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Mapping of quantitative trait loci controlling *Orobanche foetida* Poir. resistance in faba bean (*Vicia faba* L.)

Díaz-Ruiz R.[1], Torres A.[2], Gutierrez M. V.[2], Rubiales D.[3], Cubero J. I.[4], Kharrat M.[5], Satovic Z.[6] and Román B.[2]*

[1]Colegio de Postgraduados-Campus Puebla, Km 125.5 Carretera Federal Méx-Pue. C.P. 72760, Puebla, Apdo 2-12, México.
[2]IFAPA-CICE (Junta de Andalucía), CIFA "Alameda del Obispo", Área de Mejora y Biotecnología, Apdo. 3092, 14080 Córdoba, Spain.
[3]Instituto de Agricultura Sostenible, CSIC, Apdo. 4084, 14080 Córdoba, Spain,
[4]Departamento de Genética, ETSIAM, Campus de Rabanales, Ed. Gregor Mendel, Universidad de Córdoba, 14071 Córdoba, Spain.
[5] Food Legume Laboratory, INRAT, BP 10, 2049, Ariana, Tunisia.
[6]Department of Seed Science and Technology, Faculty of Agriculture, University of Zagreb, Svetosimunska 25, 10000 Zagreb, Croatia.

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Although in the Mediterranean region the most important *Orobanche* weed attacking leguminous crop is *Orobanche crenata*, recently *Orobanche foetida* has been found attacking faba bean, vetch and chickpea in Tunisia and vetch in Morocco. In this study we have identified and map the quantitative trait loci (QTL) controlling resistance to *O. foetida* in faba bean (*Vicia faba*) and studied their stability in two different environments. One hundred and forty four Recombinant Inbred Lines (RILs) derived from the cross between a susceptible and a resistant parent were analysed using isozymes, RAPD, seed protein genes, STSs, ESTs, microsatellites and SCAR markers. Two hundred and seventy seven markers segregating in the RIL population could be mapped into 21 linkage groups, 9 of them assigned to specific chromosomes. By evaluating broomrape resistance in these RILs under field conditions, two QTLs for *O. foetida* resistance were identified showing instability across environments. Whereas Of1 was located in the chromosome 1 and explained 7% of the phenotypic variation in one environment, Of2 was assigned to chromosome 3, was only identified in the second environment and explained 9% of the trait variation. Since this map has been used before to locate *O. crenata* resistance QTLs its exploitation offers the possibility of accumulating resistance genes in *V. faba* germplasm against both parasites.

Key words: *Orobanche foetida*, *Vicia faba*, parasitic plants, resistance QTLs, molecular mapping.

INTRODUCTION

The genus *Orobanche* contains over 100 species of obligate root holoparasites in both the Old and New Worlds. The genus reaches its greatest diversity in Mediterranean climates and in Western Asia. Most of the economically important parasites are Old World species. Major crop hosts for *Orobanche* are legumes crops, umbels, cole crops, lettuce and sunflower. Control is difficult due to seed longevity in the soil, small seed size, fecundity (thousands of seeds per plant), and a subterranean phase (seeds germinate beneath the soil and parasitize the host before they emerge and become evident). Moreover, the soil seed-bank can remain viable for many years and germinate only after stimulation by root exudates of the host. Damage varies with level of infestation, and total crop failures can occurred in some cases.

By far the most economically damaging parasitic plant on legumes is crenate broomrape (*Orobanche crenata* Forks.), but some other broomrapes such as *Orobanche* minor Sm. and *Orobanche aegyptiaca* Pers. can also be

*Corresponding author. E-mail: belen.roman.ext@juntadeandalucia.es. Tel: 34 957 016125. Fax: 34 957 016043.

of great importance in certain areas. *Orobanche foetida* Poir. is considered important as an agricultural parasite of faba bean (*Vicia faba*) and common vetch (*Vicia sativa*) crops in the region of Beja in Tunisia (Kharrat et al., 1992). The recent report of *O. foetida* attacking vetches in Morocco (Rubiales et al., 2005) results in great interest since it indicates the first introduction of this parasite into crops in Morocco. Apart from the reports of *O. foetida* in legume fields of Tunisia and Morocco, it has never been found infecting crops, even though it is widely distributed in the Western Mediterranean area (Portugal, Spain) parasitizing wild herbaceous *Leguminosae of the genera Anthyllis, Astragalus, Ebenus, Lotus, Medicago, Ononis, Scorpiurus* and *Trifolium* (Pujadas-Salvá, 1999).

The spread of this new parasite population on crops should be monitored as it could represent a further constraint for legume production in this area. In Tunisia, the parasite is more aggressive on faba bean than on other food legume crops and only peas (*Pisum sativum* L.) escapes to *O. foetida* attack (Kharrat, 1999). Since the appearance of *O. foetida* in crop fields in Tunisia some studies have been developed in order to identify sources of resistance among germplasm collections (Kharrat and Halila, 1994), to test faba bean varieties against different inoculum levels (Kharrat et al., 1994) or to evaluate different chemical control strategies (Kharrat and Halila, 1996). Nevertheless, the identification and location of the genomic regions controlling the trait has not been studied already.

In order to prevent legumes for severe broomrape attacks many control strategies have been applied from agronomical practices, such as hand-weeding or herbicide treatment to genetic improvement of crops through conventional breeding. Although at present, studies on agronomical and chemical practices are still under investtigation, the major effort shifted to crop genetic breeding that appears as the most appropriate and cost-effective control practice. The recent location of broom-rape resistance QTLs in legumes (Román et al., 2002; Valderrama et al., 2004) and sunflower (Pérez-Vich et al., 2004) will also help to identify molecular markers tightly linked to the genomic region of interest allowing their use in Marker-Assisted Selection breeding.

In the case of *O. crenata*, Román et al. (2002) located three resistance QTLs in an F_2 population of faba bean derived from the cross between a susceptible and a resistant parent. The availability of lines with fixed genomes offers the possibility of studying QTL stability across different environments and years. Recombinant inbred lines are the homozygous selfed progeny of the individuals of an F_2 population. Because recombinations can no longer change the genetic constitution of RILs further segregation in the progeny of such lines is absent. It is thus one major advantage that these lines constitute a permanent resource that can be replicated indefinitely and be shared by many groups in the research community. A second advantage of RILs is that because they undergo several rounds of meiosis before homozygosity is is reached, the degree of recombination is higher compared to F_2 populations. Consequently, maps debe-loped from RIL populations show a higher resolution that maps generated from F_2 populations (Burr and Burr, 1991) and the map positions of even tightly linked markers can be determined. Taking advantage of the RIL population derived from the cross used to identify and locate *O. crenata* QTLs in Vicia faba, the objectives of this study have been: (i) to develop a genetic map in the derived RILs from this cross, (ii) to identify and locate *O. foetida* QTLs in this map and (iii) to validate these QTLs across two different environments.

MATERIALS AND METHODS

Plant material

Molecular analyses were carried out using plant tissue from 165 individual F_6 plants obtained by selfing F_2 lines derived from the cross between two faba bean contrasting lines for *O. foetida* 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 to specific chromosomes. The line Vf136 originates from the cross Vf1071 x 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 well adapted to southern Spain conditions (Cubero and Hernández, 1991). The F_6-derived recombinant inbred lines (RILs) were checked for *O. foetida* 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, aconitase hydratate, (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 analysed. The genetic and chromosome location for each isoenzymatic locus were previously reported (Torres et al., 1998). A total of 57 RAPD primers were analysed. Out of these markers, 44 were selected based in the previous mapping analysis in the F_2 population (Román et al., 2002) and 13 were selected for showing polymorphic bands with other cross that shared one of the parental lines (Ávila et al., 2004). These markers were analysed as described by Williams et al. (1990) with slight modifications (Torres et al. 1993). The cross was tested for two seed-protein genes (legumin B3 and legumin B4) that produced clear and reproducible polymorphic bands in the corresponding F_2 population. The sequences of the primers used for detection of length polymerphisms among these genes were previously reported (Macas et al., 1993). Amplification conditions were similar to those used with RAPD primers with some modifications described by Vaz Pato et al. (1999) in order to maximise the amplification and the resolution of the products. A total of 5 SSR primers (GA4, GAII-8, GAII-30, GA II-59 and JF1-AG3) physically located by PCR with sorted or micromanipulated chromosomes (Pozarkova et al., 2001), were analysed in the segregant population.Amplification conditions were those described by Pozarkova et al. (2001). The segregant advanced progeny from this cross was also tested for 52 Sequence Tagged Sites (STS), from which 20 of them were specific of Psativum (Weeden et al., 1998) and 37 of *Medicago truncatula* (Choi et al., 2004). To test the cross-amplification of these markers,

the parental lines as well as 5 individuals from the progeny were used. 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 4- and 5- base seqquences. 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. Thirteen gene-based PCR primers pairs from P. sativum and eight from M. truncatula designed to amplify intron-spanning sequences from homologous exons in legumes were assayed. PCR amplification was optimised in order to obtain a single specific band that cross amplified in V. 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 MgCl2, and 1 U Taq polymerase (Biotools). Amplifications were carried out in a gradient thermocycler (T Gradient PCR, Biometra) with a 5 min initial denaturation at 95ºC, followed by 40 cycles of 95ºC for 1 min, annealing temperature ranging from 50 to 62ºC (determined for each primer pair analysed with the Oligo Software) for 1 min and extension at 72ºC for 2 min, with a final extension step at 72ºC for 8 min before cooling to 4ºC. To detect polymorphisms between the parental lines the amplification product were 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 37ºC. Three Sequence Characterized Amplified Regions (SCARs) specifically developed to tag linkage groups to chromosome 6 of V. faba, were also analysed. These SCARs were obtained from RAPD bands (OPA11, OPD15 and OPI16) previously assigned to this chromosome using trisomic segregant progenies (Vaz Pato et al., 1999). Each 25 µl amplification reaction contained: 20-40 ng of plant genomic DNA, buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 0.001% gelatine], 0.2 mM of each dNTP, 0.2 mM of primers, and 1 U of Taq DNA polymerase. After initial denaturation (94°C 4 min), products were amplified for 30 cycles (94°C 1 min, 55°C 50 s, 72°C 1 min and 30 s). Cycling was concluded with a final extension at 72°C for 8 min. The annealing temperature was specifically determined for each primer by using a gradient thermocycler being 67, 61 and 57ºC for OPA11, OPD15 and OPI16, respectively.

Field disease trials

The Vf6 x Vf136 F_6 derived progenies were tested against *O. foetida* in naturally infested fields of Beja (Tunisia) in two different years. The RIL population was grown under open-pollination conditions in Beja during 2002-03 and 2003-04 seasons, respectively (Beja-03 and Beja-04). The RILs were grown together with the susceptible control Brocal in an alpha lattice design (12 x 12) with two replications. In order to determine the infestation level, ten plants of each RIL family was sown in a row surrounded by 4 rows of 10 susceptible checks. Resistance to *O. foetida* was scored as the final number of emerged broomrape shoots per faba bean 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 broomrapes per plant of the four adjacent plots of cultivar Brocal.

Resistance scoring

Simple regression was carried out using the broomrape score in susceptible checks as an independent variable and the broomrape score in the recombinant inbred lines as a dependent variable to remove any statistically significant effects of field infestation variability. Regression corrected values (residuals) were then calcu-lated to correct for differences in broomrape seed density in the soil between plots (Roman 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).

Statistics

Each marker was tested against the expected segregation ratio using a chi-square 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 Version 2.0 (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 2.5 (Wang et al., 2005). Interval Mapping (IM) and Composite Interval Mapping (CIM) were performed. Markers to be used as cofactors for CIM were selected by Forward and Backward stepwise regression. Number of markers to control the genetic background in CIM was set to two. 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).

RESULTS AND DISCUSSION

From the 326 polymorphic markers, 317 presented adjusted segregation (1:1) and the rest (5 RAPD, 3 STS and 1 SCARs) showed distorted segregation and were discarded for further analysis. A total of 278 RAPD, 2 seed protein genes, 6 SSR, 1 SCAR, 4 isozymes, 5 STS and 21 intron-spanning markers were used to construct the map. The segregation analysis grouped 277 markers in 21 linkage groups and 9 of them were unambiguously assigned to specific chromosomes. Out of the 21 linkage groups obtained, 16 consisted of three or more markers. The map covered 2856.7 cM of the *V. faba* genome with a mean inter marker distance of 12.72 cM.

The experimental fields presented a high level of infestation as shown by the high attack suffered by the susceptible control Brocal, thus allowing the clear detection of resistance segregation in the recombinant lines. The maximum, minimum and average values of the number of broomrapes per plant in the RILs as well as in the adjacent Brocal plots are shown in Table 1. Range standardized regression residuals ranging from 0 (most susceptible) to 1 (most resistant) were considered as broomrape resistance index to be used in QTL analysis. Similar index was previously applied in a search for QTLs conferring *O. crenata* resistance in faba bean and pea (Román et al., 2002; Valderrama et al., 2004). The mean values of resistance index were 0.63 in Beja-2003 and 0.66 in Beja-2004 and the distribution of the resistance index values was skewed towards the more resistant parent as shown by a negative value of skewness cefficients

Table 1. Average number of *O. foetida* plants per faba bean plant in recombinant inbred line (RIL) plots and control plots sown with susceptible cultivar Brocal in two trails in Tunisia.

Environment	Plots	Average number of broomrapes per plant		
		Minimum	Maximum	Average
Beja-2003	RIL plots	0.11	7.58	2.47
	Control plots	1.14	10.49	5.76
Beja-2004	RIL plots	0.00	11.50	4.56
	Control plots	1.82	9.88	5.96

Table 2. Broomrape resistance index of faba bean RILs in two trials in Tunisia.

Environment	Average	sd	Skewness	Kurtosis
Beja 2003	0.63	0.17	-0.83	1.47
Beja 2004	0.66	0.17	-1.11	2.05

Table 3. Putative QTLs detected for *Orobanche foetida* resistance in faba bean (*Vicia faba* L.) in RILs generation by composite interval mapping (CIM). Paremeters were estimated from phenotypic data of 165 RILs derived from the cross between a resistant (Vf6) and a susceptible (Vf136) line.

Environment	QTL	LG	Flanking marker	LOD	Add	R^2
Beja 2003	Of1	I.A	$OPAH13_{\_475}$	2.34	-0.0466	0.07
Beja 2004	Of2	III.B	$B3_{\_1049}$ / Pis_GEN_25_2_3_1	2.37	-0.0499	0.09

LOD, Peak value of the maximum LOD test statistic; Add, additive effect; R^2, proportion of phenotypic variance explained by the respective QTL.

(Table 2). No correlation was found between broomrape resistance indexes in Beja-2003 and Beja-2004 (r = 0.03).

Two QTLs controlling *O. foetida* resistance were identified, Of1 and Of2 (Figure 1). In Beja-03 Of1 was identified in chromosome I.A, on the marker $OPAH13_{475}$ Of1 presented a LOD value of 2.34 and explained 7% of the phenotypic variance showing and additive effect of -0.046 (Table 3). The length of the 1-LOD confidence interval for this QTL was 10.45 cM. Of2 was detected in the RILs evaluated in Beja-04 on chromosome 3.B, between the legumin $LegB3_{1049}$ and the intron-spaning marker Pis_GEN_25_2_3_1. This QTL presented a LOD value of 2.37, an additive component of -0.049 and explained 9% of the phenotypic variation of the trait (Table 3). Of2 was located in a confidence interval of 15.51 cM. All the resistance enhancing alleles originated from the resistant parent Vf136 as shown by the negative values of the additive genetic effects of both QTLs, Of1 and Of2.

The development of a genetic linkage map using *V. faba* RILs derived from a cross initially generated to study *O. crenata* resistance, together with the use of an asynaptic line as a female parent, has made possible to identify, locate and assign to specific chromosomes the first *O. foetida* QTLs in legumes. The continuous distribution of the *O. foetida* resistance values in the RILs corroborated the quantitative inheritance of the resistance to this parasite in faba bean. The field resistance evaluation indicates that some of the recombinant lines showed higher levels of resistance that the parental lines, confirming the existence of transgressive segregation that could be attributable to the complementary action of genes existing in both parental lines.

Two QTLs conferring resistance to *O. foetida* Of1 and Of2 have been detected in two different environments. These QTLs have not been stable across locations and the percentage of phenotypic variation explained has been low, 7 and 9%, respectively. The only known *Orobanche* resistance source identified so far in *V. faba* is that reported in Egypt by Nassib et al. (1982) showing good levels of resistance to *O. crenata*. The resistant parental line Vf136 was used in this cross, derived from this line and thus carried the *O. crenata* selected resistance genes. As the resistant material used in this study has been selected for *O. crenata* resistance and it has not been exposed to *O. foetida* selection pressure in the breeding process, it should be possible that only part of the variation has been detected since not really extreme values for *O. foetida* resistance have been considered. An additional cross that allows us to score more extreme values in the resistance evaluation could help us to detect QTLs explaining higher levels of varia-tion. However, this does not preclude that the remaining variation could be due to the occurrence of other undetected

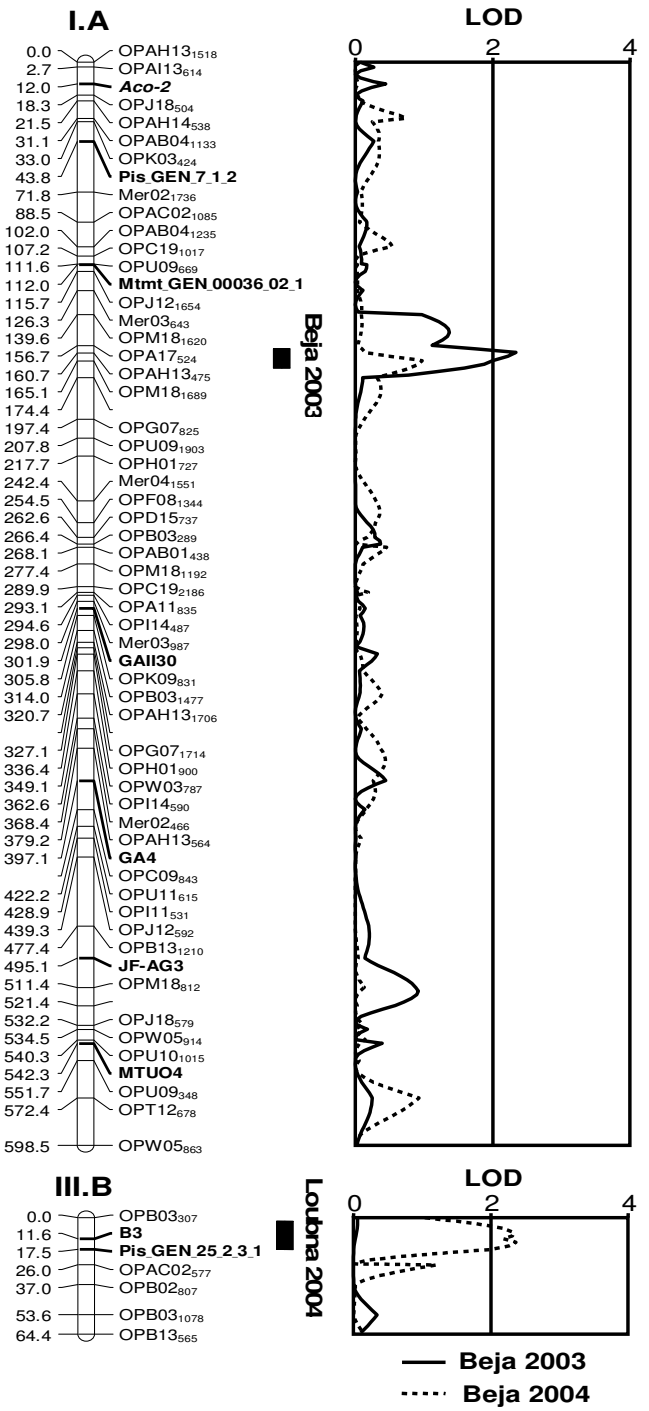

Figure 1. LOD profiles of QTL analyses of *Orobanche foetida* resistance index in faba bean (*Vicia faba* L.) from field trials in Beja 2003 and Beja 2004 as obtained by composite interval mapping in RIL population. Map positions are given in cM using the Kosambi's mapping function. QTL locaton bars corresponing to 1-LOD support interval are indicated as a box

tected *O. foetida* resistance QTLs with minor effects or, less likely, to incomplete map coverage. A more accurate quantitative scoring system of the disease reaction, may allow also the detection of additional minor QTLs accounting for the remaining unexplained variation, as well as the analysis of larger populations. Moreover, some epistatic interactions might significantly contribute to the unexplained variation.

The assignment of the two identified QTLs to specific chromosomes (Of1 to chromosome 1.A and Of2 to chromosome 3.B) has been possible through the use of the asynaptic line Vf6 as a parental line. Of1 was located in the same region of chromosome 1 where Oc5 conferring resistance to *O. crenata* was detected (Diaz et al., 2005), indicating that part of the resistance variation is the same against *O. foetida* and *O. crenata*. There arealso other studies where different genomic regions identified in the same map seemed to be controlling resistance to different species of a pathogen suggesting that some biochemical pathways are shared between different resistance responses. In this sense, Risterucci et al. (2003) detected in cocoa six genomic regions that controlled resistance to three different species of Phytophtora. Moreover, the results of a resistance QTLs study in two species of Peronosclerospora in sorghum show the existence of some QTLs controlling both species of the pathogen and some additional loci specifically involved in one of them (Nair et al., 2005). There are also successful examples where different genes of resistance have been pyramided contributing to

wide the spectrum of resistance that is resistance to *Puccinia striiformis* Westend. f. sp. hordei in barley, (Fort et al. 2003) or resistance to *Xanthomonas oryzae* pv. Oryzae in rice (Singh et al., 2001). Since pyramiding of genes results is difficult by traditional breeding methods due to the effects of dominance and epistasis, the availability of molecular markers connected with these genes could allow the identification of plants with two or three possible genes of interest. Therefore, the identification of multiple resistance QTLs to different species of *Orobanche* could offer the possibility to improve the durability of the resistance in faba bean by means of accumulating different resistance genes located in different chromosomal regions pyramided into simple genotypes.

None of the two identified QTLs remained stable since both of them were only detected in only one environment: Of1 in Beja-03 and Of2 in Beja-04. Since the two trials were set in different parts of the experimental field in Beja, the dissimilar molecular nature of the *O. foetida* populations that infested these two locations could explain these facts. Román et al. (2006) found molecular differences between *O. foetida* populations growing on chickpea and faba bean fields of Tunisia suggesting a host differentiation process. Unfortunately, we have no available information regarding the previous crop from which the parasitic population originated in the fields where the RILs were evaluated. Considering the molecular differences between O foetida populations depending on the parasitized host, the advanced lines could have been evaluated under *O. foetida* populations that differ at the molecular level. Although it is plausible that two different

race-specific *O. foetida* resistance QTLs have been detected in this study, future studies should clarify this hypothesis.

On the other hand Of2 was located in the chromosome 3 where no *Orobanche* resistance QTLs have been identified so far and could represent a specific region controlling resistance to *O. foetida*. In this same chromosome but in a different linkage group, one QTL controlling resistance to *Ascochyta fabae* has been already identified in the F_2 population (Román et al., 2003). Thus, this mapping population results interesting not only to identify genomic regions controlling resistance to different species of *Orobanche* but also to other pathogens that threaten the crop. The effective location of resistance QTLs to *O. crenata*, *O. foetida* and *A. fabae* in this population will facilitate the development of molecular markers tightly linked to different resistances and will contribute to assist breeders by Marker Assisted Selection.

When scoring the final number of emerged broomrapes per RIL in the infested fields, we are not considering any of the multiple stages of the parasite development but only the final number of attached mature parasites. As each phase of the developmental process could serve as a target to control the infection, it would be clearly interesting to determine the genes/QTLs involved in each of the metabolic stages of the process. In this sense, and considering that germination percentages of *O. foetida* seeds on *V. faba* roots varied depending on the host from which the seeds were collected (Roman et al., 2006), this mapping population would be very useful to determine if the genomic regions controlling the germination of *O. foetida* seeds collected on different host are the same. Moreover, and considering that no germination of *O. foetida* seeds has been observed with the synthetic germination stimulant GR-24 at the normal concentration used as standard to germinate *O. crenata* seeds, (Pérez-de-Luque, personal communication), these advanced lines could be also useful to determine if the regions controlling differences in germination rates of both parasite species are or not the same. Considering the molecular differences between *O. foetida* populations growing on different hosts together with the differential germination rates depending on the host on which seeds are collected (Román et al., 2006), future studies should identify and locate the genetic regions controlling germination of the parasite that seems to be crucial for the host specialisation process recently reported.

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