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ORIGINAL PAPER

# Validation of QTLs for *Orobanche crenata* resistance in faba bean (*Vicia faba* L.) across environments and generations

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**Abstract** Broomrape *(Orobanche crenata* Forsk.) is a major root–parasite of faba bean (*Vicia faba* L.), that seriously limits crop cultivation in the whole Mediterranean area. This parasitic weed is difficult to control, difficult to evaluate and the resistance identified so far is of polygenic nature. This study was conducted to identify genetic regions associated with broomrape resistance in recombinant inbred lines (RILs) and to validate their previous location in the original $F_2$ population derived from the cross between lines Vf6 and Vf136. A progeny consisting of 165 $F_6$ RILs was evaluated in three environments across two locations in 2003 and 2004. Two hundred seventy seven molecular markers were assigned to 21 linkage groups (9 of them assigned to specific chromosomes) that covered 2,856.7 cM of the *V. faba* genome. The composite interval mapping on the $F_6$ map detected more quantitative trait loci (QTL) than in the $F_2$ analysis. In this sense, four QTLs controlling *O. crenata* resistance (*Oc2–Oc5*) were identified in the RI segregant population in three different environments. Only *Oc1,* previously reported in the $F_2$ population, was not significant in the advanced lines. *Oc2* and *Oc3* were found to be associated with *O. crenata* resistance in at least two of the three environments, while the remaining two, *Oc4* and *Oc5,* were only detected in Córdoba-04 and Mengíbar-04 and seemed to be environment dependent.

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## Introduction

Parasitic plants, as is the case of broomrapes, are important agents of biotic stress in different legume species. *Orobanche crenata* (crenate broomrape) is a significant pest in faba bean (*Vicia faba*) and in many other grain and forage legumes in the Mediterranean basin, North Africa and the Middle East. This holoparasitic weed obtains carbon, nutrients and water through haustoria, which connect the parasite with the host vascular system. As a result, it can cause severe reductions in crop yield, depending on the infestation level and the planting date. Losses ranging from 50 to 80% have been reported in faba bean fields with medium and high levels of infestation, respectively (Gressel et al. 2004). Heavy *Orobanche* infestation not only can lead to crop yield reduction, but also may force farmers to abandon of traditional legume growing areas (Abu-Irmaileh 1994). The control of *Orobanche* spp. remains a challenge because fully satisfactory and economic control measures are not yet available. Several attempts have been made involving cultural practices, such as late sowing and crop rotation, chemical control, synthetic germination, stimulation,

nitrogenous fertilization and soil solarization (López-Granados and Garcia-Torres 1998; Pieterse 1991; Parker and Riches 1993; Joel 2000; Mauromicale et al. 2001; Rubiales et al. 2006), but none of them with noticeable success.

Growing genetically resistant cultivars has been largely recognized as the most appropriate and cost-effective means of managing pest and diseases (parasitic weeds among them). In faba bean breeding programs, the development of cultivars resistant to *O. crenata* is a key component and has also become a major research objective. Resistance against most parasitic weeds is, however, difficult to assess, scarce, of complex nature and of low heritability, making breeding for resistance a difficult task (Rubiales 2003). Not much resistance to *O. crenata* was available in faba bean until the appearance of the Egyptian line F402 (Nassib et al. 1982) that allowed the development of several resistant cultivars (Giza 402, Giza 429, Giza 674, Giza 843) that are being utilized in crosses to breed for *Orobanche* resistance (Khalil et al. 2004).

Broomrape resistance identified so far is of polygenic nature (Cubero and Moreno 1999). In faba bean, the resistance is controlled by a quantitative genetic system with strong additive effects where dominance, if present, is generally partial and weak (Cubero 1983). The results from studies of quantitative trait loci (QTL) for *O. crenata* resistance in legumes using molecular markers have corroborated its complex inheritance (Román et al. 2002; Valderrama et al. 2004). Although the aggressiveness of different broomrape biotypes is highly variable, plant–parasite interaction is very low suggesting an incomplete and race non-specific genetic system (Cubero 1991; Cubero and Hernández 1991). Besides, breeding programs for broomrape resistance are complicated, since selections rely primarily on field evaluations where natural parasite populations occur and homogeneity of seed distribution in the soil is difficult to achieve. Moreover, infections are highly influenced by the environmental conditions. Although evaluations in controlled environments could avoid these difficulties, they are extremely expensive and difficult to manage.

The development of genetic maps offers a new approach for improving resistance to *O. crenata* and combining it with favorable agronomic traits. Molecular markers are of great interest for dissecting quantitative resistance by identifying associated genomic regions or QTLs and molecular markers tightly linked to the trait. The development of marker-assisted selection (MAS) techniques for broad-based polygenic resistance is a particularly promising approach, since *Orobanche* resistance tests are difficult, expensive and sometimes unreliable. The first study to map QTLs controlling crenate broomrape response in *V. faba* was performed by Román et al. (2002). Three QTLs (*Oc1, Oc2* and *Oc3*) were identified using an $F_2$-derived population of a cross between the partially resistant line Vf136 and the susceptible line Vf6. All the resistance-enhancing alleles originated from the resistant parent Vf136. One of the three QTLs explained more than 35% of the phenotypic variance, whereas the others accounted for 11.2 and 25.5% of the variation. The study confirmed that broomrape resistance in faba bean can be considered a polygenic trait with major effects from a few single genes.

QTL confirmation and validation followed by high-resolution mapping is critical in determining its usefulness in MAS. QTLs detected in early generations must be of predictive value for later generations and stable in different environments. Although the effects of many QTLs seem to be consistent across environments, the size of their effects depends on environmental conditions, due to QTL × environment interactions. For this reason, replicated field tests are required to characterize accurately the effects of QTLs and to evaluate their stability across environments. These interactions remain a major constraint for the discovery of QTLs that confer a consistent advantage across a wide range of environments and should, therefore, be carefully considered in developing an effective MAS scheme (Francia et al. 2005; Collard and Mackill 2008). In plants, the use of RI populations for multiple testing is preferred because it can easily be maintained through seeds, which allows the analysis of different environmental influences and the study of multiple, even invasive or destructive, traits. The increased replication of homozygous genotypes results in an increased power for testing differences between genotypic classes and in a greater precision of trait measurement when compared with other type of progenies (Austin and Lee 1996). In addition, the effect of a QTL can depend on the genetic background. This emphasizes the importance of testing the QTL effects and the reliability of marker associations in various genetic backgrounds and, whenever possible, in parallel populations, before MAS is undertaken. In this study, QTL verification was defined as the repeated detection, at a similar position on the genetic map, of a QTL controlling broomrape resistance under more than one set of experimental conditions.

The objective of the investigation reported here was, thus, to gain a better knowledge of the genetic factors controlling faba bean resistance to broomrape by: (1) confirming the position and effects of the putative QTLs for *O. crenata* resistance identified in the $F_2$ population of the cross Vf6 × Vf136, (2) verifying the QTLs in the recombinant inbred lines (RILs) derived from this cross and (3) testing them in field trials in three different environments. The approach will facilitate the identification of the most stable QTLs as well as the most tightly linked markers to assist the introgression of the appropriate broomrape resistance alleles in faba bean breeding programs.

## Materials and methods

### Plant material

Molecular analyses were carried out using plant tissue from 165 individual $F_6$ plants obtained by the selfing of segregating lines derived from the cross between two faba bean progenitors contrasting for *O. crenata* resistance. The female parent Vf6 was susceptible to the parasite, whereas the male parent Vf136 showed resistance. Vf6 is an asynaptic line that has been used previously in mapping projects, facilitating the assignment of linkage groups (LGs) to specific chromosomes. The line Vf136 originates from the cross Vf1071 × Alameda (Cubero et al. 1992). Vf1071 is a resistant line to *O. crenata* selected from the resistant cultivar Giza 402 by Cubero and Hernández (1991) and Alameda is a commercial cultivar adapted to the conditions of southern Spain (Cubero and Hernández 1991). The $F_6$-derived RILs were checked for *O. crenata* resistance under field conditions. Genomic DNA extraction was performed on young leaves of $F_6$ individuals, using the extraction method described by Lassner et al. (1989) modified by Torres et al. (1993).

### Marker analysis

Four enzymatic systems, aconitate hydratase (ACO, E.C. 4.2.1.3), 6-phosphoglucanate dehydrogenase (6-PGD, E.C. 1.1.1.44), peroxidase (PRX, E.C. 1.11.1.7) and superoxide dismutase (SOD, E.C. 1.15.1.1) were analyzed. The genetic and chromosome location for each isoenzymatic locus were previously reported (Torres et al. 1998).

Random amplified polymorphic DNA (RAPD) analysis used in this study, was as described by Williams et al. (1990) with slight modifications (Torres et al. 1993). A total of 57 RAPD primers were analysed. Out of these markers, 44 were selected based on the previous mapping analysis in the $F_2$ population (Román et al. 2002) and 13 were selected for showing polymorphic bands with another cross that shared one of the parental lines (Ávila et al. 2004).

The cross was tested for two seed-protein genes markers (legumin B3 and legumin B4) that produced clear and reproducible polymorphic bands in the corresponding $F_2$ population. These legumins were physically located by PCR with sorted or micromanipulated chromosomes and by "in situ" hybridisation (Macas et al. 1993a, b; Fuchs and Schubert 1995). The sequences of the primers used for detection of length polymorphisms among these genes were previously reported (Macas et al. 1993a). Amplification conditions were similar to those used with RAPD primers with some modifications described by Vaz Patto et al. (1999) to maximize the amplification and the resolution of the products.

A total of five SSR primers (GA4, GAII-8, GAII-30, GA II-59 and JF1-AG3) physically located by PCR with sorted or micro-manipulated chromosomes (Pozarkova et al. 2002), were analyzed in the segregant population. Amplification conditions were those described by Pozarkova et al. (2002). When the polymorphism pattern between the parental lines was scored clearly, the analysis of the corresponding RIL populations was made on 1% Seakem agarose, 1% Nu-Sieve agarose gels. In the case of a complex pattern that prevented distinguishing the different genotypes, 2.5% metaphor agarose was used.

The segregant advanced progeny from this cross was also tested for 52 sequence tagged sites (STS), of which 20 were specific to *Pisum sativum* (Weeden et al. 1998) and 37 of *Medicago truncatula* (Choi et al. 2004). Amplification conditions were those described by Gilpin et al. (1997). When no polymorphism was detected, PCR products amplified from both parents were digested with a range of restriction endonucleases which recognize four and five base sequences. Two units of restriction enzyme were added to 10 µl of PCR reaction together with 12 µl of sterile water and 2.5 µl of the specific buffer for each enzyme. The digestions were incubated for 12 h at 37°C. Polymorphisms were analyzed on agarose gels.

Thirteen gene-based PCR primers pairs from *P. sativum* and eight from *Medicago truncatula* designed to amplify intron-spanning sequences from homologous exons in legumes and created by the European Grain Legumes Integrated Project (Seres et al. 2007) were also assayed. PCR amplification was optimized to obtain a single specific band that cross amplified in *Vicia faba*, using the parental lines of this cross. Reaction mixtures of 20 µl contained 10 mM Tris–HCl (pH 8), 50 mM KCl, EDTA 1 mM, 0.1 % Triton X-100, 50% (v/v) glycerol, 30 ng of template DNA, 0.6 µM of each forward and reverse primer, 2 mM $MgCl_2$ and 1 U *Taq* polymerase (Biotools). Amplifications were carried out in a gradient thermocycler (TGradient PCR, Biometra) with a 5 min initial denaturation at 95°C, followed by 40 cycles of 95°C for 1 min, annealing temperature ranging from 50 to 62°C (determined for each primer pair analyzed with the Oligo Software) for 1 min and extension at 72°C for 2 min, with a final extension step at 72°C for 8 min before cooling to 4°C. To detect polymorphisms between the parental lines, the EST amplified product was separated on agarose gels or restricted with 20 different restriction enzymes to develop CAPs markers (0.2 units of restriction enzyme were added to 1–2 µl of PCR amplification together with 2.5 µl of the specific buffer for each enzyme and miliQ water until 10 µl of total volume). The digestion was incubated for 12 h at 37°C.

The genetic linkage map developed in $F_2$ generation and used in previously reported QTL analysis for broomrape resistance (Román et al. 2002) has been improved by including more markers. The final version consisted of 121 markers (112 RAPDs, 3 isozymes, 1 SCAR, 3 SSRs, 2 STSs) mapped into 15 LGs covering 1,245.42 cM of the faba bean genome. The $F_2$ molecular marker data reported in Román et al. (2002) were re-analyzed, but no considerable differences in QTL positions nor effects were observed.

### Field disease trials

The Vf6 × Vf 136 $F_6$ progenies were tested against *O. crenata* in naturally infested fields at three different environments in Spain. The RIL population was grown under open-pollination conditions in two locations in Southern Spain (Córdoba and Mengibar) during two consecutive seasons 2002–2003 and 2003–2004. During the first season, the resistance evaluation was scored in Córdoba (Córdoba 2003). During the second season, the field trials were located at Córdoba (Córdoba 2004) and Mengibar (Mengibar 2004). The RILs were evaluated for resistance together with the susceptible control Brocal in an alpha lattice design. The trials consisted of two replications in a field plot naturally infested with *O. crenata* seeds. To determine the infestation level, ten plants of each RIL family was sown in a 1 m row surrounded by four rows of ten susceptible checks each. Resistance to *O. crenata* was scored as the final number of emerged broomrape shoots per host individual at plant maturity by considering total number of faba bean plants and total number of emerged broomrapes per family. For each segregant line, the mean number of broomrapes per plant was calculated as well as the mean number of *O. crenata* individuals per plant of the four adjacent plots of cultivar Brocal.

### Resistance scoring

Simple regression has been carried out using the broomrape score in susceptible checks as an independent variable and the broomrape score in the RILs as a dependent variable to remove any statistically significant effects of field infestation variability. Regression corrected values (residuals) were then calculated to correct for differences in broomrape seed density in the soil between plots (Román et al. 2002; Valderrama et al. 2004). Regression residuals were range standardized and multiplied by −1 to construct the broomrape resistance index ranging from 0 (most susceptible RI line) to 1 (most resistant RI line).

### QTL mapping

Each marker was tested against the expected segregation ratio using a $\chi^2$ goodness of fit. The markers not showing normal diploid segregation ($P < 0.01$) were excluded from further analysis. The linkage map was constructed by MAPMAKER V2.0 (Lander et al. 1987) using a LOD score of 4.0 as the threshold for considering significant linkage. Recombination fractions were converted to centimorgans (cM) using the mapping function of Kosambi (1944). Genotypes from the linkage map and quantitative data for resistance index were used for input into Windows QTL Cartographer version 2.5 (Wang et al. 2005). Interval mapping (IM) and composite interval mapping (CIM) were performed. The threshold for the detection of a QTL was fixed at a LOD value of 2 (LR = 9.21). For each LOD peak, the 1-LOD support intervals were determined (Van Ooijen 1992).

### Epistatic interaction

Two-way interactions between putative epistatic QTLs were tested among all pairwise combinations of the markers using EPISTAT (Chase et al. 1997). A total of 106 trails were used in the Monte Carlo simulation to establish the statistical significance of the log-likelihood ratios of the interactions detected. The $P$ values found by the Monte Carlo simulation were transformed into 1 $(1 - P)^n$, thus, adjusting for the number of loci ($n$) searched (Lark et al. 1995).

## Results

### Segregation analysis and linkage map

Out of 317 markers showing normal diploid segregation in the RIL population, 277 markers (238 RAPDs, 4 isozymes, 5 ESTs, 1 SCAR, 6 SSRs, 2 STSs and 21 intron-spanning markers), mapped into 21 LGs (mean inter-marker distance of 12.72 cM) and covered 2,856.7 cM, giving rise to the most saturated faba bean map to date. Nine out of 21 LGs could be assigned to specific chromosomes because markers were common with previous studies (Torres et al. 1993; Satovic et al. 1996; Vaz Patto et al. 1999) that used primary trisomics to establish the chromosomal location of markers and/or genes as summarized in Román et al. (2004).

### Field trait data

The experimental fields showed a high level of infestation, as illustrated by the severe attacks observed in the

susceptible Brocal checks, revealing a clear segregation of the resistance among the RILs. In case of Córdoba-03, the average number of broomrape shoots per *Vicia faba* plant in RIL plots ranged from 0 (most resistant RIL; resistance index = 1) to 5.36 (most susceptible RIL; resistance index = 0), in Córdoba 2004 from 0 to 12.3, and in Mengíbar 2004 from 0 to 11.1.

The average values of the resistance index of the RILs in the three environments considered are shown in Table 1. The mean values of resistance index were 0.66 in Córdoba 2003 and Córdoba 2004 and 0.63 in Mengibar 2004. In all the cases, the distribution of the resistance index values was skewed towards the more resistant parent as shown by a negative value of skewness coefficients. The correlations among the index values in the three environments considered were significant at $P < 0.01$.

## QTL analyses

### *Broomrape resistance QTLs in the updated F2 map*

Román et al. (2002) identified three QTLs (*Oc1, Oc2* and *Oc3*) using the $F_2$ population from the cross Vf6 × Vf136. After the saturation of this map, a high level of conformity with the previous QTL analysis was observed. Nevertheless, although the three QTLs were again detected, slight differences in the LGs layout, map distances, QTL positions and genetic effects estimates were also apparent (Table 2; Fig. 1). In the case of the QTL *Oc1*, after including new markers, the RAPDs $OPJ13_{686}$ and $OPAC02_{730}$, flanking the QTL in the $F_2$, constituted an independent LG in the $F_6$ map. As in the former study, the major-effect QTL, *Oc1*, was located in the position of the marker $OPJ13_{686}$ (Table 2), but explained a higher proportion of the phenotypic variance (43 vs. 37% in 2002). *Oc2* was again identified on chromosome VI, and the inclusion of new markers common to a reference composite map already reported (Román et al. 2004), facilitated the ascription of the LG bearing *Oc3* to chromosome II. The percentage of phenotypic variance explained by *Oc2* and *Oc3* was just the same in the two analysis (11% for *Oc2* and 25% for *Oc3*). Markers flanking the broomrape resistance QTLs are given in Table 2. When compared with *Oc2* and *Oc3*, *Oc1* revealed considerable dominant effects. In this case, the dominance/additive (*d*/*a*) ratio was greater than 1.0 indicating that, on average, the heterozygous individuals for *Oc1* are more resistant than the dominant homozygotes.

**Table 1** Broomrape resistance index of faba bean RILs in three different environments

| Environment | Average | SD | Skewness | Kurtosis |
|---|---|---|---|---|
| Córdoba 2003 | 0.66 | 0.20 | −0.92 | 0.91 |
| Córdoba 2004 | 0.66 | 0.19 | −0.88 | 1.19 |
| Mengíbar 2004 | 0.63 | 0.17 | −1.08 | 1.78 |

*SD* standard deviation

### *Broomrape resistance QTLs in the RILs map*

The CIM on the more dense $F_6$ map detected more QTLs than in the $F_2$ analysis. Four QTLs controlling *O. crenata* resistance (*Oc2–Oc5*) were identified in the RI segregant population in three different environments by SIM using QTL CARTOGRAPHER ver. 2.5 (Table 2). Only *Oc1,* previously reported in the $F_2$ population (Román et al. 2002) and identified as well in the more comprehensive $F_2$ map, was not significant in the advanced lines. *Oc2* and *Oc3* were found to be associated with *O. crenata* resistance in at least two of the three environments, while the remaining two, *Oc4* and *Oc5,* were only detected in Córdoba 2004 and Mengíbar 2004 explaining individually 17 and 9% of the phenotypic variation, respectively. The analysis was not improved with the use of cofactors. As expected, *Oc2* mapped in chromosome VI, *Oc3* in chromosome II while the remaining two QTLs, *Oc4* and *Oc5,* were ascribed to the large metacentric chromosome 1. Additive effects were significant in the four QTLs. All the resistance-enhancing alleles originated from the resistant parent Vf136 as shown by the negative values of the additive genetic effects (Table 2). *Oc4* in Córdoba 2004 had the largest effect increasing the level of the resistance index by 0.0789 units, whereas *Oc2* in Mengíbar 2004 presented the lowest effect only increasing the index value by 0.0493 units. The genetic effects, LOD values and flanking markers for each QTL across environments are described in Table 2.

### *Epistatic interaction*

A single significant epistatic interaction was found in Córdoba 2003 as well as in Mengíbar 2004, while six were detected in Córdoba 2004 at $P < 0.01$ after adjustment for the number of loci ($n = 317$). In all cases, the interaction between alleles from both parents increased the resistance to *Orobanche crenata.* All the interactions involved loci located on the LG II.A., where the QTL Oc3 was detected, but none of the EQTLs was found in the vicinity of the QTL. Five out of six interactions identified in Córdoba 2004 included loci located on LG II.A. ($OPM18_{1093}$ and mtmt_GEN_00103_01_1) and those located on the LG V ($OPT12_{389}$, $OPR16_{917}$, $OPJ011_{641}$, $OPI14_{1199}$) (Table 3). As $OPM18_{1093}$ and mtmt_GEN_00103_01_1 are linked as well as $OPT12_{389}$, $OPJ011_{641}$ and $OPI14_{1199}$, multiple interactions may actually represent a single epistatic pair (Malmberg et al. 2005).

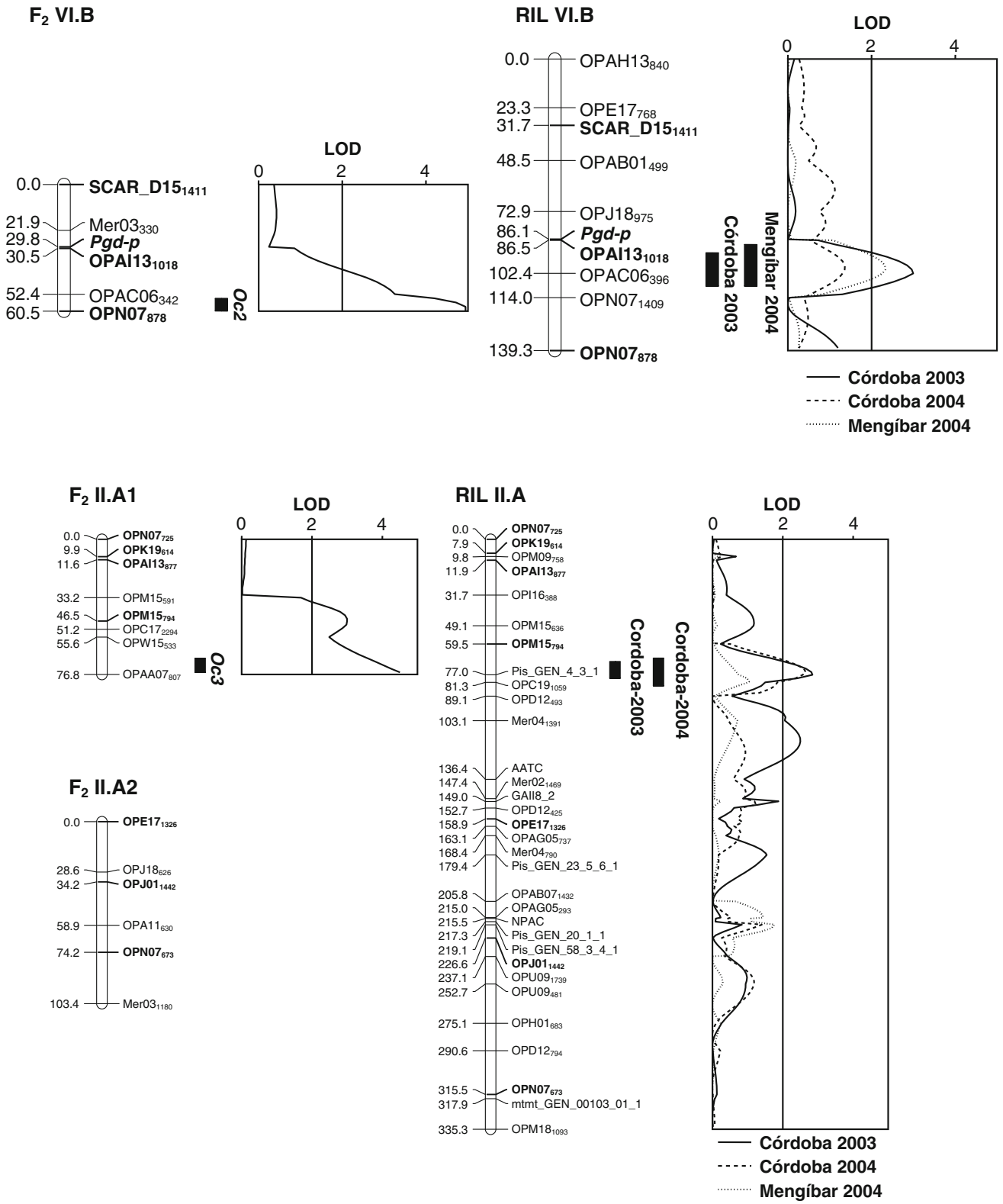


**Fig. 1** Comparison of LOD profiles of QTL analyses of *Orobanche creanta* resistance in faba bean (*Vicia faba* L.) as obtained by composite interval mapping in $F_2$ and RIL populations (in Córdoba 2003, Córdoba 2004 and Mengíbar 2004). Map positions are given in cM using the Kosambi's mapping function. Common markers are shown in *bold*. QTL location bars corresponding to 1-LOD interval are indicated as a *box*. The software used has been QTL Cartographer ver. 2.5

**Table 2** Putative QTLs detected for broomrape resistance in faba bean (*Vicia faba* L.) in $F_2$ and RILs generation by composite interval mapping (CIM) using QTL Cartographer

| QTLs | Population | Environment | LG | Flanking markers | LOD | Add | Dom | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| *Oc1* | $F_2$ | | LG03 | $OPJ13_{686}$/$OPAC02_{730}$ | 5.89 | −0.1267 | 0.1626 | 0.43 |
| *Oc1* | RILs | Córdoba 2003 | | | | | | |
| *Oc1* | RILs | Córdoba 2004 | | | | | | |
| *Oc1* | RILs | Mengíbar 2004 | | | | | | |
| *Oc2* | $F_2$ | | VI.B | $OPN07_{878}$ | 4.95 | −0.0702 | 0.0341 | 0.11 |
| *Oc2* | RILs | Córdoba 2003 | VI.B | $OPAC06_{396}$ | 3.00 | −0.0587 | na | 0.08 |
| *Oc2* | RILs | Córdoba 2004 | | | | | | |
| *Oc2* | RILs | Mengíbar 2004 | VI.B | $OPAI13_{1018}$/$OPAC06_{396}$ | 2.33 | −0.0493 | na | 0.09 |
| *Oc3* | $F_2$ | | II.A1 | $OPAA07_{807}$ | 4.50 | −0.0982 | 0.0787 | 0.25 |
| *Oc3* | RILs | Córdoba 2003 | II.A | Pis_GEN_4_3_1 | 2.85 | −0.0576 | na | 0.08 |
| *Oc3* | RILs | Córdoba 2004 | II.A | OPM15_794/Pis_GEN_4_3_1 | 2.66 | −0.0582 | na | 0.09 |
| *Oc3* | RILs | Mengíbar 2004 | | | | | | |
| *Oc4* | RILs | Córdoba 2004 | I.A | OPAB01_438/OPM18_1192 | 4.63 | −0.0789 | | 0.17 |
| *Oc5* | RILs | Mengíbar 2004 | I.A | OPM18_1620/OPA17_524 | 2.25 | −0.0523 | | 0.09 |

Trait: broomrape resistance index

Parameters were estimated from phenotypic data of 196 $F_2$-derived $F_3$ families as well as of 165 RILs derived from the cross between a susceptible (Vf6) and a resistant (Vf136) line

*LG* linkage group, *LOD* peak value of the maximum LOD test statistic, *Add* additive effect, *Dom* dominance effect, $R^2$ proportion of phenotypic variance explained by the respective QTL

## Discussion

Faba bean resistance to broomrape is a particularly challenging character to study in terms of host genetics and the complexity of the disease, making the trait an ideal candidate for MAS. Nevertheless, validation is essential prior to using QTL information in MAS. The objectives in this study were to confirm the position and effects of the 3 QTLs for *O. crenata* resistance (*Oc1, Oc2* and *Oc3*), identified in the $F_2$ population of the cross Vf6 × Vf136 (Román et al. 2002). To do so, validation experiments were carried out using the advanced progeny of this cross ($F_6$) in three different environments and years (Córdoba 2003, Córdoba 2004 and Mengíbar 2004).

### Comparison of QTLs detected in $F_2$ and $F_6$ generations

Differences between the number of QTLs detected in the $F_2$ and RIL population have been found in this study. The increased power of the RI population, afforded by the increase in homozygosity, has allow the detection of more QTLs (*Oc4* and *Oc5*) with smaller effects. Thus, three QTLs (*Oc1, Oc2* and *Oc3*) were declared in the $F_2$, whereas four (*Oc2–Oc5*) were significant in the RI population. Out of them, *Oc2* and *Oc3* were consistent with those reported in the original $F_2$ (Román et al. 2002) thereby confirming their environmental stability and pointing to their appropriateness as targets for MAS for broomrape faba bean resistant varieties. The lower percentage of phenotypic variation explained by *Oc2* and *Oc3* in the RIL population compared with the $F_2$ (11% vs. a mean value of 8.5% in the case of *Oc2* and 25 vs. 8.5% for *Oc3*), could be explained by the absence of the dominance effects in the advanced material in contrast with the early generation.

The major QTL, *Oc1*, identified in the $F_2$ and explaining the highest percentage of the phenotypic variance, was originally mapped between flanking markers $OPJ13_{686}$ and $OPAC02_{730}$ that were also used in genotyping of RILs. Moreover, six additional markers were mapped in the vicinity of markers flanking *Oc1*, but no QTL signal was detected in the RIL population in any of the three environments under study. There are several possible reasons for this lack of conformity between the $F_2$ and $F_6$ results. The first hypothesis can be justified by the overdominance displayed by *Oc1* in the $F_2$ (Table 2), since dominant effects are known to be important in this generation, but are absent in RILs. Moreover, some QTLs detected in early generations of maximum linkage disequilibrium are indeed due to multiple, linked genes that may be separated via recombination (Austin and Lee 1996). Lastly, *Oc1* may be environment specific.

The higher marker density of the RI map compared with that of the $F_2$ has increased the accuracy in the position and effects of *Oc2* and *Oc3* (Fig. 1). In the case of *Oc2* (chromosome VI), the comparison between LGs was made possible by the presence of four common markers, 2

**Table 3** Epistatic interactions between unlinked markers

| Environment | EQTL1/LG | EQTL2/LG | | LLR | $P_t$ |
|---|---|---|---|---|---|
| | | A | B | | |
| Córdoba 2003 | $OPJ01_{1442}$/II.A | OPAA07_1043/unlinked | | | |
| | A | 0.725 | 0.605 | 13.20 | 0.0038 |
| | B | 0.571 | 0.743 | | |
| Córdoba 2004 | $OPM18_{1093}$/II.A | $OPT12_{389}$/V | | | |
| | A | 0.702 | 0.569 | 13.46 | 0.0013 |
| | B | 0.587 | 0.758 | | |
| Córdoba 2004 | $OPM18_{1093}$/II.A | $OPR16_{917}$/V | | | |
| | A | 0.724 | 0.567 | 15.00 | 0.0016 |
| | B | 0.587 | 0.736 | | |
| Córdoba 2004 | $OPM18_{1093}$/II.A | $OPJ13_{1193}$/LG06a | | | |
| | A | 0.593 | 0.716 | 11.80 | 0.0044 |
| | B | 0.755 | 0.597 | | |
| Córdoba 2004 | $OPM18_{1093}$/II.A | $OPJ01_{1641}$/V | | | |
| | A | 0.719 | 0.573 | 15.14 | 0.0003 |
| | B | 0.593 | 0.759 | | |
| Córdoba 2004 | mtmt_GEN_00103_01_1 / II.A | $OPJ01_{1641}$/V | | | |
| | A | 0.695 | 0.579 | 12.47 | 0.0032 |
| | B | 0.585 | 0.768 | | |
| Córdoba 2004 | $OPM18_{1093}$/II.A | $OPI14_{1199}$/V | | | |
| | A | 0.720 | 0.601 | 10.56 | 0.0088 |
| | B | 0.590 | 0.737 | | |
| Mengíbar 2004 | AATC/II.A | $OPD06_{1583}$/LG09 | | | |
| | A | 0.583 | 0.722 | 15.88 | 0.0088 |
| | B | 0.669 | 0.645 | | |

The epistatic quantitative trait loci (EQTL1 and EQTL2) and the linkage groups are given

The mean phenotypic values for all four marker classes are listed: *A* being an allele from susceptible parent (Vf6), *B* being an allele from resistant parent (Vf136)

LLR represents the log-likelihood ratio if an epistatic model is compared with an additive model

Additive *P* values associated with the LLRs derived from the Monte Carlo simulation were adjusted for the number of loci searched ($P_t$)

RAPDs, 1 SCAR and 1 isozyme. The higher saturation of the region bearing *Oc2* in the RI population has modified the previous location of this QTL in the distal part of the group (Fig. 1). Concerning *Oc3* located in chromosome II, the analysis of seven common markers have allowed the integration of two unlinked $F_2$ LGs in the $F_6$ map (Fig. 1), thus improving the precision of estimating the QTL position previously reported (Román et al. 2002).

Our study has confirmed the clear advantage of the RILs over the $F_3$ families for the number and resolution of QTLs controlling *O. crenata* resistance. RI populations have additional recombination between loci and an increased power for detecting QTLs. Moreover, as mentioned by Chaïb et al. (2006), new QTLs may appear in this populations because of the fixation of the previously segregating major QTLs. Apart from these reasons, the inclusion of new markers in the $F_6$ map favoured the map reorganization with a gain in marker density that could have helped to identify a false-positive QTL and to detect new QTLs with minor effects. The identification of these QTLs, undetected in the $F_2$ generation, could be also attributed to the higher statistical strength of the advanced RIL material when using mainly dominant markers, as was in our case.

Further studies are needed to understand the genetics of resistance to broomrape in faba bean. Particularly, the usefulness of the resistance alleles identified in Vf136 will have to be verified in other Mediterranean environments where the parasite is particularly severe and damaging. The identification of new resistance loci from other sources of resistance should be also an obvious objective to accumulate multiple resistance alleles in a genotype and increase the level of resistance to this destructive parasite.

## Stability of QTLs across environments

By combining the results over the two locations and years, we have identified four genomic regions associated with *O. crenata* resistance in faba bean. Moreover, multiple environment testing has been very helpful in assessing the stability of two of the QTLs associated with the trait. Thus, *Oc2* and *Oc3* were identified in at least two of the three environments while *Oc4* and *Oc5* were expressed only in

Córdoba 2004 and Mengíbar 2004, respectively. Differences in the level of infestation and/or weather conditions could have resulted in the expression of different genes in each location. Although research on QTL tends to emphasize their validation in different environments, in a real selection scheme, there are genes/QTLs of interest which are specific to a certain environment. In this case, environment sensitive QTLs, such as *Oc4* and *Oc5*, can be useful in specific locations for MAS.

The stability of *Oc2* and *Oc3* detected in at least two different locations (Córdoba 2003/Mengíbar 2004 and Córdoba 2003/Córdoba 2004, respectively), could also be supported by the genetic homogeneity of the parasite populations. A molecular study considering *O. crenata* populations from both locations (Córdoba and Mengíbar) (Roman et al. 2001) determined low genetic differences among six *O. crenata* populations from Andalusia. The evaluation of resistance under a similar parasite population could be favouring the detection of *Oc2* and *Oc3* in these two environments.

The total variance explained by all QTLs detected in the $F_6$ population was 16, 18–28% in Córdoba 2003, Mengibar 2004 and Córdoba 2004, respectively. The unexplained variation in broomrape resistance may be attributable to factors such as minor effect QTLs, incomplete map coverage and environmental effects. Moreover, epistatic interaction between additive QTLs could also be contributing to the unexplained variation.

Finally, the complexity of the trait could also give rise this outcome. Broomrape resistance is a high complex trait, since the number of emerged shoots per faba bean plant is the consequence of the success of the parasite to overcome a chain of escape factors and/or resistance mechanisms acting at different phases of the infection process (Pérez-de-Luque et al. 2005). These factors could imply avoidance of the parasite by root architecture (Ter Borg 1999), low induction of broomrape seed germination (Sillero et al. 1999; Rubiales 2003) and resistance to the parasite penetration or to the establishment of tubercles (Ter Borg 1999; Rubiales 2003; Pérez-de-Luque et al. 2005). As a consequence, differences in resistance caused by diverse mechanisms, which could account for the remaining variation, might be under-represented by the simple scoring of the final number of emerged *O. crenata* shoots.

## Implications for MAS

Our study has identified two QTLs (*Oc2* and *Oc3*), controlling faba bean resistance that were fairly consistent across two locations and environments. The efficiency of marker-assisted backcrossing to introgress a QTL from a donor line into a recipient line depends on the stability of QTL expression. For this reason, the information reported here is a step towards the use of molecular markers for indirect selection of broomrape-resistant genotypes. Depending on their relative effects and position, some of the QTLs reported could be targeted for MAS, providing opportunity to accelerate faba bean breeding programmes. Because resistance against *O. crenata* is a trait difficult to evaluate, we consider that increasing the selection intensity by MAS pre-selection of genotypes and subsequent phenotypic selection will lead to an improved selection gain. Nevertheless, before using this information in MAS schemes, the genomic regions containing these QTLs should be saturated to refine their position and identify the marker most closely linked to the resistance genes.

Isolating the genes underlying a QTL is, however, an enormous endeavor in species with small genomes that can be even harder in crops such as faba bean with one of the largest genomes among legumes (~13,000 Mb). In this study, Pis_GEN_4_3_1, an EST from *P. sativum*, was one of the flanking markers falling within 1-LOD of the *Oc3* region. This marker identifies a gene homolog to a G protein $\beta$ subunit that participates in signal transduction regulating multiple cellular processes. Co-localization/association of ESTs and QTLs represents a possible strategy for the identification of genes underlying agriculturally important traits (Pflieger et al. 2001; Wright et al. 2005). The positional information for 21 *M. truncatula* and *P. sativum* ESTs provided here is, thus, a valuable starting resource for candidate gene studies in faba bean. Nevertheless, further EST saturation of the faba bean genomic regions involved in broomrape resistance is required, prior to identifying plausible functional links between genes and their function.

The present development in legumes of different tools in functional genomics such as the microarray technology and EST analyses could facilitate the identification of candidate genes expressed during the different steps of the infection process. The RIL population used in this study will be a valuable material to correlate the expression of candidate genes identified in model species as *M. truncatula* (Dita et al. 2005) with physiological plant response under broomrape attack. Thus, the information generated in models can be, therefore, transferred to other legumes gaining insight into the physiological and functional aspects of the QTLs detected so far. Integrating functional, comparative and structural genomics with molecular breeding should offer a comprehensive research strategy to allow a more efficient selection for faba bean broomrape resistance in the near future.

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