R^2 = 10–35 %), 10 QTL controlling morphological traits (R^2 = 11–23 %) and 2 QTL of WFD (R^2 = 9–19 %). The Caméor allele was associated with early flowering and the China alleles confer late flowering with winter frost tolerance and higher NB, NBB, yield and yield components, except TSW; (iii) The third cluster, located on the bottom of LGIII (III.3), spanned between markers Ps000433 and TE002G22: 8 QTL of morphological traits controlling plant height, number of branches and leaf area (R^2 = 12–64 %) co-located with 11 QTL associated with phenological traits (R^2 = 11–30 %), 3 QTL of WFD (R^2 = 11–15 %) and 14 QTL controlling yield, yield components and seed protein content (R^2 = 12–47). Positive additive effects were shared between the two parents. Greater plant height, higher PN, SN, SW, SDW was associated with the China allele while winter frost tolerance, higher SPC, Chloro, NBB and HI were brought by the Caméor allele.

Other notable QTL are worth mentioning: Two major QTL explaining respectively 31 % at Cler10 and 32 % at Dij08 of the WFD variation were clustered on top of

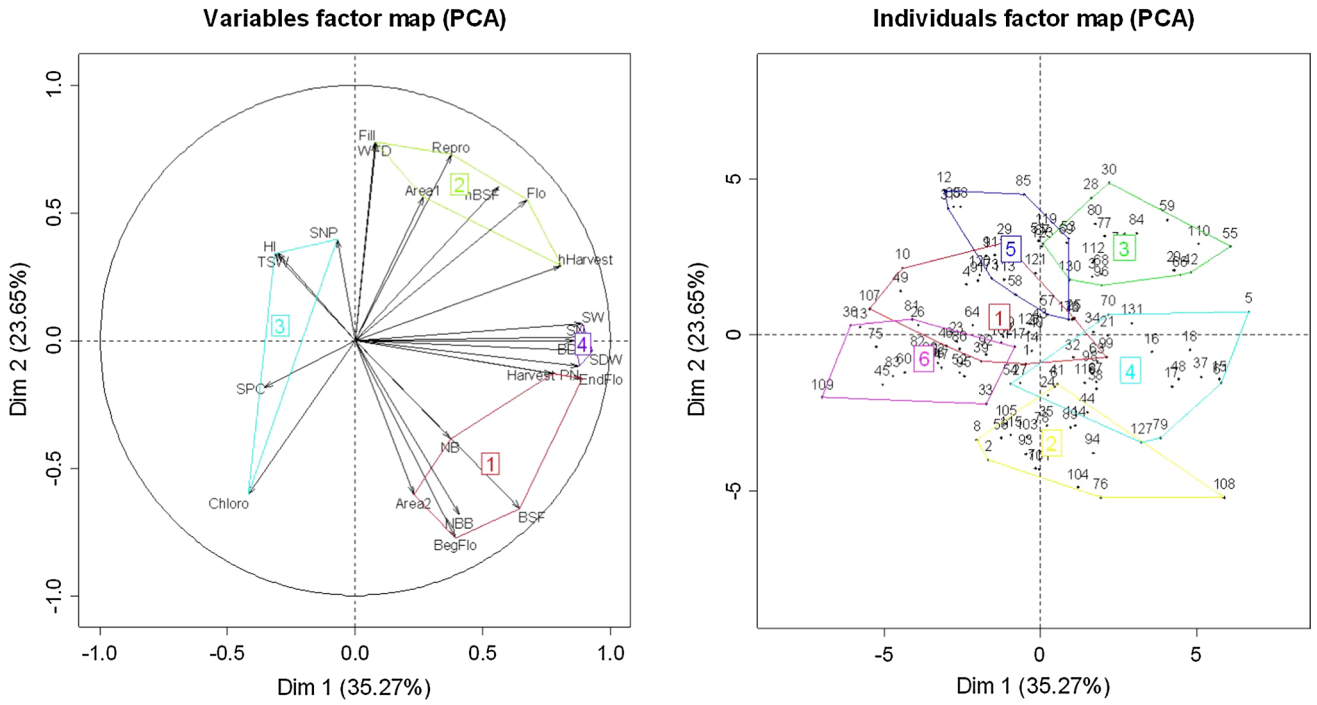

Fig. 3 Principal component analysis of phenotypics traits (Fig. 3a) and recombinant inbred lines (Fig. 3b) observed on one-row field trial between 2005 and 2010 at INRA Dijon and INRA Clermont-Ferrand. *BegFlo* beginning of flowering, *BSF* beginning of seed filling, *EndFlo* end of the flowering, *Harvest* maturity, *Flo* flowering period, *Fill* filling period, *Repro* reproductive period, *WFD* winter frost damage, *NB* branching type, *Area1* and *Area2* leaf projected area before and after the winter respectively, *Chloro* SPAD chlorophyll measurements, *hBSF* height at BSF, *hHarvest* height at harvest, *NBB* number of basal branches per plant, *PN* pod number per plant, *SN* seed number per plant, *SNP* seed number per pod, *SW* seed weight per plant, *TSW* thousand seed weight, *SDW* straw dry weight per plant, *BDW* biomass dry weight per plant, *HI* harvest index and *SPC* seed protein content (color figure online)

Table 1 QTL detected in Pop9 from 2005 to 2010 at INRA Dijon and INRA Clermont-Ferrand

Trial[a]	*N* QTL detected[b]	*N* trait[c]	Sowing	Year
Dij08	40 QTL	19 traits	Autumn sowing	2007–2008
Dij08S	34 QTL	15 traits	Spring sowing	2008
Dij09	28 QTL	17 traits	Autumn sowing	2008–2009
Dij10	51 QTL	22 traits	Autumn sowing	2009–2010
Cler06	2 QTL	1 trait	Autumn sowing	2005–2006
Cler10	6 QTL	2 traits	Autumn sowing	2009–2010

[a] *Dij08* Dijon autumn sowing in 2007, *Dij08S* Dijon spring sowing in 2008, *Dij09* Dijon autumn sowing in 2008, *Dij10* Dijon autumn sowing in 2009, *Cler06* Clermont-Ferrand autumn sowing in 2005 and *Cler10* Clermont-Ferrand autumn sowing in 2009

[b] Number of QTL detected in each trial

[c] Number of phenotypic trait associated with QTL

LGV (V.1). The winter frost tolerance allele was brought by China. In the same linkage group between Ps001644 and Ps001336 were clustered three other QTL of WFD (V.2, R^2 = 13–23 %). TSW QTL were detected on LGI (I.1) across all winter environments in Dijon from Ps001480 to Ps001408 and explained 18–22 % of the phenotypic variation with a positive effect from China's allele. Three SW, SDW, SPC QTL (R^2 = 14–17 %) detected only in the spring sowing were detected on LGI (I.2) between Ps001067 and Ps001136. Three QTL of flowering time (R^2 = 16–29 %) and a QTL of EndFlo (R^2 = 23 %) with positive effects from Caméor's allele were detected on the LGII (II.1) in the genomic region of *TFL1c* = *lf (late flowering)*, a gene involved in the genetic control of the flowering (Foucher et al. 2003; Weller et al. 2009). Three QTL of seed protein content (R^2 = 13–18 %) on the LGIII (III.2) were identified in the genomic region of *DiPeptIV* encoding a Dipeptidyl Peptidase IV-like protein and *PsAAP1*, an amino acid permease I. In this region, Caméor's allele conferred a higher seed protein. Finally, a region controlling the seed number per pod in all winter environments in Dijon (R^2 = 19–22 %) linked with the seed protein content in Dij08S and Dij10 (R^2 = 15–17 %) was identified on LGVII (VII.1) from Ps000937 to Ps000366, in the PPT2 region. The Caméor allele confers a higher SPC but a lower SNP.

Discussion

Toward a comprehensive picture of freezing tolerance QTL in pea

Using the Pop2 RIL population, derived from a cross between the winter frost tolerant line Champagne and the sensitive line Terese, Lejeune-Hénaut et al. (2008) and Dumont et al. (2009) detected 9 QTL regions for frost tolerance, among which four were consistently identified in five to thirteen different field conditions. The frost tolerance data collected in the present study from new field experiments and a different genetic background allowed confirming the same four consistent WFD QTL positions III.1, III.3, V.2 and VI. Common markers between Pop2 and Pop9 located within or flanking their confidence intervals support the collinearity of these four consistent QTL: respectively AA175 for III.1, Bfruct for III.3, kdsa and RNAH for V.2 which are shown to surround Tri and DHPS1 in Bordat et al. (2011), Cabb for VI.1 which is shown to be common between Pop2 and Pop9 thanks to Tayeh et al. (2013a). Furthermore, this study also confirmed the meaning of the

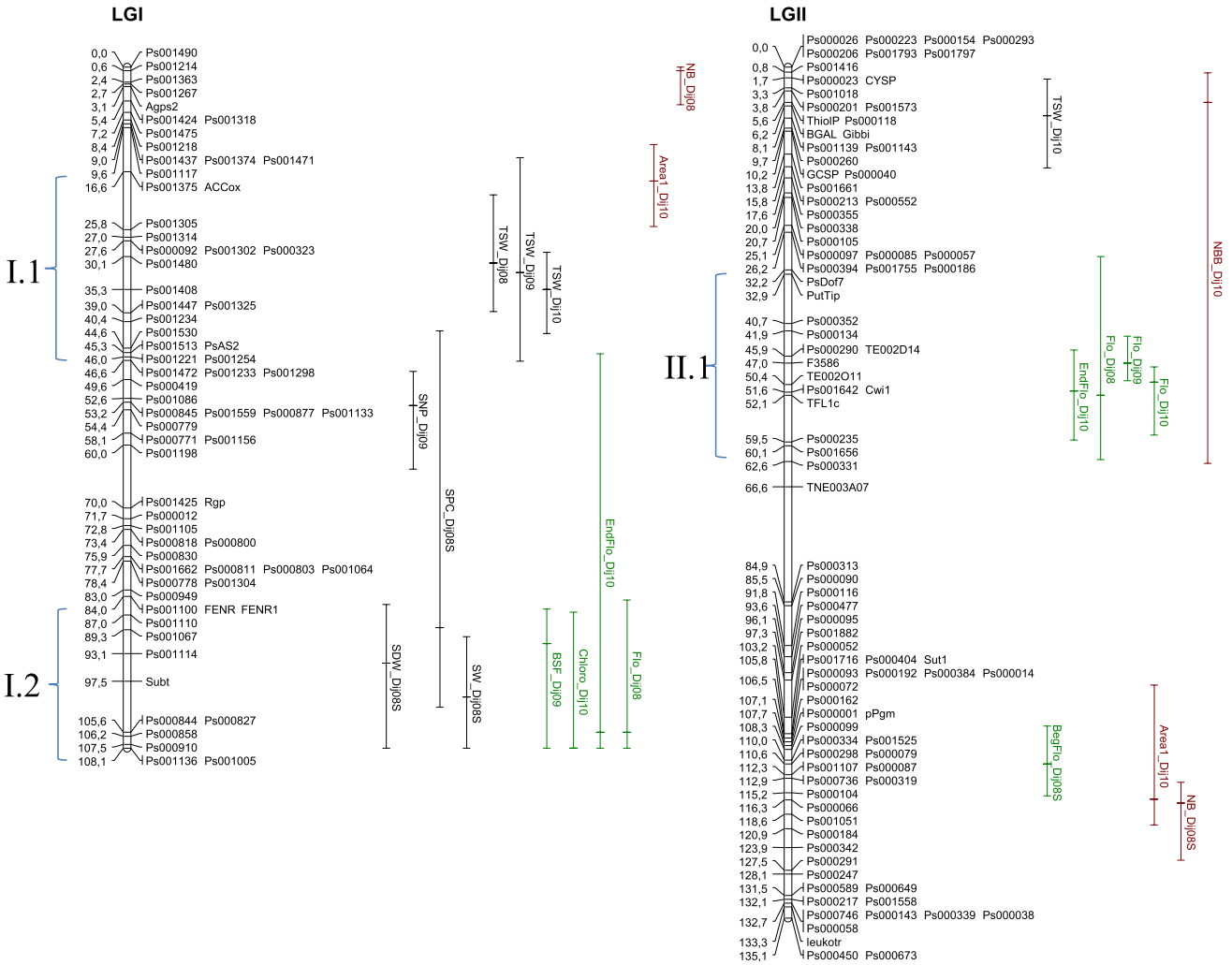

Fig. 4 QTL mapping in Pop9 detected from 2005 to 2010 at INRA Dijon and INRA Clermont-Ferrand. Each QTL is identified by (a) traits—*green*: phenologic trait *BegFlo* beginning of flowering, *BSF* beginning of seed filling, *EndFlo* end of the flowering, *Harvest* maturity, *Flo* flowering period, *Fill* filling period, *Repro* reproductive period—*red* : morphologic trait *NB* branching type, *Area1*, *Area2* leaf projected area before and after the winter respectively, *hBSF* height at BSF, *hHarvest* height at harvest, *NBB* number of basal branches per plant—*blue*: physiologic trait *WFD* winter frost damage—*black*: yield and yield components trait *PN* pod number per plant, *SN* seed number per plant, *SNP* seed number per pod, *SW* seed weight per plant, *TSW* thousand seed weight, *SDW* straw dry weight per plant, *BDW* biomass dry weight per plant, *HI* harvest index, *Chloro* SPAD chlorophyll measurements, *SPC* seed protein content (b) field trials: *Cler06* Clermont-Ferrand autumn sowing in 2005, *Cler10* Clermont-Ferrand autumn sowing in 2009, *Dij08* Dijon autumn sowing in 2007, *Dij08S* Dijon spring sowing in 2008, *Dij09* Dijon autumn sowing in 2008, *Dij10* Dijon autumn sowing in 2009 (c) support intervals and peaks of QTL assigned on the RIL9 genetic map (in cM Kosambi) (color figure online)

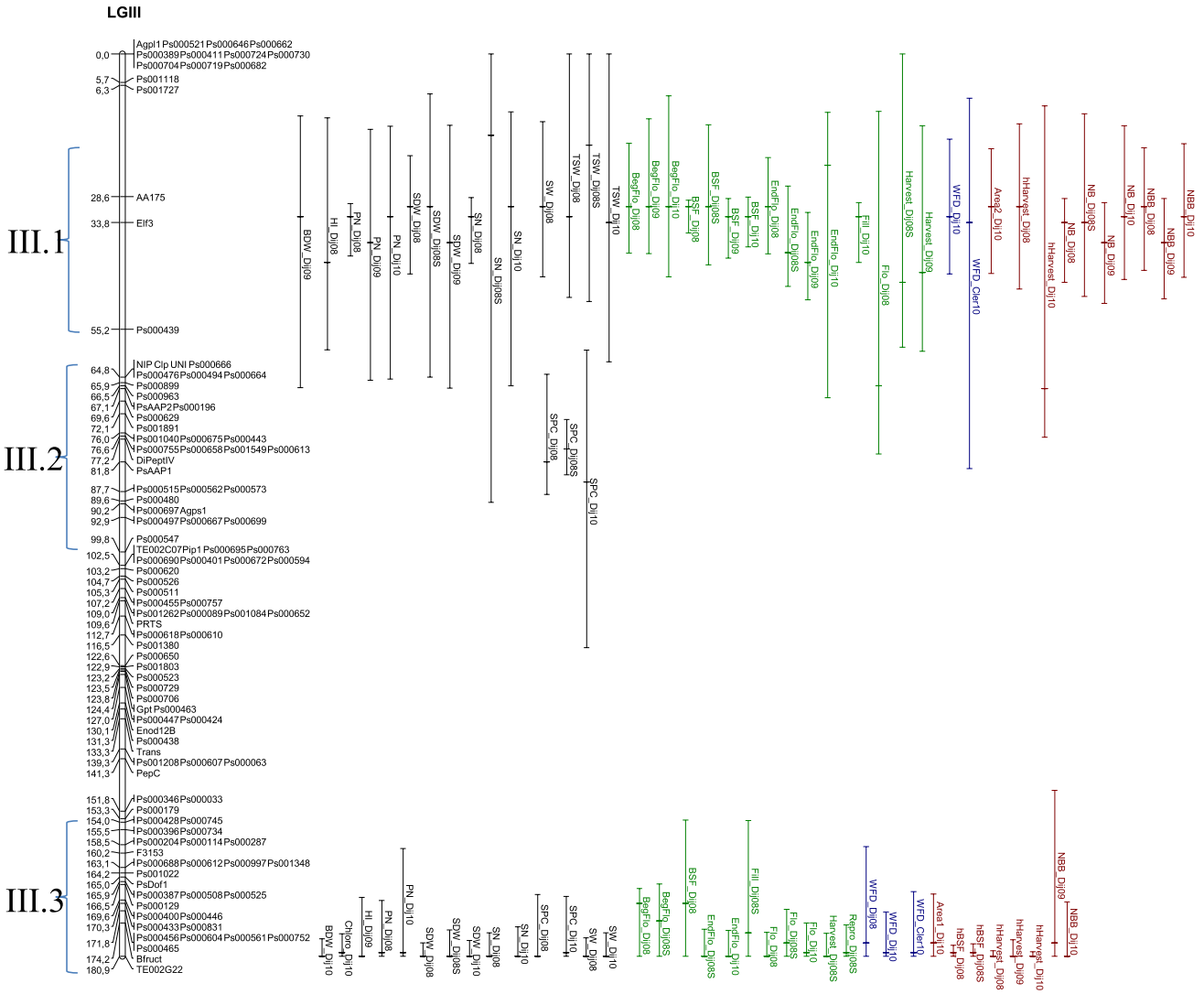

Fig. 4 continued

allelic value of these 4 QTL regions, between the forage frost tolerant parent and the sensitive parent. For three of these QTL, the forage line, i.e. Champagne for Pop2 and China for Pop9, brought a favorable allele for frost tolerance. For 1 QTL (III.3), the sensitive parent of both Pop2 and Pop9, respectively Terese and Caméor, carried the favorable allele. Additionally to these four common positions with Pop2, Pop9 revealed a novel QTL region, V.1, where two WFD QTL were detected in the Dij08 and the Cler10 environments and for which China brought the favorable allele. A one-environment QTL was also detected in Pop9 in Dij10 on linkage group VII (VII.2). Its projection on the functional map proposed by Bordat et al. (2011) shows partial overlapping, at the level of marker D24 and co-localizes with the FD.c frost tolerance QTL detected in controlled conditions by Dumont et al. (2009). For these QTL, the favorable alleles are brought by the forage lines in both Pop2 and Pop9. Experiments conducted during the past seven years at the high altitude site of INRA Dijon at Chaux-des-Prés, France (46°30′N, 5°51′E, altitude 872 m) have shown that Champagne and China are among the most frost tolerant lines identified in the pea French genetic resources collection, both being able, through a highest cold acclimation, to survive without frost damage up to −23 °C and without snow cover. However, it was also shown that these two lines differ in their cold acclimation rate, China being able to acclimate faster than Champagne (C. Lecomte, personal communication). Further experiments would be necessary to test the involvement of the V.1 QTL region evidenced exclusively in Pop9, in the rate of acclimation of pea relative to autumnal temperature conditions. The same hypothesis should be tested for the VII.1 QTL region highlighted in Pop9 in an environment (Dij10) characterized by a mild autumn and an early frost event in

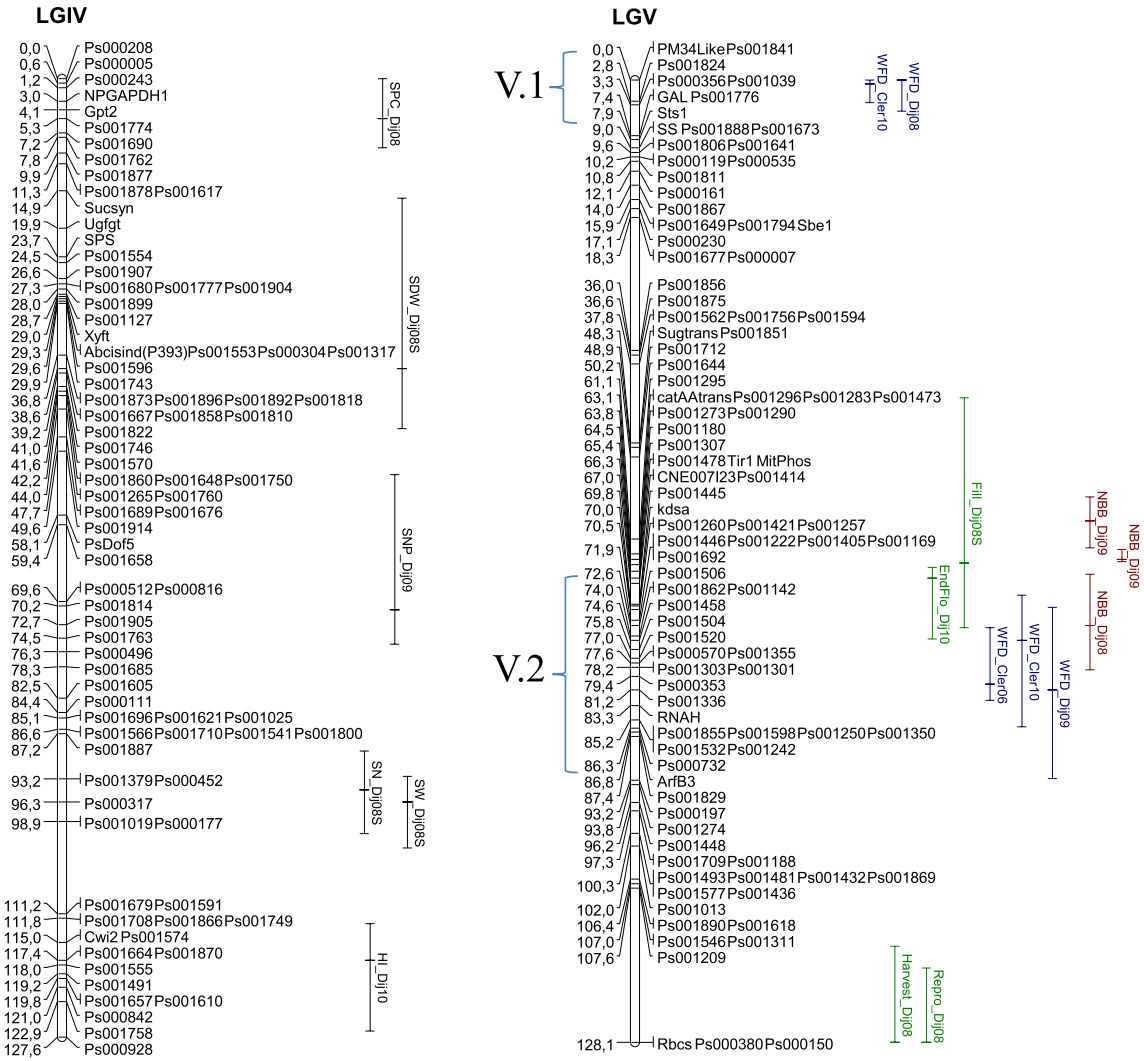

Fig. 4 continued

December (−20.2 °C) during which one can assume that cold acclimation has not been optimal.

Different QTL suggest various mechanisms underlying frost tolerance

The assessment of phenological and morphological traits associated with the genetic map including major loci known to control flowering and architecture has allowed to specify the main genetic relationship between frost tolerance, plant phenology and morphology. Three main clusters of QTL (III.1, III.3 and VI.1) gather the three types of traits showing the genetic relationship between frost tolerance, plant phenology and morphology. The III.1 cluster co-localizes with *Elf3*, a gene involved in circadian cycle signaling that has recently been proposed to correspond to the *Hr* locus in pea (Weller et al. 2012). In Pop9, China brings at this position favorable alleles for higher frost tolerance, later beginning of flowering and higher number of basal branches. Similarly, *Hr* was also shown to be associated with a frost tolerance QTL in Pop2 and most of the lines bearing the dominant *Hr* allele in this population were characterized by a marked rosette type, i.e. profuse basal branching in autumn and winter field conditions (Lejeune-Hénaut et al. 2008). The flowering allele *Hr* has long been described as enhancing the capacity of pea photoperiodic lines to produce basal laterals (Murfet and Reid, 1993). Whether this reflects genetic linkage or a pleiotropic effect of the *Hr-Elf3* gene cannot yet be determined. The *Hr-Elf3* locus thus impacts the phenotypes of Pop2 and Pop9 in a similar manner. However, a main difference concerns the relative importance of this QTL region according to its coefficient of determination (R^2). If the *Hr-Elf3* region is the most explanatory of frost tolerance variation in Pop2,

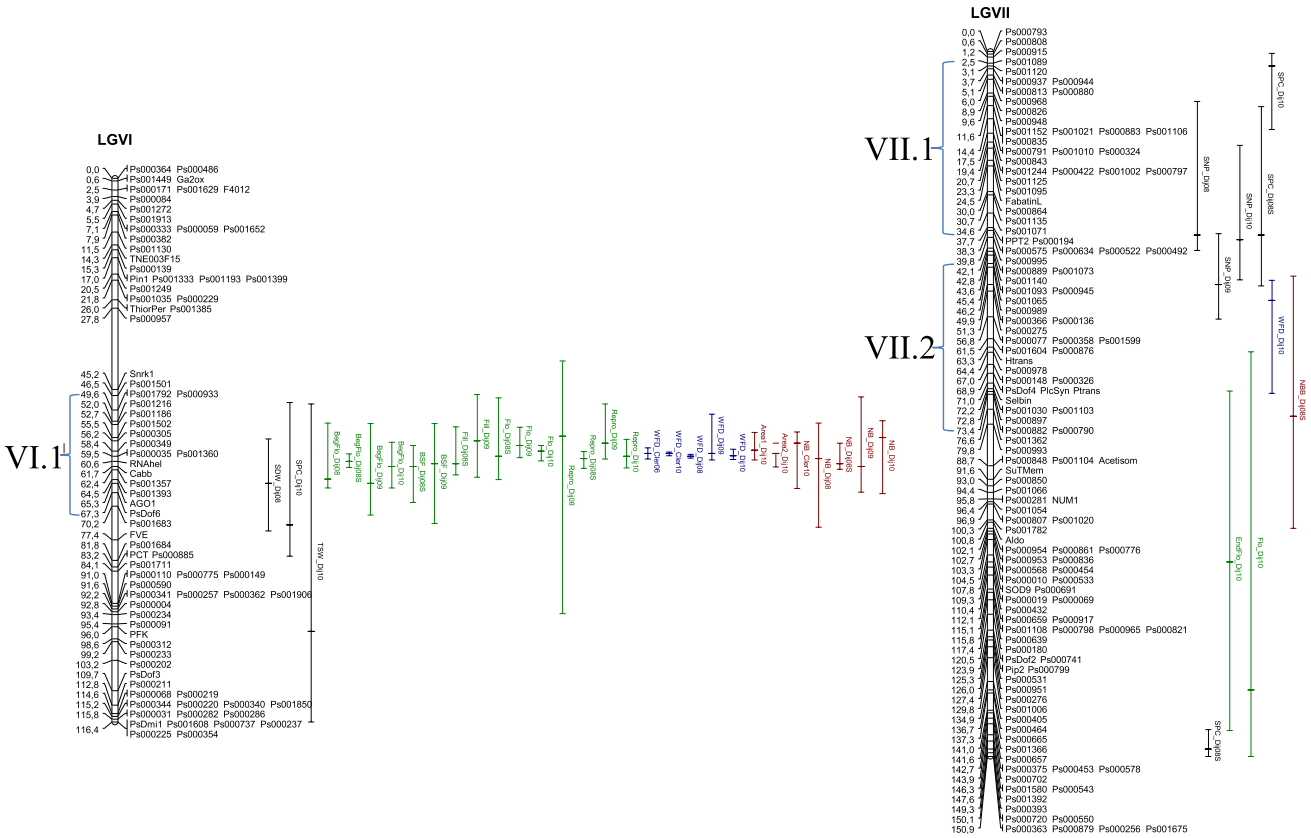

Fig. 4 continued

with a mean R^2 value of 0.39 over the eleven environments tested, the most important QTL region in Pop9 is the one located at the middle of linkage group VI, with a mean R^2 value of 0.55 over the five environments tested. The lower effect of this locus on Pop9 phenotypes as compared to Pop2 could be due to different alleles at this locus in the two genotypes. Indeed, based on *Elf3* gene sequencing data, Weller et al. (2012) have shown that China and Champagne carry different haplotypes of this gene. The genetic background could also modulate the effect of the *Hr-Elf3* locus. The *Lf* flowering locus on LGII has previously been shown to interact for determining flowering time and growth habit. Early alleles at the *Lf* locus have been shown to confer earlier flowering in photoperiod-responsive genetic backgrounds under short days (Murfet 1985). Another photoperiod response locus on LGVI (QTL6 in Weller et al. 2012) has also been shown to interact for determining flowering time: the domesticated allele at QTL6 has been shown to counteract the *Hr* allele and promote flowering under short days (Weller et al. 2012). The VI.1 QTL cluster located on the middle of LGVI corresponds, in Pop9, to the major frost tolerance QTL. It is detected in all five winter field trials and explains from 38 to 71 % of the phenotypic variation of frost tolerance. This locus also impacts, but to a lower extent, the date and duration of flowering (BegFlo, Flo) and branching (NB). The lower determination of this region on flowering traits suggests either that two different genes may be acting in this region, one on flowering (corresponding to QTL6 described in Weller et al. 2012) and one on frost tolerance or alternatively, that a same gene has pleiotropic and/or epistatic effects on frost tolerance and flowering time. The syntenic region of this pea frost tolerance QTL also holds a QTL of frost tolerance in *M.truncatula* (Tayeh et al. 2013a). Several cold induced binding factors have been found in the QTL vicinity in *M.truncatula* (Tayeh et al. 2013b). Furthermore, Dong et al. (2011) have shown in Arabidopsis that the circadian clock components CCA1 and LHY have a role in cold induction of the CBF (C-REPEAT BINDING FACTOR) pathway and that they are required to attain maximum freezing tolerance. As *Hr-Elf3* has been shown to influence the rhythmic expression of LHY in pea (Weller et al. 2012), this locus could have a pleiotropic effect on frost tolerance.

The last QTL cluster (III.2) coincides with the well-known nanism locus Le (Lejeune-Hénaut et al. 2008, Bordat et al. 2011). Consistently, in Pop9, this cluster included high determination coefficient QTL for plant height. In both Pop2 and Pop9, the sensitive parent, respectively

Terese and Caméor, carries the favorable allele at this QTL. As for the VI.1 QTL, frost tolerance is associated with a smaller leaf area before winter and late flowering. For III.3, Caméor brings frost tolerance and small leaf area, whereas for VI.1, China brings these characteristics. This raises two hypotheses: the smaller leaf area before winter could trigger lower thermic exchange surface, freezing and dehydration, or lower leaf area could be associated with higher sugar or anti-freeze molecule concentration. Altogether, our results suggest close physiological links between flowering, branching and frost tolerance. However, neither epistasis nor pleiotropy or even linkage can be ruled out. Increased branching could favour growth resumption after frost damage; delayed flowering due to photoperiod could allow avoidance of frost; and signaling genes involved in flowering and branching could also regulate cold associated genes.

For two other frost tolerance QTL clusters (V.1 and V.2) no direct link can be established with morphology and/or phenology: the V.i region was found to specifically control frost tolerance in two winter trials (Dij08 and Cler10) with high determination coefficients (R^2 from 0.31 to 0.32). Two candidates in this region, *Gal*, the gene encoding galactinol synthase and *Sts*, a gene encoding a stachyose synthase have been described as possibly involved in anti-freeze metabolism during frost stress (Zhuo et al. 2013; Nishizawa et al. 2008; Cunningham et al. 2003; Taji et al. 2002). This QTL could correspond to intrinsic frost tolerance possibly linked with anti-freeze component synthesis. The other region (V.2) controlled frost tolerance in three winter trials (Cler06, Cler10 and Dij09) but with lower determination coefficients (R^2 from 0.13 to 0.23). This frost tolerance QTL region was also detected in Pop2 (Lejeune-Hénaut et al. 2008).

Genetic relationship between frost tolerance, seed productivity and quality

At first, it should be noted that the lowest plant and seed biomass was obtained in the Dij08S only spring field trial, confirming that increasing the plant life cycle had a beneficial effect on plant productivity as long as the plants survived winter. However, our results suggested little interactions between seed productivity and frost tolerance, apart from the two major QTL clusters corresponding to major developmental gene loci (*Hr* and *Le*). Frost tolerance was linked with architecture type and phenological traits which have in turn an impact on seed yield. Rosette-type architecture provided an efficient seed yield. Major developmental gene loci (*Hr* and *Le*) impacted the whole plant physiology including seed productivity traits, as observed in Burstin et al. (2007), whether in spring or winter trials. Interestingly, other QTL more specifically associated to seed traits were also detected. Our results confirm a high genetic and environmental stability for a genomic region controlling a seed weight trait. Indeed, a TSW QTL (R^2 = 18–22 %) was detected in three winter environments on the top of LGI in Pop9, similarly detected in Krajewski et al. (2011), Bourion et al. (2010) and (Timmerman-Vaughan et al. 2005) in spring pea population. Another productivity QTL (SW) was detected on LGI but only in the spring field trial. In this genomic region lies an interesting candidate: the *subti* marker corresponds to a gene which encodes a subtilisin protein described to be involved in the control of size seed (D'Erfurth et al. 2012). On LGIII, 3 QTL of seed protein content (R^2 = 13–18 %) common among two winter trials and a spring trial were identified in the genomic region of *DiPeptIV* encoding a Dipeptidyl Peptidase IV-like protein and PsAAP1, an amino acid permease I. In this region, Caméor's allele confers higher seed protein content. A QTL for seed number per pod was detected on LGVII during winter trials that corresponded in spring to a QTL for SPC suggesting that this regions could act through assimilate availability on early seed onset in winter conditions and on later seed filling in spring conditions (Larmure and Munier-Jolain 2004). In conclusion, our results suggest that winter frost tolerance can be selected in pea independently to seed productivity and quality.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The experiments comply with the current laws of the country in which they were performed.

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