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Mapping of quantitative trait loci controlling partial resistance against rust incited by *Uromyces pisi* (Pers.) Wint. in a *Pisum fulvum* L. intraspecific cross

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Abstract A quantitative trait loci (QTL) associated with resistance to pea rust, caused by the fungus *Uromyces pisi* (Pers.) Wint., has been identified in a F_2 population derived from an intraspecific cross between two wild pea (*Pisum fulvum* L.) accessions, IFPI3260 (resistant) and IFPI3251 (susceptible). Both parental lines and all the segregating population displayed a fully compatible interaction (high infection type), which indicates absence of hypersensitive response. Nevertheless, differences on the percentage of symptomatic area of the whole plant (disease severity) were observed. A genetic map was developed covering 1283.3 cM and including 146 markers (144 random amplified polymorphic DNA (RAPDs) and two sequence tagged sites (STSs) markers) distributed in 9 linkage groups. A QTL explaining 63% of the total phenotypic variation was located in linkage group 3. RAPDs markers ($OPY11_{1316}$ and $OPV17_{1078}$) flanking this QTL should allow, after their conversion in SCARs, a reliable marker-assisted selection for rust resistance.

Keywords RAPD marker · STS markers · Pea rust · *Uromyces pisi*

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Introduction

Pea rust has become an important pathogen of dry pea from the mid-1980s and is distributed in Europe, North and South America, Asia, Australia and New Zealand. The pathogen develops in warm, humid weather and the disease usually appears during mid-spring when the crop is at flowering or podding stage (EPPO 2009). The activity of the rust generally results in the break of physiological and biochemical processes in the plant, and photosynthesis is particularly reduced (EPPO 2009). In years of epidemics, rust strongly affects leaves dry up. Most of them fall down and pods remain undeveloped, which consequently yield losses higher than 30%.

In tropical and subtropical regions as in India and China, pea rust has been reported to be caused by the fungus *Uromyces viciae-fabae* (Pers.) J. Schröt (syn. *U. fabae* (Pers.) de Bary), since the warm humid weather is suitable for the appearance of both the uredial and the aecidial stage (Pal et al. 1980; Singh et al. 2004; Kushwaha et al. 2006). However, in temperate regions, pea seedlings can be infected by

U. viciae-fabae, but the pathogen hardly settle down and progress in field conditions, being *U. pisi* (Pers.) Wint. the main responsible for the disease (Emeran et al. 2005; Barilli et al. 2009a, c).

U. pisi is a heteroecious macrocyclic fungus that completes its life cycle on the spontaneous *Euphorbia cyparissias* L. (cypress spurge) (Pilet 1952), which becomes severely infected (Pfunder and Roy 2000). The fungus overwinters on vegetation residues as teliospores and on rhizomes of *Euphorbia* as mycelium.

Only recently, sources of incomplete resistance to *U. pisi* from a *Pisum* spp. collection have been identified in field conditions and further characterized macro and microscopically in controlled environment (Barilli et al. 2009b, c). Higher levels of resistance have been identified in the wild species *Pisum fulvum* L. (Barilli et al. 2009a), but such resistance has not yet been efficiently used in breeding programs. The wild *P. fulvum* accession IFPI3260 has shown to have very good levels of resistance in both, field and controlled conditions (Barilli et al. 2009a, b), consisting of a pre penetration resistance not associated with host cell death.

Knowledge on the genetic system controlling resistance to *U. pisi* in accession IFPI3260 would facilitate gene transfer to pea cultivars. To date, the only study regarding the genetic nature of the resistance in peas against rust have been carried out on *P. sativum* accessions using *U. viciae-fabae* as pathogen (Katiyar and Ram 1987; Vijayalakshmi et al. 2005). On this pathosystem, the dominant nature of the partial resistance, assayed as number of pustules cm^{-2}, has been justified as the expression of a single major gene for which the symbol *Ruf* was proposed (Katiyar and Ram 1987; Vijayalakshmi et al. 2005). Further, this trait seems to be affected by some polygenes in addition to the proposed oligogene *Ruf* (Singh and Ram 2001). Two random amplified polymorphic DNA (RAPD) markers, $SC10\text{-}82_{360}$ and $SCRI\text{-}71_{1000}$ were detected flanking the gene *Ruf* 10.8 and 24.5 cM apart, respectively, but these markers were not close enough to allow a reliable marker-assisted selection (MAS) approach for rust resistance improvement (Vijayalakshmi et al. 2005).

The use of molecular markers could accelerate plant breeding in relation to certain agronomic traits, including disease resistance, enabling combinations of resistance genes to be assembled. However, no published reports on molecular mapping of *U. pisi* resistance genes are available in *Pisum* spp. so far. Therefore, the goals of the preset work have been: (1) to develop a genetic map of *P. fulvum* using both RAPD and sequence tagged sites (STS) markers; (2) to detect quantitative trait loci (QTLs) for *U. pisi* resistance with the aim to study the genetic inheritance of the resistance to this pathogen; (3) to identify molecular markers tightly linked to the responsible gene(s) which might be useful for marker assisted selection (MAS) purposes.

Materials and methods

Plant material

The cross IFPI3260 (resistant) × IFPI3251 (susceptible) was selected for the study because the parental lines have shown a consistent reaction to rust for more than four years. Furthermore, both parents were also resistant to powdery mildew (*Erysiphe pisi* Syd.) and broomrape (*Orobanche crenata* Forsk.) (Rubiales et al. 2005; Fondevilla et al. 2007), two other important pathogens under Mediterranean conditions (Rubiales et al. 2009). This fact might facilitate future crop improvement by producing new pea cultivars with multiple resistance specificities using molecular breeding strategies. Ninety-four F_2 plants were sown in the field at the beginning of February together with the susceptible control cv. "Messire". In June, when pods were at maturity, 25 seeds per plants were collected and sown under controlled condition to obtain the subsequent F_3 families.

These 94 F_3 families were grown in pots (35 × 35 cm), two plants per pot which were filled with a 1:1 mixture of sand and peat. Three replicas were performed with eight plants per replica in a completely randomized design. Seedlings were inoculated when the third leaf was completely expanded. Inoculation was carried out by dusting the plants with *U. pisi* isolate Up-Co01 urediospores (2 mg spores $plant^{-1}$) diluted in pure talc (1:10, v:v) using a spore settling tower. Plants were incubated for 24 h at 20°C in complete darkness and 100% relative humidity, then transferred to a growth chamber at 20°C, with a photoperiod of 14/10 h day/night regime, with 148 μmol m^{-2} s^{-1} irradiance at plant canopy.

Infection Type (IT) and Disease Severity (DS) were observed 15 days after inoculation. DS was visually estimated as the percentage of symptomatic area of the whole plant. IT was assessed using the 0-4 scale of Stackman et al. (1962), where IT 0 = no symptoms, IT; = necrotic flecks, IT 1 = minute pustules barely sporulating; IT 2 = necrotic halo surrounding small pustules, IT 3 = chlorotic halo and IT 4 = well-formed pustules with no associated chlorosis and necrosis.

PCR analysis

For DNA extraction, about 100 mg of young leaf tissue from the F_2 plants was excised, immediately frozen in liquid nitrogen and stored at −80°C. DNA was isolated using the CTAB method (Lassner et al. 1989), with slight modifications (Torres et al. 1993). In the initial screening, a total of 660 RAPD decamer primers (prefix "OP-" from Operon Technologies, Alameda, CA, USA) were tested on the parents for polymorphism. Once a polymorphism was found, the corresponding primer was genotyped in the F_2 population and the two parents for linkage analysis. Optimal reaction conditions were established according to Cobos et al. (2005). Amplification was performed in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in 25-µl volumes including 20-40 ng plant genomic DNA, buffer (50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100), 2 mM $MgCl_2$, 2.5 mM of each dNTP, 0.2 µM of each primer and 1 unit of *Taq* DNA polymerase (Biotools B&M Labs, Madrid, Spain). Forty cycles denaturation at 94°C for 20 s, annealing for 1 min at 36°C and 1 min elongation at 72°C, with a final extension at 72°C for 8 min were performed.

In addition 10 STS markers reported by Gilpin et al. (1997) were surveyed for polymorphism using their protocol. The 25-µl reaction volumes included 20 ng plant genomic DNA in buffer (10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100), 2.5 mM Mg Cl_2, 200 µM each dNTP, 1.0 µM primer and 0.625 U *Taq* DNA polymerase (Bioline Ltd, London, UK). After DNA denaturation for 1 min at 95°C, the reaction mixture was subjected to 40 cycles of the following temperature profile: 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 8 min. When no polymorphism was detected, PCR products from both parents were digested with a range of restriction endonucleases which recognized 4- and 5- base sequences (Bio-$Labs_{inc}$, Ipswich, MA, USA). A 0.2 µl aliquot of restriction enzyme, 12 µl of sterile water and 2.5 µl of the buffer required for each enzyme were added to 10 µl of the PCR reaction, and the digestion was incubated overnight at 37°C.

Amplification products from the RAPD and STSs were separated in gels composed of a mixture of 1% agarose and 1% Nu-Sieve agarose (Hispanlab, Spain) in one-time Tris–borate-EDTA (TBE) buffer containing 0.5 µg/ml ethidium bromide (Sambrook et al. 1989) for 3 h at 105 V. The gel was scanned using Kodak Digital Science 1D (ver. 2.0 and 3.5) Image Analysis Software (New Haven, CT, USA).

Statistical analysis

Segregation data from the F_2 population together with the parental accessions IFPI3260 and IFPI3251 were used to obtain a map. Goodness-of-fit to the expected 3:1 and 1:2:1 segregation ratio of the RAPD and STS markers, respectively, was calculated by χ^2 tests. Linkage analysis was performed using MapMaker ver. 2.0 (Lander et al. 1987). A LOD score threshold of 3 and a maximum recombination fraction of 0.3 were employed as general linkage criteria to establish linkage groups (LGs). Kosambi's function was applied to estimate map unit distances (Kosambi 1944).

The phenotypic DS values measured in controlled conditions were normalized by arcsine transformation [y = arcsine ($\sqrt{}$DS)], and used for QTL analysis. Skewness and Kurtosis coefficients were calculated following Lynch and Walsh procedure (1997). QTL analysis was performed using QTL Cartographer, ver. 2.5 (Wang et al. 2005). The threshold for the detection of a QTL appropriate for a sparse-map case (Lander and Botstein 1989) was calculated by a Bonferroni correction to ensure the experiment-wise significance level of 95%. If n independent tests (number of marker intervals) with significance level α are conducted, the probability that at least one test being false positive is (Lynch and Walsh 1997):

$$\gamma = 1 - (1 - \alpha)n$$

Bonferroni's correction for multiple comparisons states that an overall significance level γ requires that each individual test be based on a significance level of:

$$\alpha = 1 - -(1-\gamma)1/n$$

In our case, 137 marker intervals were tested and the experiment-wise type-I error probability was set to $\alpha = 0.05$. Thus, comparison-wise type-I error probability using Bonferroni correction was calculated ($g = 0.000374$) corresponding to an LR threshold of 18.34 (LOD = 3.98) with 3 degrees of freedom (additive and dominance effects for a QTL are fitted).

The coefficient of determination (R^2) for the marker most tightly linked to a QTL was used to estimate the proportion of the total phenotypic variation explained by the QTL.

Results

Disease assessment

Confirming results in previous studies (Barilli et al. 2009a, b), the parental accessions showed a compatible interaction (high IT) against *U. pisi* although they differed in DS. IFPI3260 displayed an incomplete resistance with a DS value of 1% (arcsine transformed DS value = 0.10). In contrast, the parental IFPI3251 was highly susceptible, showing a DS value of 19.4% (arcsine transformed DS value = 0.46) (Fig. 1). Disease assessment in the F_3 population followed a normal distribution (Lynch and Walsh normality test, $P > 0.01$), with DS values ranking between 0.4 and 14.9% (arcsine transformed DS values between 0.06 and 0.4). Highly significant differences between families (ANOVA $P < 0.0001$) were observed. The coefficient of Skewness in controlled conditions was of 0.86, indicating that the population distribution tends to the resistance as did the parent IFPI3260. In addition, five lines more resistant than IFPI3260 were observed, but differences were not significant (Dunnet test, $P > 0.05$).

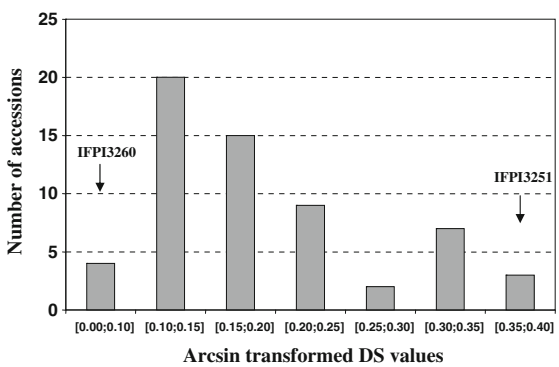

Fig. 1 Frequency distribution for rust resistance estimated as the arcsin transformed DS values in the pea F2 population IFPI3260 × IFPI3251 under controlled conditions

Linkage map

Eighteen RAPD primers out of the 660 tested in the parental lines, revealed the existence of polymorphism and were screened in the whole F_2 population originating 168 clear and scorable bands. Segregation deviation from the expected 3:1 ratio was determined based on a chi-squared test at $P < 0.05$ and $P < 0.01$ levels. At $P < 0.05$ level, only two RAPD markers ($OPAB17_{519}$ and $OPAD11_{1277}$) showed distorted segregation.

Only two of the 10 STSs analyzed showed a direct length polymorphism between the parental lines while the remaining 8 had to be digested with different restriction endonucleases to reveal six putative codominant STSs (P202, Q500 and P393 digested with *HindI*; M27 and Q363 with *RSAI*; P628 digested with *AluI*). However, the amplification patterns achieved in most cases were not precise and repeatable enough to genotype unambiguously the whole population. As a result, only 2 STSs (P482 and Q363) could be properly amplified in the F_2 population and included in the

Table 1 Linkage group (LG), number of RAPD markers, lenght (cM) and distance average between markers (cM) of the linkage map developed from molecular data of 94 F_2 accessions from the cross IFPI3260 × IFPI3251

LG	Number of markers	Length (cM)	Distance between markers	STS
LG01	36	250.8	7.17	
LG02	29	331.7	11.85	
LG03	24	314.2	13.66	
LG04	17	87.3	5.46	
LG05	16	61.9	4.13	P482
LG06	9	57.6	7.20	P363
LG07	8	109.9	15.70	
LG08	5	63.3	15.83	
LG09	2	6.6	6.60	
Not linked	24			
Total mapped	146	1283.3		
Average			9.73	

linkage analysis. The map covered 1283.3 cM of the wild pea genome with an average inter-marker distance of 9.73 cM (Table 1). The use of two common markers (P482 and Q363) with the international pea consensus genetic map (Weeden et al. 1998) enabled two of the linkage groups (LGs 5 and 6) to be assigned to the pea chromosome VI (Fig. 2).

QTL mapping

Quantitative trait loci analysis with composite interval mapping (CIM) method revealed a single genomic region associated with resistance to *U. pisi* in controlled conditions located in LG 3. The putative QTL *Up1* showed a LOD score of 4.83 and explained the 63% of the phenotypic variation. *Up1* was localized at 32.9 cM from the beginning of the LG03, between the RAPD markers $OPY11_{1316}$ and $OPV17_{1078}$. The distance to these flanking markers was of 6 and 13.4 cM, respectively. The QTL distance to $OPV17_{1078}$ might be even smaller considering the 1-LOD support interval which range between 32.9 and 38.9 cM (Table 2; Fig. 3). Both markers derive from the susceptible parent IFPI3251. The resistance-enhancing allele originates from the resistant parent IFPI3260 as shown by the negative value of the additive genetic effect (-0.094); the dominant effect was of -0.056 (Table 2). *Up1* showed the dominance/additive (*d/a*) ratio of 0.6 indicating a partially dominant gene action.

Discussion

In the present study, a linkage map was developed using a F_2 population derived from the intraspecific

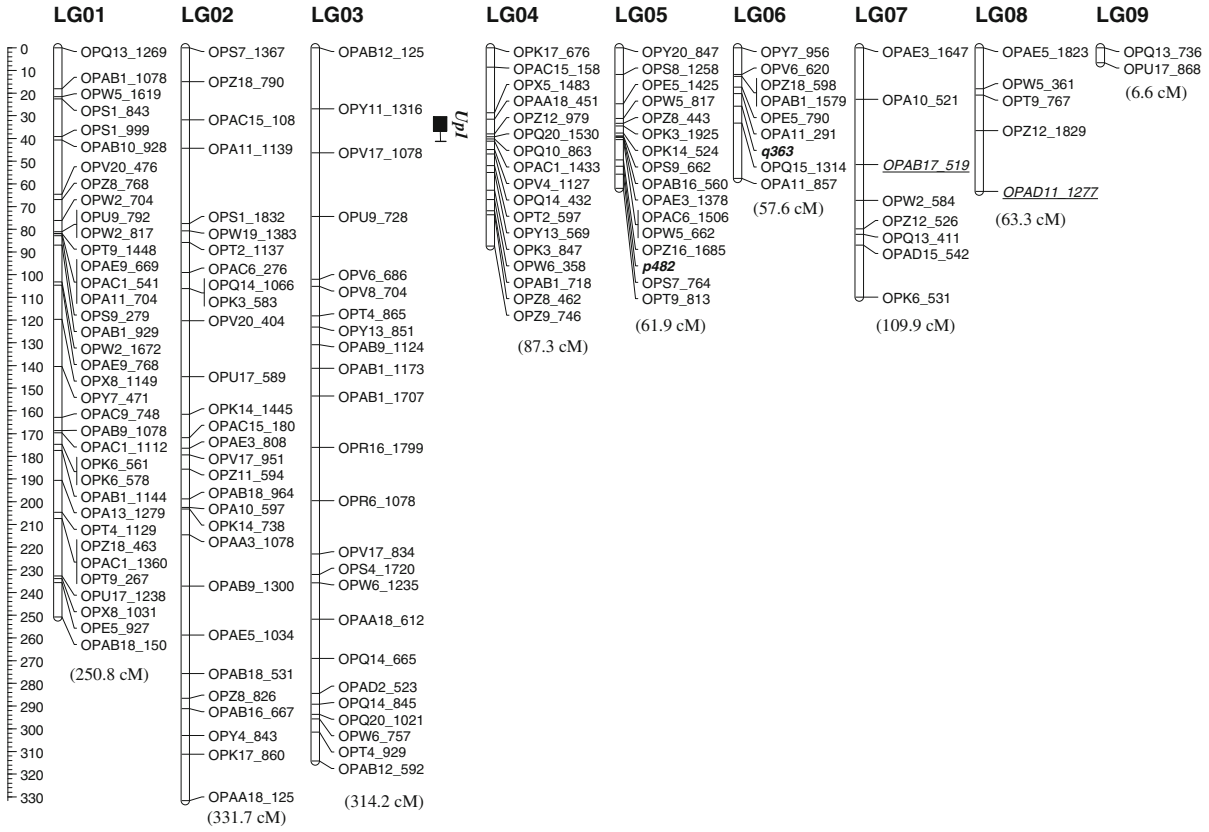

Fig. 2 Genetic linkage map from 94 F2 plants from the cross IFPI3260 × IFPI3251. The size of each group is indicated in centimorgans (cM). Bar position indicate the location of the QTL for resistance to *U. pisi* in controlled conditions. Outer and inner interval corresponding to 1-LOD and 2-LOD support interval are indicated as a *full box* and a *single line*, respectively. Markers in *bold italics* are anchored to other published pea genetic maps. Markers showing distorted segregation are *underlined*

Table 2 Putative QTL for *U. pisi* resistance in controlled conditions detected in the F_2 pea population by composite interval mapping (CIM)

Carácter	LG[a]	QTL position[b]	1-LOD support interval[c]	Flanking markers		LOD	a[d]	d[e]	R^{2f}
Disease Severity	3	32.9	32.9-38.9	OPY11_1316	OPV17_1078	4.83	−0.0937	−0.0563	0.63

[a] Linkage group in which the QTL is located

[b] QTL position from the beginning of the LG (cM)

[c] Refers to the region flanking the QTL peak in which the LOD score decline by one

[d] Additive effect of the QTL

[e] Dominant effect of the QTL

[f] Proportion of phenotypic variance explained by the QTL

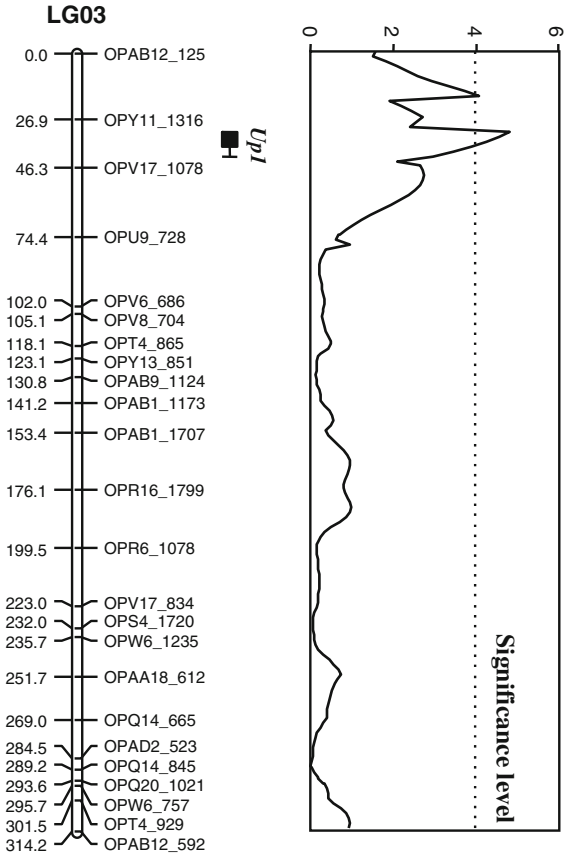

Fig. 3 LOD profile of the QTL analysis of rust resistance obtained in the pea F_2 population IFPI3260 × IFPI3251 using CIM

cross between two *Pisum fulvum* lines. The approach has enabled, for the first time, the identification of a QTL for resistance to pea rust incited by *U. pisi* in wild pea.

The high level of resistance showed by the *P. fulvum* accession IFPI3260, previously reported by Barilli et al. (2009a, b), has been confirmed in the present study. This outcome points out the scoring mode for rust resistance, reported in this study, as a reliable method. Both parental lines and the derived progeny showed a compatible interaction with no associated cell death. Resistance was based on the reduction of the disease severity. This is in agreement with previous studies in which only partial resistance was described against both *U. viciae-fabae* (Pal et al. 1980; Singh and Sokhi 1980; Xue and Warkentin 2001; Chand et al. 2006) and *U. pisi* (Barilli et al. 2009a, b, c). In other legume-rust pathosystems, quantitative non-hypersensitive types of resistance are predominant (reviewed in Sillero et al. 2006), e.g. in pea—*U. viciae-fabae* (Pal et al. 1980), in chickpea—*U. ciceris-arietini* (Madrid et al. 2008) and in groundnut—*Puccinia arachidis* (Subrahmanyam et al. 1993). However, the hypersensitive reaction is found on certain legumes species such as *Phaseolus vulgaris*, against *U. appendiculatus* (Stavely et al. 1989), faba bean against *U. viciae-fabae* (Sillero et al. 2000) or soybean against *Phakopsora pachyrhizi* (Li 2009), but the great disadvantage of this type of resistance is that it is often ephemeral (Niks and Rubiales 2002), due to the evolution of virulent fungal isolates.

The map developed from the F_2 population, covered a genetic distance of 1283.3 cM and contained 146 markers, distributed in nine linkage groups. The extent of genome coverage is comparable with that of previous pea maps developed in *P. sativum* by Gilpin et al. (1997) (1,330 cM), and higher than the maps reported by Tar'an et al. (2003) (1,274 cM), Prioul et al. (2004) and Timmermann-Vaughan et al. (2004) (1,061 and 930 cM, respectively) and Fondevilla et al. (2008) (1,214 cM). Unfortunately, our map is only

connected to the pea consensus map of Weeden et al. 1998 (nearly 800 cM) by two common STS markers. The transferability to these markers allowed us to assign two linkage groups to a specific pea chromosome. In particular, the STSs P482 and Q363 located on the LG 5 and 6, respectively, enabled us to assign these LGs to the chromosome 6 of the *P. sativum* consensus map reported by Weeden et al. (1998).

A single QTL (*Up1*) was identified on LG 3. Frequency distribution for rust resistance in the pea F_2 population IFPI3260 × IFPI3251 tended towards a bimodal distribution of DS values suggesting an oligogenic control of resistance in these materials. The involvement of a major genetic factor for partial resistance to rust would explain such behaviour as supported by the high R^2 (0.63) revealed for the only QTL detected. The remaining variation not explained by *Up1* could be due to undetected additive QTLs with weak effects or to incomplete map coverage. Analysis of a larger populations or a more precise quantitative scoring system of the disease reaction may allow the detection of additional minor QTLs that account for the remaining unexplained variance.

The bimodal distribution of the resistant trait in the progeny and the identification of a single genomic region associated with resistance to *U. pisi* points towards the quantitative nature of rust resistance in cross IFPI3260 × IFPI3251, and identify molecular markers associated with partial resistance. Partial resistance is usually assumed to be poligenically inherited. However, this is not always the case as single genes, causing partial resistance to rust, have been described in pea (Vijayalakshmi et al. 2005), chickpea (Madrid et al. 2008) and wheat, e.g. *Lr34* (Rubiales and Niks 1995) and *Lr46* (Singh et al. 1998; Martínez et al. 2001), always related to pre-haustorial resistance.

Our results indicate that the QTL explained a very high percentage of the phenotypic variation throughout the population, suggesting that effective selection could be possible with a few markers tightly linked to the QTL. The RAPD markers $OPY11_{1316}$ and $OPV17_{1078}$ are located at the position 26.9 and 46.3 cM respectively, delimiting a region fairly close to *Up1*. Therefore the RAPD markers $OPY11_{1316}$ and $OPV17_{1078}$ should allow, after their conversion in SCARs markers, a reliable marker-assisted selection for rust resistance.

Nevertheless, QTL mapping is only the first step in MAS, because QTL analysis needs to be refined to determine the precise position and effects of the QTLs involved. Future research will be focused on the saturation of the region bearing this QTL with more robust and transferable markers such as new STSs, microsatellites (Tauz and Renz 1984; Loridon et al. 2005) with the aim to identify one in coupling phase with the resistant parent IFPI3260, tightly linked to *Up1*.

Markers associated with rust resistance in *P. fulvum* could be also assayed in other *P. sativum* populations (Vijayalakshmi et al. 2005) or even in other legume species. DNA markers that allow cross-species mapping (referred as anchor markers) are critical for comparative genome analysis. The approach has revealed significant transferability between species (Gutiérrez et al. 2005; Madrid et al. 2008). This transferability could help us to verify if the same region is presented in this model specie whose genome is being sequenced.

Moreover, ESTs (Expressed Sequence Tagged Sites) derived from protein-encoding regions in a number of model species are being extensively used to detected orthologous loci in multiple legume genomes (Choi et al. 2006). Efforts to integrate the informative of anchor markers into crop legumes linkage maps are also in progress (Sato et al. 2005; Nelson et al. 2006; Phan et al. 2007; Ellwood et al. 2008). It is anticipated that the inclusion in this maps of genes of known function, will help us to verify if any of the mapped genes is a good functional or positional candidate for the rust resistance.

Our results for rust resistance must be validated across diverse locations and genetic backgrounds before the application of MAS in pea breeding programs. For this reason, we are at present developing the recombinant inbred lines (RILs) derived from the cross, as well as between *U. pisi*-resistant *P. sativum* lines and susceptible high-yielding commercial varieties, in order to analyze QTL stability in different environments and pedigrees. As susceptibility of *Pisum* spp. accessions to faba bean rust (*U. viciae-fabae*) has been already described (Pal et al. 1980; Xue and Warkentin 2001; Chand et al. 2006), the availability of RILs is also of great importance to evaluate the fixed material with different pathogen species.

In conclusion, the present study is the first report on the detection of a QTL controlling a high percentage of the resistance against *U. pisi* in *P. fulvum* and therefore may be useful to predict the location of the homologous region in related species. The inclusion of new transferable markers and the transformation into SCARs of the RAPDs tightly linked to the detected QTL will enhance the possibility of developing a map useful not only for MAS purposes in wild and cultivated peas but also for comparative mapping studies among different legumes.

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