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ORIGINAL PAPER

Comparative genomics to bridge *Vicia faba* with model and closely-related legume species: stability of QTLs for flowering and yield-related traits

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Abstract This study presents the development of an enhanced map in faba bean. The map contains 258 loci, mostly gene-based markers, organized in 16 linkage groups that expand 1,875 cM, with an average inter-marker distance of 7.26 cM. The combination of EST-derived markers with a number of markers physically located or previously ascribed to chromosomes by trisomic segregation, allowed the allocation of eight linkage groups (229 markers), to specific chromosomes. Moreover, this approach provided anchor points to establish a global homology among the faba bean chromosomes and those of closely-related legumes species. The map was used to identify and validate, for the first time, QTLs controlling five flowering and reproductive traits: days to flowering, flowering length, pod length, number of seeds per pod and number of ovules per pod. Twelve QTLs stable in the 2 years of evaluation were identified in chromosomes II, V and VI. Comparative mapping suggested the conservation of one of the faba bean genomic regions controlling the character days to flowering in other five legume species (*Medicago*, *Lotus*, pea, lupine, chickpea). Additional syntenic co-localizations of QTLs controlling pod length and number of seeds per pod between faba bean and *Lotus japonicus* are likely. The new genetic map opens the way for further translational studies between faba bean and related legume species, and provides an efficient tool for breeding applications such as QTL analysis and marker-assisted selection.

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Introduction

Faba bean (*Vicia faba* L.), a diploid plant with $2n = 2x = 12$ chromosomes, possesses one of the largest genomes among crops (~13,000 Mb), comparable to the hexaploid genome of wheat. This fact complicates the development of genetic and physical maps, as well as map-based cloning. Faba bean is currently one of the most important cool-season food legumes in the world together with dry beans (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.), providing an important source of dietary protein in the human diet and animal feed (FAOSTAT 2009). As a leguminous crop, faba bean plays an essential role in the management of soil fertility through crop rotation, thus contributing to sustainable agriculture.

No wild progenitor is known for this crop and all the attempts of hybridization with other *Vicia* species have proved unsuccessful (Cubero 1982). The traditional faba bean groups are (1) *major* (broad bean, flattened seeds, 1.0–2.0 g/seed), (2) *equina* (horse bean, field bean, flattened seeds, 0.6–1.0 g/seed), (3) *minor* (tic bean, ellypsoidal seeds, 0.4–0.6 g/seed) and (4) *paucijuga*, a primitive form supposedly close to a putative wild progenitor (Cubero 1974) with 0.31–0.40 g/seed. Considered as a relic, crosses involving this primitive form provide huge variability in morphological, yield-related traits, root structure and autofertility to existing germplasm collections.

In faba bean, different markers (morphological, isozyme, RFLPs, RAPDs, seed protein genes and SSRs) have produced genetic maps that identified loci controlling genetically complex traits as seed weight, resistance to crenate broomrape (*Orobanche crenata* Forsk.) and ascochyta blight (Vaz Patto et al. 1999; Roman et al. 2002, 2003; Avila et al. 2004; Diaz-Ruiz et al. 2009; Diaz et al. 2010). An extended review of the faba bean maps and QTLs published so far is summarized in (Torres et al. 2006, 2010 and references therein).

Most of these maps are of low to moderate density, with a minimum marker spacing of around 12–14 cM, and each is composed of between 50 and 317 markers (Torres et al. 2006; Ellwood et al. 2008; Diaz-Ruiz et al. 2009; Diaz et al. 2010). High density faba bean maps composed of robust and transferrable markers are essential for the selection and cloning of agronomically important genes. Moreover, to obtain a useful map for scientific and breeding purposes, linkage and cytogenetic information should be combined. In *V. faba*, this is possible due to the availability of trisomics obtained from crosses with asynaptic mutants (Gonzalez and Martin 1983). The analysis of their respective F_2 progenies has allowed the unequivocal assignment of the first linkage groups (LGs) or molecular markers to specific chromosomes (Torres et al. 1995, 1998; Satovic et al. 1996; Vaz Patto et al. 1999).

The first molecular evidence for the existence of macrosynteny between legumes was reported by Weeden et al. (1992) and Simon and Muehlbauer (1997), by comparing lentil and chickpea with pea linkage maps. After that Choi et al. (2004) and Zhu et al. (2005) published the most comprehensive macrosynteny analysis between legume genomes. Cross-species gene specific markers were used to identify homologous genome segments among eight legume species (*M. truncatula*, alfalfa, *L. japonicus*, pea, chickpea, soybean, mungbean and common bean). These studies were continued by further analysis of synteny among the fabaceae family (Kalo et al. 2004, 2011; Stracke et al. 2004; Mudge et al. 2005; Nelson et al. 2006; Cannon et al. 2006; Phan et al. 2006, 2007; Gutierrez et al. 2005; Ellwood et al. 2008). To provide cross-species amplification, legume consensus sequences have been generated from expressed genes representing single copy of orthologous genes. As a result, several functional genomic resources are available today in both model and important crop legumes (Cannon et al. 2009; Young and Udvardi 2009).

Sequences targeting expressed genes have a significant potential for future identification of tightly linked markers useful in marker-assisted selection (MAS) and map-based isolation of candidate genes. Once a trait of interest is mapped in the crop plant, microsynteny analysis can identify the corresponding region in the model genome, enabling candidate genes to be cloned (Young and Udvardi 2009). Since the EST-PCR primers designed for one species can be used in related species, the cost involved in developing markers is reduced significantly. This fact has special importance for faba bean, a crop where genomic resources are still rather limited.

Recently, Ellwood et al. (2008) reported the first exclusively gene-based genetic linkage map of faba bean using an F_6 population of the cross Vf6 × Vf27 involving an *equina* and a *paucijuga* type, respectively. Intron-targeted amplified polymorphic (ITAP) markers based on alignments between *M. truncatula*, *Lupinus albus*, and *Glycine max* and GLIP markers (Grain Legumes Integrated Project; FP6-FOOD-CT-2004-506223) derived from *M. truncatula* and pea, were used for this purpose. Macrosyntenic relationship between faba bean, lentil and *M. truncatula* was reported; however, none of the faba bean LGs (noted as FB) could be then assigned to a specific chromosome.

Control of flowering time is a major target in plant breeding to produce novel varieties that better adapted to a particular environment (Jung and Müller 2009). Floral transition has been extensively studied in crop and model species and, in recent years, a growing number of related genes and QTLs have been identified (Kumar et al. 2011). As in other crops, flowering time is critical in faba bean to balance the risk of late frost damage against the need for adequate water supply for grain filling at middle and lower latitudes (Nelson et al. 2010) and the need for early maturity at high latitudes (Stoddard et al. 2009). To date, no QTLs controlling faba bean flowering time have been reported.

This study was carried out with the following aims: (1) to develop the most detailed genetic map reported yet in faba bean and refine the order and distance among markers; (2) to assign the gene-based LGs to specific chromosomes; (3) to establish a global level of correspondence among *V. faba* chromosomes and LGs with those of closely-related legume species as a first step towards the comparison of wide syntenic genome regions; and (4) identify and

validate, for the first time, QTLs for flowering time and life cycle-related traits, which is a prerequisite for targeted genetic modification of flowering behaviour in breeding programs.

Materials and methods

Population development and DNA extraction

A population of 124 F_6 RILs derived from a cross between Vf6 × Vf27 was obtained by single-seed descent at IFAPA (Instituto de Investigación y Formación Agraria y Pesquera, Córdoba, Spain). The F_2 progeny of these accessions was used in previous genetic studies by the group (Torres et al. 1995; Vaz Patto et al. 1999; Roman et al. 2004) and later on Ellwood et al. (2008) used the F_6 progeny to develop the first exclusively gene-based genetic linkage map in the species. The female parent, Vf6, is a *equina* medium-seeded field bean with beige seed coat while Vf27 is a black and small-seeded *paucijuga* form. Genomic DNA was extracted from young leaves using liquid nitrogen. The extraction procedure was that described by Torres et al. (1993).

RAPD markers

A total of 34 RAPD primers (OPERON Technologies, Alameda, California, USA) were analysed. These markers were selected for being highly polymorphic in the F_2 maps of the Vf6 × Vf27 cross (Satovic et al. 1996; Vaz Patto et al. 1999).

Microsatellite markers (SSR)

Twenty-three SSR primers, some of which were physically located in chromosome I by PCR with sorted or micromanipulated chromosomes (Pozarkova et al. 2002), were also analysed in the segregating population using the conditions described by Pozarkova et al. (2002).

Isozyme markers

Three enzymatic systems, aspartate amino transferase, (AAT, E.C. 2.6.1.1), peroxidase (PRX, E C 1.11.1.7) and superoxide dismutase (SOD, E.C. 1.15.1.1) that were polymorphic in the F_2 progeny were genotyped by horizontal starch-gel electrophoresis as described by Gottlieb (1973). All the assay solutions were adapted from those reported by Wendel and Weeden (1990). The genetic and chromosomal location for each isoenzyme locus have been reported previously (Torres et al. 1998).

Seed-protein genes

The RIL population was tested for five seed-protein genes coding for viciline, legumin A, legumin B3, legumin B4, and USP (unknown seed protein). The sequences of the primers used for detection of length polymorphisms among these genes were previously reported (Macas et al. 1993; Fuchs and Schubert 1995). Amplification conditions were similar to those used with RAPD primers with some modifications described by Vaz Patto et al. (1999), to maximise the amplification and the resolution of the products.

EST (expressed sequence tag) derived markers

In the former Vf6 × Vf27 study (Ellwood et al. 2008), 131 markers generated the first gene-based faba bean map. In this study, 72 new GLIP and MP (derived after *M. truncatula* and *P. sativum* alignments) markers were assayed. PCR amplification was optimized to obtain a single specific band that cross-amplified in *V. faba* using the parental lines. Markers shared with other faba bean maps used the amplification protocol reported by Diaz-Ruiz et al. (2009). For new markers, the PCR conditions were set up for each new primer pair as follows. Reaction mixtures of 20 µL contained 30 ng of template DNA, 1× PCR buffer, 0.6 mM of each forward and reverse primer, 200 µM of dNTPs, 2 mM $MgCl_2$, and 1 U Taq polymerase (Biotools). Amplifications were carried out in a Eppendorf Thermal Cycler with 40 cycles of 95 °C for 1 min, annealing temperature of 54–60 °C (determined for each primer pair analysed with the Oligo Software for 1 min, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 8 min before cooling to 4 °C.

To test for polymorphism, parental lines together with five F_6 individuals were genotyped. When no polymorphism was detected on agarose gels, PCR products amplified from both parents were treated with a range of restriction endonucleases (36) according to manufacturer's instructions.

RGA (resistance gene analogues)

Ten RGA classes were tested in the parental lines by Palomino et al. (2006) being monomorphic for the 10 primer combinations used. To obtain CAP (Cleaved Amplified Polymorphism) markers, amplification products for each RGA class were digested with restriction enzymes as described in the previous section. PCR products of polymorphic markers were resolved on agarose gels (RGA08 and RGA09 on 2 % and RGA01 on 2.5 % agarose gels), and visualized by ethidium bromide staining.

Morphological traits

One morphological trait (black seed-coat, *Sc/sc*), present in line Vf27, was analysed in the RIL population. This trait was previously reported as being a single dominant in faba bean (Picard 1979; Sjödin 1971; Cabrera 1988).

Field evaluation of life cycle and yield-related traits

The evaluations were conducted at the centre IFAPA in Cordoba, Spain (geographical coordinates 37°51′42″N and 4°48′00″W, altitude 117 m). Average annual temperatures at this location are 24.6 °C and annual rainfall is of 536 mm, mostly concentrated from December to February. The specific conditions of temperature and rainfall along the two seasons of evaluation (2007 and 2008), are detailed in the supplementary data file S4.

The RILs were evaluated using a completely randomized design with two replications. Each replication consisted of 10 seeds per F_7 or F_8 line sown in 1 m rows separated by 0.70 m. Each experiment included the parents Vf6 and Vf27. Sowings were performed on December 7, 2006 and December 13, 2007 and plants were irrigated between flowering and grain filling.

Days to flowering (DF) and flowering length (FL) were recorded in both 2007 and 2008 being each of the rows an experimental unit. DF was scored as the number of days from the sowing until the appearance of the first flower and FL was recorded as the difference of days between the start and the end of flowering (when no flowers were present in the plants).

Besides flowering timing, three reproductive traits related with the fruit set were recorded: pod length (PL) and number of seeds and ovules per pod (NSP and NOP). PL was scored in five plants of each row as the average length (cm) of five pods located at the central part of the main stem. Number of seeds per pod (NSP) was considered as the average number of seeds in five pods of the main stem of five plants. Finally, number of ovules per pod (NOP) stands for the mean number of ovules recorded in the same experimental unit.

Statistics and linkage analysis

Each marker was tested against the expected segregation ratio using an χ^2 goodness of fit. Markers showing normal diploid segregation ($p > 0.01$) and those common to previous maps were selected for further analysis. The linkage map was constructed by MAPMAKER Version 2.0 (Lander et al. 1987) using a LOD score of 3.0 as the threshold for significant linkage. Recombination fractions were converted to centimorgans (cM) using the mapping function of Kosambi (1944). Association between each LG and the putative QTL regions related to flowering time and yield-related traits were determined by interval mapping using QTL Cartographer software v. 2.5 (Wang et al. 2007). Composite interval mapping (CIM) (Churchill and Doerge (1994); Doerge and Churchill (1996) and multiple interval mapping (MIM) (Kao et al. 1999; Zeng et al. 1999) were also performed. Markers to be used as cofactors for CIM were selected by forward and backward stepwise regression. The number of markers controlling the genetic background in CIM was set to five. The threshold for the detection of a QTL was estimated by permutations analysis (Churchill and Doerge 1994) using 1,000 permutations. Intervals of 1-LOD and 2-LOD for position of each QTL were calculated according to Darvasi and Soller (1997).

Results

General features of the genetic map

Segregation analysis of the Vf6 × Vf27 recombinant inbred population revealed 346 polymorphic markers (Table 1). Forty-two of them showing significant segregation distortion or missing data (>25 %) were rejected to avoid bias and false linkages. Therefore, the final linkage analysis generates a map with 258 markers (46 remained unlinked), joined in 16 LGs and covering 1,875.1 cM.

Linkage groups were composed of 2–45 loci with an average inter-marker distance of 7.26 cM (Table 2; Fig. 1). Among these, 199 [47 RAPDs, 11 microsatellites, 2 storage seed proteins, 3 isozymes, 1 morphological marker (black seed colour *Sc/sc*), and 135 ESTs] (supplementary data file S1), were in common with previous mapping studies (Sjödin 1971; Torres et al. 1993; Satovic et al. 1996; Vaz Patto et al. 1999; Pozarkova et al. 2002; Gutierrez et al. 2005; Ellwood et al. 2008; Diaz-Ruiz et al. 2009; Diaz et al. 2010). This allowed the construction of a much more detailed map and the allocation of 8 LGs (229 markers) to specific chromosomes (supplementary data file S2). The remaining 29 markers were joined into 8 unassigned LGs with 7 (LG09), 6 (LG10 and 11) and 2 markers (LG12 to LG16). Markers with slight distorted segregation were distributed among 11 LGs, the largest number of them in LG07 with 8 markers (Fig. 1). Linkage group density was fairly similar, with LGs 1, 3 and 5 being the most saturated.

The inclusion of new markers that are evenly distributed throughout the genome facilitated the integration of several LGs (listed as FB by Ellwood et al. 2008) in the same chromosome. Thus, LG02 integrates three small FB groups (FB-5, FB-9, FB-10) while FB-6 and FB-4 join together to become LG04 in the present study (Fig. 1). The current map shows the highest level of saturation among the faba bean maps described yet. The analysis of 124 versus the 94

Table 1 Number and type of genetic markers used for the construction of the enhanced Vf6 × Vf27 map

Types of marker	References	No. markers analyzed	No. of loci		
			Polymorphic	Mapped	Unlinked
RAPDs		34	141	71	30
ESTs	Ellwood et al. (2008); Seres et al. (2007)[a]	855/379[b]	179	167	12
RGAs	Palomino et al. (2006)	6	3	3	0
Microsatellites	Pozarkova et al. (2002)	23	16	11	2
Isozymes	Torres et al. (1993)	3	3	2	1
Seed protein genes	Macas et al. (1993)	3	3	3	0
Morfological (Sc/sc)	Sjödin (1971)	1	1	1	1
Total		449	346	258	46

The table compiles the ESTs reported in Ellwood et al. (2008) and the new markers analysed in this study

[a] Primers developed by Dr. G. Kiss's group in Gödöllo, Hungary within the framework of the Grain Legume Integrated Project (GLIP) (http://bioweb.abc.hu/cgi-mt/pisprim/pisprim.pl)

[b] Summary of ESTs primer pairs analyzed by Ellwood et al. (2008) and this study: 379 out of 855 ESTs-derived markers, cross-amplified in faba bean

Table 2 Characteristics and distribution of the markers assigned to linkage groups (LGs)

LGs	V. faba chrom[a]	Length (cM)	Average marker spacing (cM)	No. of ESTs derived markers[b]					RGAs[c]	SSRs[d]	RAPDs	Isozymes	Seed protein genes	Morfol.	No. of loci mapped
				GLIP		MP	ML	MLG							
				Mt	Ps										
LG01	lll	290.3	6.45	8	2	9	5	9	0	1	8	1	2	0	45
LG02	ll	389.1	9.26	5	2	6	4	8	1	2	13	0	0	1	42
LG03	l.B	232.5	6.11	8	1	9	3	12	0	0	5	0	0	0	38
LG04	l.A	242.8	6.93	8	1	6	3	3	0	4	10	0	0	0	35
LG05	Vl	179.2	5.97	11	0	6	3	1	2	0	7	0	0	0	30
LG06	l.A	90.2	6.44	1	1	1	1	4	0	0	6	0	0	0	14
LG07	V	100.3	7.71	3	1	3	1	1	0	1	2	1	1	0	13
LG08	l.A	121.7	10.14	2	0	0	0	0	0	3	6	0	0	0	12
LG09		51.0	7.60	0	0	1	0	2	0	0	4	0	0	0	7
LG10		64.8	11.40	0	0	1	1	1	0	0	3	0	0	0	6
LG11		58.6	9.76	2	0	0	1	1	0	0	2	0	0	0	6
LG12		19.5	9.75	0	0	1	0	0	0	0	1	0	0	0	2
LG13		3.5	1.75	0	0	0	0	1	0	0	1	0	0	0	2
LG14		10.9	5.45	0	1	1	0	0	0	0	0	0	0	0	2
LG15		9.8	4.90	0	0	0	0	0	0	0	2	0	0	0	2
LG16		10.9	5.45	1	0	0	0	0	0	0	1	0	0	0	2
Total		1875.1	7.26	49	9	44	22	43	3	11	71	2	3	1	258

cM centimorgans

[a] Chromosome of *Vicia faba*

[b] Gene markers derived from *Medicago truncatula (*Mt) *or Pisum sativum* (Ps), designed within the framework of the *GLIP* Project (Seres et al.2007); *MP*, *MLG* y *ML* gene markers designed by Phan et al. (2007)

[c] *RGAs* resistance gene analogues (Palomino et al. 2006)

[d] *SSR* microsatellites designed by Pozarkova et al. (2002)

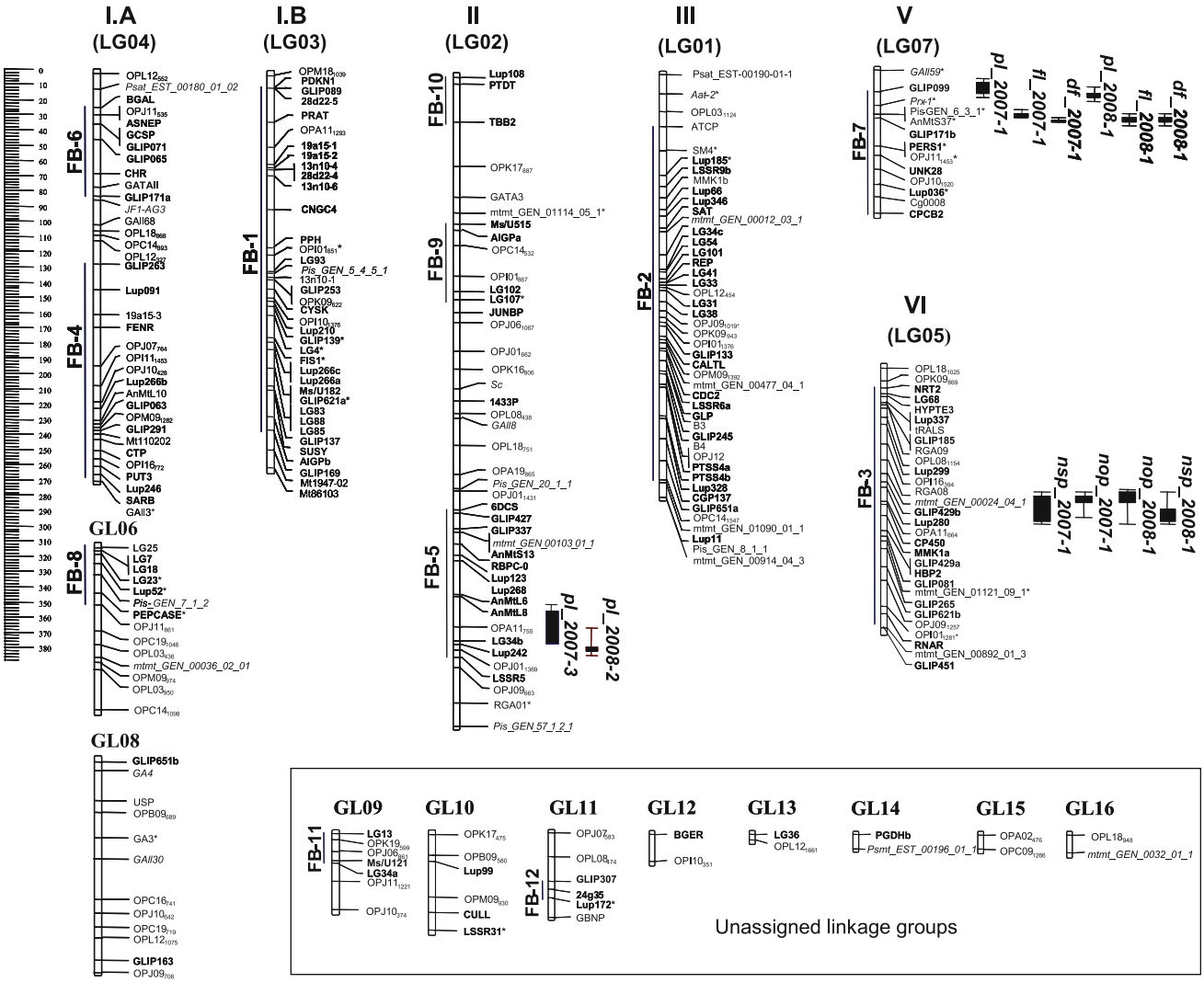

Fig. 1 Genetic linkage map developed in the faba bean (*Vicia faba* L.) recombinant inbred population Vf6 × Vf27 and position of the stable QTLs detected for days to flowering (DF), flowering length (FL), pod length (PL), number of ovules per pod (NOP) and number of seeds per pod (NSP) in 2007 and 2008. Genetic markers and linkage groups (noted as FB) common to Ellwood et al. (2008) are shown in *bold*. Markers common to Diaz-Ruiz et al. (2009) and Diaz et al. (2010) are shown in *italics*. *Bars* represent the 1-LOD (*full bar*) and 2-LOD (*simple bar*) confidence intervals for the QTL peaks obtained by CIM and MIM method, respectively. Marker distance is given in cM. *Asterisk* slightly distorted markers

individuals used in the previous study has certainly contributed to precise the marker order and distance in the respective LGs. As a result, marker order was mostly consistent as only five markers displayed an inverted position relative to the previous map.

Assignment of LGs to specific faba bean chromosomes

The correspondence of eight LGs with their respective chromosomes (Fig. 1) was established by means of markers with known chromosomal locations or linked to previously assigned markers.

Twenty-three SSR markers assigned to chromosome I (Pozarkova et al. 2002) were assayed in this population, 16 revealed polymorphisms and 11 were integrated in the map. Location of GAll68, GATAll, JFI-AG3 and GA4 in chr. I agreed with their predicted location (Pozarkova et al. 2002) and previous assignments (Roman et al. 2004; Avila et al. 2004; Diaz-Ruiz et al. 2009). GA3, GAll3 and GAll30 further confirmed their location in this chr. by linkage with USP, a seed storage protein, assigned to the metacentric chr. I by Macas et al. (1993). In contrast GAll8 and GATA3 were assigned to chr. II (LG02), as was SM4 in chr. III (LG01). These outcomes point towards a contamination of the sorted fractions used for the construction of the original chr. I library, as verified in case of microsatellite GAII8 by Pozarkova et al. (2002). Markers AG6, AG2 and GATA2 remained unlinked. Based on this

information the association between LGs and chromosomes were as follows:

Chromosome I

Microsatellites GATAII, JF1-AG3, GAII68, GAII3, GA4, GA3 and GAII30 together with the seed storage protein USP and the GLIP marker mtmt_GEN_00036_02_1 analyzed by Gutierrez et al. (2005) and Diaz et al. (2010), identified LGs 4, 6 and 8 as part of the faba bean metacentric chr. I (IA). Another fraction of the same chromosome (chr. IB) was identified by the marker Pis-Gen5_4_5_1 mapped by Gutierrez et al. (2005).

Chromosome II

The black seed-coat colour (*Sc*) was ascribed by Sjödin (1971) to the long arm of chromosome II. RAPD markers, linked to the *Sc/sc* gen by Vaz Patto et al. (1999), and different EST-derived markers (Fig. 1), used in previous studies (Gutierrez et al. 2005; Diaz et al. 2010), allowed the unambiguous assignment of LG02 to this chr.

Chromosome III

The isozyme *Aat-2* and the legumines B3 and B4 (the only seed protein genes producing clear and reproducible polymorphic bands), allocated by Torres et al. (1995, 1998) and Vaz Patto et al. (1999), identified LG01 as a chr. III segment.

Chromosome IV

No clear-cut LG assignation to faba bean chr. IV has been possible to date. The heterogeneity of heterochromatin distribution reported in this chr. (Greilhuber 1975) may have prevented the accomplishment of this objective.

Chromosome V

The isozyme *Prx-1* located by trisomy on chromosome V (Torres et al. 1995), allowed a clear assignment of LG07 to chr. V.

Chromosome VI

LG05 was assigned to chr. VI by means of the RAPD markers $OPL08_{1154}$, linked with $OPI16_{394}$, which showed trisomic segregation in a Vf6 × Vf27 F2 family derived from an individual trisomic for this chromosome (Vaz Patto et al. 1999). Furthermore, Mtmt_GEN_00024_04_01 linked with the two previous RAPDs and with the isozyme *Sod-1* by Gutierrez et al. (2005), further corroborate this localization, since isozymes Sod-*1* and *Pgd-p* were previously ascribed to chr. VI (Torres et al. 1998).

Macrosynteny with related legume species

In this study, the enrichment of the Ellwood et al. (2008) map by analysing 72 new EST-derived markers from *Medicago*, pea, lupin and soja has facilitated new comparisons with the genetic and physical maps of important legume crops (Weeden et al. 1992; Choi et al. 2004; Kalo et al. 2004; Zhu et al. 2005; Aubert et al. 2006; Phan et al. 2006, 2007; Ellwood et al. 2008), which are detailed in Table 3.

Macrosynteny studies between faba bean and *M. truncatula* (Ellwood et al. 2008 and this study) or between *L. culinaris* and *M. truncatula* (Phan et al. 2007) using common orthologous markers, revealed a high level of colinearity and a similar pattern of homology among the three species (Figs. 2, 3). Faba bean chr. I.B (FB-1) and Lc-II were virtually syntenic to Mt-8, and shared six markers which were fairly uniformly distributed in the corresponding LGs. Moreover, chr. III (FB-2) and Len-III, with five markers in common, were syntenic with Mt-1 (Figs. 2, 3). Other examples include chr. VI (FB-3), Len-l and Mt-4 and FB-5, FB-9 and FB-10 (merged in chr. II), which are collinear with Len-III, Len-VI and Len-VII, and these in turn with Mt-3, according to Ellwood et al. (2008) and Kalo et al. (2011). In fact, differences in chromosome number between the genomes of *L. culinaris* ssp. culinaris and *M. truncatula* ($n = 7$ and $n = 8$, respectively) can be explained by this moderate level of rearrangements (Phan et al. 2006), although the same did not occur between *Vicia* and *Lupinus albus* ($n = 6$ and $n = 25$).

The inclusion of only 12 pea EST-derived markers prevented us from performing detailed *V. faba* and *Pisum* sp. comparisons. Nevertheless, as reported by Choi et al. (2004) and Kalo et al. (2011), large-scale synteny blocks between chromosomes and chromosome segments of *Medicago*, pea and faba bean were evident (Table 3; Fig. 2), as expected from three Galegoid clade members, belonging to the tribe Viceae.

QTL mapping analysis

In this study, the genetics of the flowering traits, days to flowering (DF) and flowering length (FL), was explored, together with the reproductive traits pod length (PL) and number of seeds and ovules per pod (NSP and NOP). The descriptive statistics of these traits are presented in Table 4 and the QTL analysis outcomes were the following:

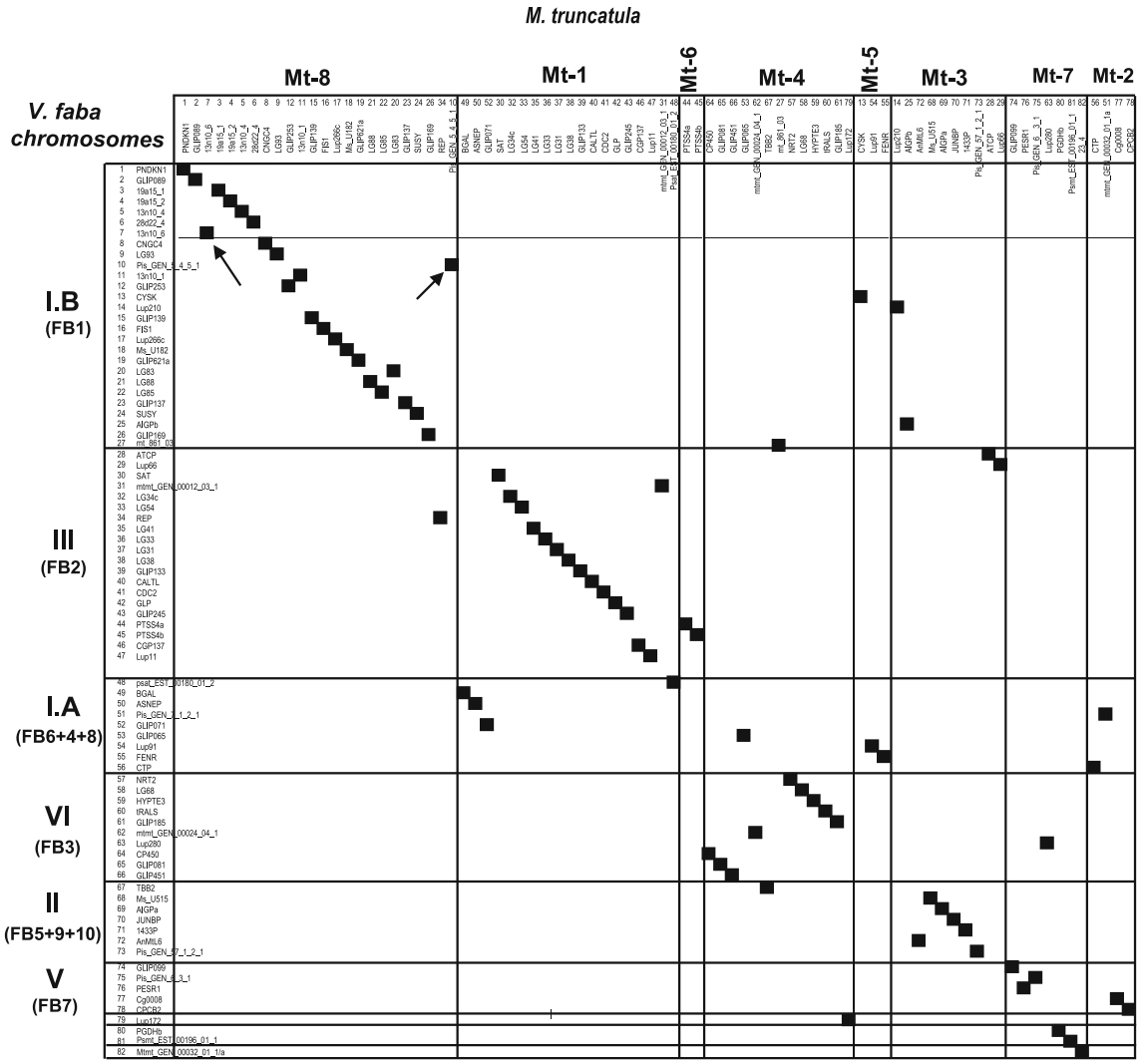

Fig. 2 Matrix dot-plot of the common gene-base markers mapped in faba bean and *M. truncatula*. The loci are listed vertically and horizontally, respectively, according to the orders in their linkage groups

Days to flowering (DF)

QTL analysis for DF revealed 5 QTLs in 2007 (Table 5). The QTL with the highest LOD value (*df-2007-1*) was located in LG07 (chr. V), and explained 27.5 % of the phenotypic variation (Table 5; Fig. 1). *Df-2008-1* was also detected in 2008 in a similar position of the map explaining a similar percentage of the variance (Table 5). As shown in Table 3, the faba bean chr. V corresponds to the *Medicago* chr. VII.

The same can be inferred for QTLs *df-2007-3* and *df-2008-2*, identified in LG04 (chr. IA), since they share the sign and value of the additive genetic effects, and explain a similar percentage of variation for this trait (Table 5). Nevertheless, their relative distant location on the array (around of 30 cM) has prevented confirmation of the homology so far. Three additional QTLs were detected in 2007, two in LG03/chr. IB (*df-2007-2* and *4*) and *df-2007-5* in LG01 (chr. III), that were not stable in the following season.

Flowering length (FL)

Chromosome V was related as well to FL (Fig. 1). One QTL controlling the trait was detected in the 2 years of study (*fl-2007-1* and fl-*2008-1*). The position of these QTLs almost coincides with that detected for DF (Fig. 1), which explains the high correlation observed between both characters (data not shown). In 2008, a second QTL (*if-2008-2*) was detected in LG04 (chr. IA), matching with the position of a second QTL for DF (*df-2008-2*). The MIM method did not detect interaction between these QTLs. In both years the model explained a similar percentage of the phenotypic variation of the character (approx. 30 %).

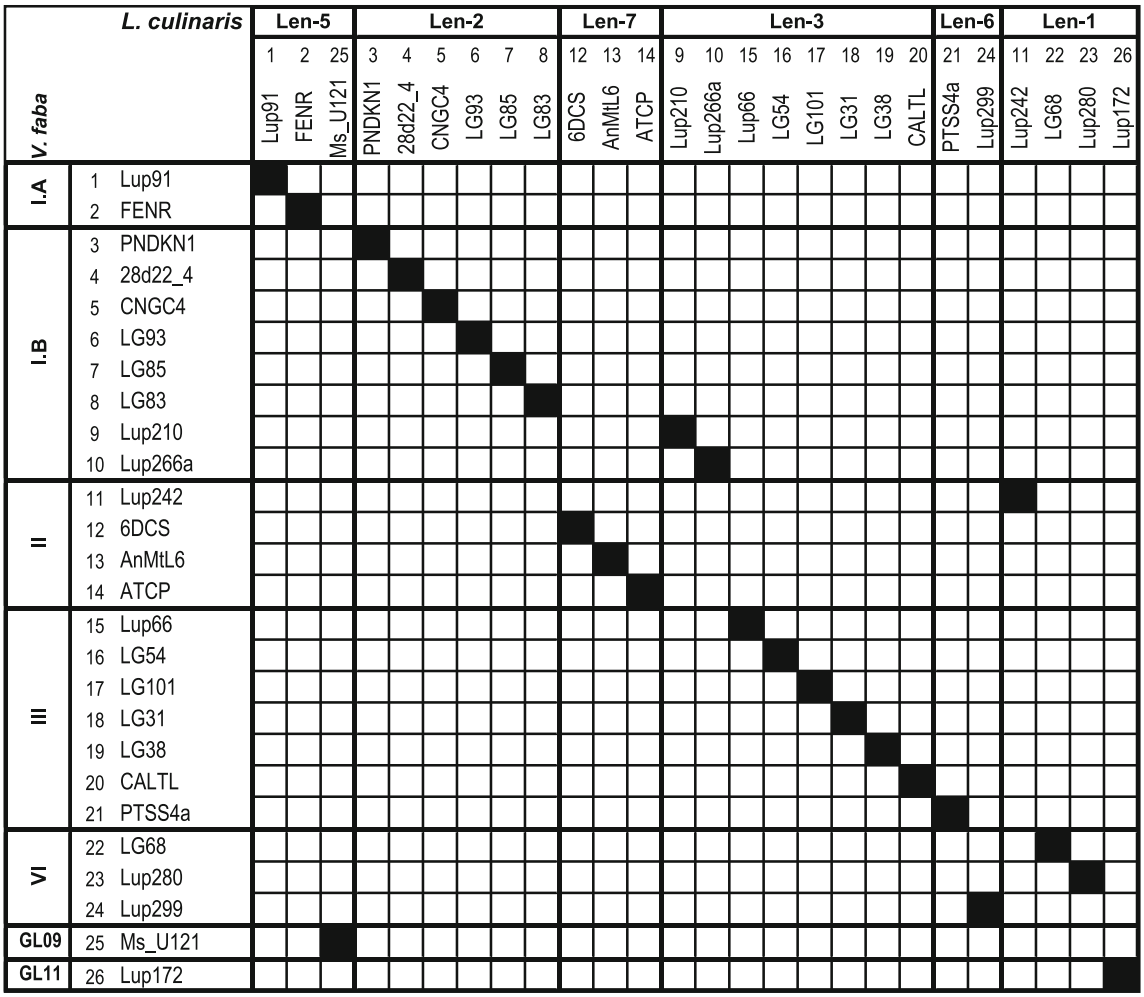

Fig. 3 Matrix dot-plot of the common gene-base markers mapped in faba bean and lentil. The loci are listed vertically and horizontally, respectively, according to the orders in their linkage groups

Pod length (PL)

Two QTLs (*pl*-2007-1 and *p*l-2007-3) located in chr. V and II, respectively, remained stable in the analysis of 2008 (*pl-2008-1* and *pl-2008-2*) and explained a similar percentage of the variation along the years (Table 5). Avila et al. (2005) detected 4 QTLs for this trait in the F_2 population of a different cross, and two of them were assigned to specific chromosomes. Thus, pl-1 mapped in chromosome III, whereas pl-4 was on chromosome V, adjacent to the isozyme marker *Prx-1*. Figure 2 shows how the QTL for pod length, identified on chromosome V and stable in the two agronomic seasons, also mapped close to this marker. The result points towards the possible conservation of this QTL in different faba bean genetic backgrounds. Gondo et al. (2007) evaluated this character in the model species *L. japonicus*, identifying 3 QTLs in chromosomes 1, 5 and 6 corresponding to chromosomes II + V, III and IA, respectively, in faba bean (Table 2). The QTL in chromosome 1 was detected in the 2 years, explaining 16 and 22 % of the variation of the character, as was the case with the PL QTLs in our study. This further supports the hypothesis of conservation of the *L. japonicus* QTL in chromosome 1 in different species, since *pl*-2007-1 and *pl*-2007-3 (or *pl*-2008-1 and *pl*-2008-2) located in the faba bean chromosomes II and V were validated as well in two environments and explained a similar percentage of variation (from 17 to 26 %). As discussed in previous sections, this premise should be checked by including in both maps new common markers linked to this trait.

Number of ovules per pod (NOP)

A single QTL was identified on chr. VI in the 2 years (*nov*-2007-1 and *nov*-2008-1), explaining a similar percentage of the trait variation (26.5 and 22.4, respectively). To our knowledge no equivalent QTLs have been reported so far in faba bean or other related legume crops.

Table 3 Llinkage groups or chromosomes correspondences among *Vicia faba, Lotus japonicus, Pisum sativum, Cicer arietinum, Phaseolus vulgaris, Glycine max, Vigna radiata, Lens culinaris, Lupinus albus* and the model species *Medicago truncatula* (based on Phan et al. 2006)

	Chromosome	Linkage group/chr.								
	M. truncatula[a]	*V. faba*[b]	*L. japonicus*[a]	*P. sativum*[c, e]	*C. arietinum*[d]	*P. vulgaris*[d]	*G. max*[d]	*V. radiata*[d]	*L. culinaris*[f]	*L. albus*[f]
	I	III.A + III.B	5	II	4	A	d1, i	4	III	15
	II	I.A	3, 6	III, VI	1	G, K	h, e, l, g	7, 8	VI	16
	III	II.A + II.B	1, 3	III	5	K, F, H	b2, c1, c2	6, 7	III, V, VII	7, 28
	IV	VI.A	4	VII	6	J	a2, c2, b1, h	5	I	3, 5, 13
	V	–	2	I	8, 2	D	a1, b1, d1, q	3	IV	10, 11
	VI	–	2	VI	2	–	–	–	III	
	VII	V	1	V	3	F, H	g, j, e	2	I	1, 17
	VIII	I.B	4	IV	7	C	f, k, j, e	1, 11	II	19
Chromosome no.	$2n = 2x = 16$	$2n = 2x = 12$	$2n = 2x = 16$	$2n = 2x = 14$	$2n = 2x = 16$	$2n = 2x = 22$	$2n = 4x = 40$	$2n = 2x = 22$	$2n = 2x = 14$	$2n = 2x = 50$
Genome size (Mbp[g])	466	13.059	466	4.337	931	588	1.103	515	4.116	588

[a] Choi et al. (2004)

[b] Gutierrez et al. (2005) and present study

[c] Kalo et al. (2004)

[d] Zhu et al. (2005)

[e] Aubert et al. (2006)

[f] Phan et al. (2007)

[g] Million of base pairs

Table 4 Descriptive statistics of the five traits recorded in the population in 2 years

Trait	Years	Parental		RILs population								
		Vf6	*Vf27*	*N*	Average	SD	Min.	Max.	CV	Skewness	Kurtosis	*P*(W)
DF (days)	2007	108.0	97.7	114	106.7	8.3	87.0	131.0	7.8	−0.19	0.36	0.091
	2008	102.2	82.2	114	101.3	9.1	79.1	125.0	9.0	−0.14	0.39	0.109
FL (days)	2007	n.a.	57.0	93	51.7	8.0	30.3	69.6	15.5	0.25	−0.05	0.081
	2008	47.5	57.0	114	42.8	8.6	20.0	66.0	20.1	0.19	0.32	0.145
PL (cm)	2007	3.9	3.2	109	3.8	0.8	2.3	7.0	20.8	1.75	4.52	0.000
	2008	3.5	4.2	114	3.9	0.9	2.3	7.5	24.0	1.77	3.72	0.000
NOP	2007	2.4	2.7	109	2.6	0.4	1.4	3.3	14.4	−0.74	0.33	0.002
	2008	2.1	3.1	114	2.6	0.4	1.8	3.6	14.8	−0.12	−0.48	0.397
NSP	2007	2.4	2.6	109	2.5	0.4	1.4	3.3	16.2	−0.57	−0.23	0.007
	2008	1.7	3.0	114	2.3	0.4	1.3	3.4	18.5	0.07	−0.19	0.647

DF beginning of flowering, *FL* flowering length, *PL* pod length, *NOP* number of ovules per pod, *NSP* number of seed per pod, *N* number of *RILs*, *SD* standard deviation, *CV* coefficient of variation (%), *P*(W) Shapiro–Wilk test for normality and *p* value, *n.a.* not available

Number of seeds per pod (NSP)

Number of seeds per pod is an important breeding index which relates to NOP and the success rates of seed set. Four QTLs were identified in 2007, which jointly accounted for 64 % of the variation of this character (Table 5). QTL *nsp*-2007-1 in chr. VI, explaining between 26 and 33 % of the NSP genetic variation, was the only one detected in both years. Studies in *L. japonicus* (Gondo et al. 2007) revealed seven QTLs for this trait in chromosomes 1, 4, 5 and 2 that might roughly correspond to chromosomes V + II, I.B + VI and III in faba bean, respectively (Table 3). However, regions bearing NSP QTLs require further saturation with orthologous common markers to support the hypothesis of homology between some of these QTLs in both species.

Discussion

The development of an enhanced map in the faba bean RIL population Vf6 × Vf27, including 167 gene-derived markers as well as 91 microsatellite, isozyme, RAPD and morphological markers (some of them with known location), allowed us to establish the correspondence between faba bean LGs and chromosomes, but also to incorporate for the first time *V. faba* in a consensus comparative array. This constitutes a key step towards the comparison of extensive faba bean heterologous genomic regions.

Several microsatellites, seed protein genes, isozymes and the morphological marker *Sc*, with previous chromosomal assignments (Macas et al. 1993; Vaz Patto et al. 1999; Pozarkova et al. 2002; Roman et al. 2004; Avila et al. 2004; Diaz-Ruiz et al. 2009) have been greatly useful in allocating several LGs to chromosomes. The only discrepancy with previous maps was the location of GAll59 in chr. V (LG07) while Roman et al. (2004); Avila et al. (2004) and Diaz-Ruiz et al. 2009, allocate this SSR in chromosome VI. Saturation of the adjacent region bearing the SSR GAll59 should be pursued to settle this inconsistency.

In addition, the map included three RGAs, which were isolated and characterized in *V. faba* and *C. arietinum* (Palomino et al. 2006, 2009). This strategy represents a tool to identify candidate resistance genes that can be used in the development of molecular markers for MAS (Deng et al. 2000). In the present study three RGAs revealed polymorphism, RGA01 was located in chr. II (LG02), while RGA08 and RGA09 were assigned to Chr. VI (LG05). Their possible association with resistance genes in the species remains to be determined.

The genome coverage (1,875.1 cM) is higher than that obtained in most of the previous studies (ranging from 1,200 to 1,700 cM). Nonetheless, it was lower than in the Diaz-Ruiz et al. (2009) map, which included 277 markers in 21 LGs and covered 2,856.7 cM with an average distance of 12.72 cM. Since a similar number of markers was used in the two studies (277 vs. 258 in this work), the type of markers used by Diaz-Ruiz et al. (2009), mostly RAPDS, could have led to some overestimation of the genetic distance. RAPD markers are likely to produce higher rates of genotyping error than the robust and reproducible EST-derived markers primarily used in the present work (Pompanon et al. 2005).

The map was used to identify and validate, for the first time, QTLs controlling five flowering time and reproductive traits. Although manipulating of flowering time is a primary breeding objective to modify the phenological adaptation of the crops to the environment, the genetics of faba bean flowering loci has remained largely unexplored. By contrast, genetic variation and control of flowering are

Table 5 Putative QTLs for flowering timing and yield-related traits detected in the faba bean RIL population Vf6 × Vf27 by composite interval mapping (CIM) and multiple interval mapping (MIM)

QTL[a]	Chr./LG	Marker interval	CIM				MIM		
			Peak	LOD	Add	R^2	Peak	Add	R^2
Days to flowering									
df-2007-1	V 07	AnMtS37	29.36	11.75	5.26	25.33	29.32	5.01	27.50
df-2007-2	I.B 03	Mt1947_02	223.41	5.11	−2.64	9.41	223.41	−2.98	13.49
df-2007-3	I.A 04	$OPI11_{1453}$	181.70	4.43	−2.43	8.07	180.67	−2.50	5.83
df-2007-4	I.B 03	CNGC4–PPH	101.40	3.11	2.14	6.29	99.40	2.50	11.66
df-2007-5	III 01	Lup328–CGP137	221.83	3.04	2.49	8.65	221.83	2.44	9.29
Total									67.77
df-2008-1	V 07	Pis-GEN-6-3-1	29.32	7.77	5.53	21.95	29.36	5.37	23.68
df-2008-2	I.A 04	Mt110202	208.68	3.52	−2.73	8.50	208.68	−2.66	9.49
Total									33.17
Flowering length									
fl-2007-1	V 07	Prx-1–Pis-GEN-6-3-1	27.77	7.31	−5.30	28.78	29.36	−5.20	30.61
Total									30.61
fl-2008-1	V 07	Pis-GEN-6-3-1	29.32	5.07	−4.18	13.69	29.32	−4.79	20.25
fl-2008-2	I.A 04	Mt110202	208.68	4.43	2.93	11.01	208.68	2.79	11.25
Total									31.50
Pod length									
pl-2007-1	V 07	GAII59–GLIP099	14.01	7.53	−0.58	23.53	15.38	−0.56	24.99
pl-2007-2	I.A 04	GLIP065	39.36	5.81	0.32	15.32	39.36	0.33	16.67
pl-2007-3	II 02	$OPA11_{755}$–LG34b	329.66	4.48	0.27	11.43	328.66	0.30	11.97
pl-2007-4	I.B 03	PRAT	33.95	4.14	0.26	10.45	31.05	0.24	11.68
Total									63.31
pl-2008-1	V 07	GLIP099	15.38	4.30	−0.52	13.92	11.01	−0.58	25.74
pl-2008-2	II 02	Lup242	338.39	3.84	0.32	10.53	330.66	0.69	25.80
Total									65.21
Number of ovules per pod									
nop-2007-1	VI 05	Lup280	79.43	8.86	−0.20	26.78	78.99	−0.20	26.54
Total									26.54
nop-2008-1	VI 05	Lup280	79.43	3.81	−0.14	11.36	77.99	−0.19	22.43
Total									22.43
Number of seeds per pod									
nsp-2007-1	VI 05	$OPA11_{664}$–CP450	84.53	8.17	−0.21	25.22	80.43	−0.21	26.02
nsp-2007-2	I.A 04	GLIP071–GLIP065	36.19	4.71	0.19	17.98	39.19	0.15	8.81
nsp-2007-3	I.A 04	CHR	59.95	3.41	−0.15	9.95	59.95	−0.14	8.13
nsp-2007-4	II 02	$OPL18_{751}$–$OPA19_{865}$	230.63	3.39	0.14	11.51	231.63	0.16	15.21
Total									64.07
nsp-2008-1	VI 05	$OPA11_{664}$–CP450	88.53	8.52	−0.27	37.93	88.53	−0.25	33.46
Total									33.46

Parameters were estimated from phenotypic data of 124 F_6 individuals derived from this cross. Peak QTL position on chromosome of the maximum LOD (cM); Add, additive effect was determined with respect to VF27. The negative signs indicates that the contributing alleles were from VF6; R^2, proportion of phenotypic variance explained by the respective QTL (%)

QTL quantitative trait locus, *RIL* recombinant inbred lines, *df* days to flowering, *fl* flowering length, *p*l pod length, *nop* number of ovules per pod, *nsp* number of seeds per pod, *Chr./LG* chromosome and linkage group, *CIM* composite interval mapping, *MIM* multiple interval mapping

[a] QTL designations (e.g., *df-2007*-1) are indicated by first letters of trait name, continued of by the year of detection and the number of QTLs detected this year

well documented in model and crop legume species (Pierre et al. 2008; Maloof 2010; De Mita et al. 2011). Over the last decade, work in rabidopsis (Ehrenreich et al. 2009), rice (Izawa 2007; Maloof 2010) and pea (Weller et al. 2009) has produced major insights into the genes and genetic mechanisms controlling responses to photoperiod, flower development, and others. Pea is the legume crop for which the genetic control of flowering is best understood (Weller et al. 2009), but genetic variation has also been documented in other legume species and several flowering loci or major-effect QTLs have been identified in soybean, common bean, chickpea, lentil, clover and Medicago (Sarker et al. 1999; Cogan et al. 2006; Millan et al. 2006; Phan et al. 2007; Pierre et al. 2008).

In this study, six QTLs identified in chromosomes II, V and VI (Fig. 1) were stable in the 2 years of evaluation: one QTL for days to flowering (DF), one for flowering length (FL), two controlling pod length (PL), one for number of seeds per pod (NSP) and one for number of ovules per pod (NOP), representing attractive targets for future faba bean breeding applications. Interestingly, marker Pis-GEN-6-3-1, closely linked to the most important DF QTL, amplifies a putative candidate gene in pea (gpa1), coding for a glyceraldehyde-3-phosphate dehydrogenase (G3PD) subunit A. G3PD activity has been implicated in floral induction in different plant species (Bonzon et al. 1987; Orr 1987) offering the possibility of application in current faba bean breeding programs.

In *Lotus japonicus*, Gondo et al. (2007), identified 2 QTLs for DF in chr. 1 that explained around 14 % of the total genetic variation of this trait. Simultaneously, DF QTLs were reported in lentil LGs I and X (Fratini et al. 2007), although the lack of common orthologous markers in both maps prevents the establishment of homologies, at present. In lupine, early flowering is controlled by the dominant allele *Ku* (Gladstones 1967) located in a LG1 region syntenic with *M. truncatula* chromosome VII (Nelson et al. 2006). In chickpea, the genetic basis of flowering time has been reported as being digenic (Gumber and Sarvjeet 1996), with two major loci (*ppd* and *elf-1)* controlling the trait. Moreover, some QTLs related with flowering date have been mapped in LG3 (Choi et al. 2004; Cobos et al. 2009; Aryamanesh et al. 2010) and LG4 (Cobos et al. 2007). There is evidence that the chromosomic region in LG3, bearing the QTL "days to flowering" ($_{DF3}$), is syntenic with a region on *Medicago* chr. 7 and on pea LG5, which contains a conserved major regulator of the flowering and vernalization response (Nayak et al. 2010; Millan et al. 2010; Hecht et al. 2011). Finally, Julier et al. (2007), also detected different QTLs controlling this trait on *M. truncatula* chromosome VII.

The syntenic relationships derived from this study and the information reported in other legume species allows us to establish a clear correspondence between chromosomes V in faba bean, I in Lotus, V in pea, LG1 in lupine, III in chickpea and VII in *Medicago* (Table 3), thus, suggesting the conservation of at least one of the genomic regions responsible for the character DF in the six legume species. Nevertheless, it is compulsory to add new common orthologous markers or candidate genes linked to flowering genes in the faba bean map to verify this hypothesis.

Additional syntenic co-localizations between faba bean and *L. japonicus* PL and NSP QTLs were observed. Thus, Gondo et al. (2007) identified an stable PL QTL in *L. japonicus* chr. 1 as occurred in faba bean chr. V (Fig. 1). The same study revealed seven QTLs for NSP in *L. japonicus* chr. 1, 2, 4 and 5. Based on the established chromosome/LG correspondences (Table 3), QTLs for NSP in *L. japonicus* chr. 4 can be regarded as potentially orthologous QTL to the faba bean NSP QTL in chr. VI.

Mapping studies examining complex and correlated traits often identify coincident QTLs. Thus, 10 of the reported QTLs were not randomly distributed but collocated in clusters in chr. V and VI. Cluster formation was reported in grass crops (Cai and Morishima 2002; Glémin and Bataillon 2009) and legumes such as *P. sativum* (Bourion et al. 2002), *P. vulgaris* (Tar'an et al. 2002), *Lotus* (Gondo et al. 2007) and soybean (Liu et al. 2007), revealing a trend for domestication-related traits to colocalize in specific genomic areas. As reported by Cai and Morishima (2002), the cluster phenomenon can be considered "multifactorial linkages", followed by natural selection favouring co-adapted traits, while some cases of clustering may be attributable to pleiotropic effects of individual genes. In faba bean, chromosome VI contains two stable yield-related QTLs (NOP and NSP). Vaz Patto et al. (1999) analysing the F_2 population of this crop, reported putative QTLs for seed weight in the same chromosome which further support the mentioned cluster formation.

Our study contributes to the future identification of candidate genes associated with traits of interest in faba bean. Syntenic regions could be used to identify orthologous genes controlling traits of agronomic interest in *V. faba*, as reported in other crop legumes. The availability of functional genomic resources (ESTs and other gene sequences) from model or related species, combined with QTL analysis using a positional candidate gene approach, will certainly promote the development of perfect markers in this crop, as they are developed from putative coding sequences with known function and complete association with the key gene or QTL. The information reported, together with the availability of extensive sequence databases and the established synteny between faba bean and closely related legume crops, opens new avenues for future molecular analysis of the flowering process and other yield-related traits in this crop.

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