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C. M. Avila · J. C. Sillero · D. Rubiales · M. T. Moreno · A. M. Torres

Identification of RAPD markers linked to the Uvf-1 gene conferring hypersensitive resistance against rust (*Uromyces viciae-fabae*) in *Vicia faba* L.

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Abstract Bulk segregant analysis was used to identify random amplified polymorphic DNA (RAPD) markers linked to a gene determining hypersensitive resistance in *Vicia faba* line 2N52 against race 1 of the rust fungus *Uromyces viciae-fabae*. The monogenic nature of the resistance was determined by analyzing the F_2 population from a cross between resistant line 2N52 and susceptible line VF-176, and further confirmed in the $F_{2:3}$-derived families. Linkage of the RAPD markers was confirmed by screening 55 F_2 plants segregating for resistance. Three RAPD markers ($OPD13_{736}$, $OPL18_{1032}$ and $OPI20_{900}$) were mapped in coupling phase to the resistance gene for race 1 (*Uvf-1*). No recombinants between $OPI20_{900}$ and *Uvf-1* were detected. Two additional markers ($OPP02_{1172}$ and $OPR07_{930}$) were linked to the gene in repulsion phase at a distance of 9.9 and 11.5 cM, respectively. The application of marker-assisted selection to develop new faba bean varieties with rust resistance genes is discussed.

Keywords *Vicia faba* · Rust · *Uromyces viciae-fabae* · Resistance genes · Marker-assisted selection

Introduction

Uromyces viciae-fabae (Pers.) J. Shört. is the causal agent of faba bean rust, a disease found worldwide that can cause up to a 70% loss in yield, particularly when the infection starts early in the season. Several methods for rust control have been developed, including biological control (Gaunt 1983), systemic induced resistance (Murray and Walters 1992; Sillero 1999) and cross protection (Yarwood 1956), but these have proved to be inapplicable at the commercial level (Stoddard and Herath 2001). Chemical control of the disease has been reported (Williams 1978; Gaunt 1983; Yeoman et al. 1987; Marcellos et al. 1995; Emeran and Rubiales 2001), but the extensive use of fungicides is not desirable in sustainable production systems of grain legumes where the most efficient use of chemicals is pursued. For an increased efficiency in their use, chemicals should be combined with partial disease resistance. Thus, the development of resistant cultivars seems to be the most practical and cost-efficient method for the control of diseases affecting grain legumes.

Several faba bean sources showing two different types of incomplete resistance to *U. viciae-fabae* have been reported. In one type, resistance is expressed as a reduction of disease severity without any macroscopically visible necrosis (Bernier and Conner 1982; Rashid and Bernier 1986a, b, 1991; Sillero et al. 2000). In the other, the hypersensitive resistance has been described as an incomplete resistance associated with late-acting necrosis of the host tissue, resulting in a reduction of the infection type (Sillero et al. 2000). Both types of incomplete resistance differ only in the presence or absence of macroscopically visible necrosis (Sillero et al. 2000; Sillero and Rubiales 2002).

With respect to the genetic basis of hypersensitive resistance, Sillero et al. (2000) suggested the monogenic inheritance of the character. In another study, Emeran et al. (2001) grouped 23 isolates of *U. viciae-fabae* from various regions around the world into 16 different races, based on the presence or absence of necrosis as discrimination criteria. The authors suggested the race-specific nature of the hypersensitive resistance. This evidence of physiological specialization in *U. viciae-fabae* implies that the use of single resistance genes in cultivars would likely not result in long-term rust control. Among the strategies proposed to increase the durability of this type of resistance is the incorporation of different resistance

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C. M. Avila (✉) · J. C. Sillero · M. T. Moreno · A. M. Torres
Departamento de Mejora y Agronomía, CIFA-Alameda del Obispo, Apdo 3092, 14080 Córdoba, Spain
e-mail: ge2avgoc@uco.es
Fax: +34-957-016043

D. Rubiales
Departamento de Agronomía y Mejora Genética Vegetal, CSIC-Instituto de Agricultura Sostenible, Apdo. 4084, 14080 Córdoba, Spain

genes into a single agronomically interesting cultivar, a process labelled resistant gene pyramiding (Duvick 1996).

Selection for disease resistance in breeding programs implies a disease screening process that in some cases is difficult or unreliable. Likewise, the obligate parasitic nature of *U. viciae-fabae* makes it difficult to maintain the pathogen in culture and to apply it to screen segregating progenies under controlled growth conditions. Moreover, the natural occurrence of the disease under field conditions is dependent upon the occurrence of appropriate environmental conditions, which further complicates the breeding program. The race-specific nature of the resistance emphasizes the necessity of screening plants simultaneously or even sequentially with several races, and this process is difficult or impossible. Therefore, the development of reliable and user-friendly molecular markers closely linked to the resistance genes provides a useful tool for breeders for gene pyramiding. This approach can be exploited for the indirect selection of interesting genotypes in a breeding program. Progress has been made in mapping and tagging many agriculturally important genes with molecular markers, forming the foundation for marker-assisted selection (MAS) in crop plants. Different molecular markers and several strategies have been developed. The choice depends on the population type under study and the inheritance type of the character.

The main goal of investigation reported here was to detect markers tightly linked to the gene(s) involved in hypersensitive resistance to rust in the *V. faba* inbred line 2N52, characterized as resistant by Sillero et al. (2000). Previously, it was necessary to study the genetic basis of this type of resistance against one of the races described by Emeran et al. (2001) (race 1). Once linked markers were identified, their selection efficiency was determined. The results can be used to develop a MAS program for rust resistance in faba bean.

Materials and methods

Inheritance of the resistance

Inheritance studies were performed on F_2 and $F_{2:3}$ progeny of the cross between the *Vicia faba* L. inbred line 2N52, showing hypersensitive resistance to faba bean rust, and line VF-176, characterized as susceptible (Sillero et al. 2000). F_2 plants were grown in a growth chamber in pots filled with a 1:1 mixture of sand and peat. After the evaluation, the F_2 plants were grown in the field to obtain the $F_{2:3}$ families. Single pustule isolate 96-Cord-2 of *Uromyces viciae-fabae*, characterized as race 1 of the pathogen (Emeran et al. 2001), was used in all inoculations. Inoculum was produced on plants of the susceptible line VF-176 and collected 24 h before inoculation. Seedlings were inoculated when the third leaf was completely expanded. Inoculation was carried out by dusting the plants with rust urediospores (2 mg spores/plant) diluted in pure talc (1:10) using a spore settling tower. The plants were then incubated for 24 h at 20 °C in complete darkness and 100% relative humidity. After this period, they were transferred to a growth chamber maintained at 20 °C under a 14/10-h (light/dark) photoperiod with a light intensity of 148 μmol/m^2 per second at the leaf canopy. The disease response was evaluated by scoring the infection type (IT) 10–15 days after inoculation using the IT scale of Stakman et al. (1962), where 0 = no symptoms, ; = necrotic flecks, 1 = minute pustules barely sporulating, 2 = necrotic halo surrounding small pustules, 3 = chlorotic halo and 4 = well-formed pustules with no associated chlorosis or necrosis. Plants showing IT values lower than 3 were considered resistant (R), while those having IT values higher than or equal to 3 were considered susceptible (S). Fifty-five F_2 plants and 51 $F_{2:3}$ families (15 plants per family) were inoculated and scored as described above. Analysis of disease reaction in $F_{2:3}$ families was used to identify homozygous resistant and susceptible F_2 lines.

Random amplified polymorphic DNA (RAPD) analysis

The bulk segregant analysis technique (BSA) (Michelmore et al. 1991) was used to identify RAPD markers linked to the resistance gene(s) against race 1 of *U. viciae-fabae*. Molecular analyses were carried out using leaf tissue from 55 F_2 plants. Homozygosity of F_2 resistant and susceptible plants to be used in the bulks was determined by inoculating 15 plants of each of 51 $F_{2:3}$ families. Based on these results, two contrasting bulks were prepared, each containing an equal amount of DNA from six homozygous resistant or susceptible F_2 individuals. Templates for polymerase chain reactions (PCRs) consisted of 20–40 ng of DNA. Amplifications were performed as described by Williams et al. (1990) and Welsh and McClelland (1990) with slight modifications (Torres et al. 1993). A total of 460 RAPD primers from Operon Technologies (Alameda, Calif.) were surveyed between the pools. Primers generating marker polymorphisms between the resistant and susceptible bulk DNAs were subsequently tested individually on DNA from the six homozygous resistant and susceptible F_2 plants used in the bulk. When the polymorphism was maintained, the primers were screened on the F_2 population for linkage analysis.

Data analysis

The segregation of the locus for rust resistance and for each marker was tested against the expected segregation ratio using a chi-square goodness of fit in the F_2 and/or F_3 generations. The linkage map was constructed using MAPMAKER v. 3.0 (Lander et al. 1987). A LOD threshold of 3 was established to consider significant linkage. Recombination fractions were converted to centiMorgans (cM) using the Kosambi mapping function (1944).

Selection efficiency

The selection efficiency for the identification of resistant/susceptible plants using selected RAPD markers was determined by analyzing phenotypic data obtained from the 55 F_2 plants inoculated with race 1. The segregation of the $F_{2:3}$ families was used to test the selection efficiency for the simultaneous use of two markers, one in coupling phase to resistant gene and the other in repulsion phase, to allow their use as a single codominant marker (Haley et al. 1994; Young and Kelly 1997).

Results

The segregation for resistance to faba bean rust race 1 gave a good fit to a 3:1 ratio resistant:susceptible ($\chi^2_{0.05}$ = 0.297) in F_2 plants originated from the cross 2N52 × VF-176 (Table 1). Consequently, it was hypothesized that a single dominant gene (formerly designed *Uvf-1*) controlled the specific resistance against the rust race 1. This hypothesis was confirmed in the $F_{2:3}$ families based on a satisfactory fit to a 1:2:1 ratio (number of families non-segregating for resistance:segregating for resistance and

Table 1 The chi-square test for segregation of resistance and susceptibility of 55 F_2 plants and 51 F_3 families to the rust race 1 from faba bean cross 2N52 (resistant) × VF 176 (susceptible)

Generation	Number of plants			Expected ratio	χ^2	*P*
F_2	R[a]	S[b]		3:1	0.297	0.586
Observed	43	12				
Expected	41.25	13.75				
F_3	R	Segregating	S	1:2:1	0.372	0.830
Observed	14	26	11			
Expected	12.75	25.5	12.75			

[a] R, resistant: infection type ≤ 2
[b] S, susceptible: infection type >2

susceptibility:non-segregating for susceptibility) (Table 1).

Twenty-three polymorphic markers were initially identified in resistant and susceptible pools from the screening of 460 Operon primers. Of these, only five ($OPI20_{900}$, $OPD13_{736}$, $OPL18_{1032}$, $OPP02_{1172}$ and $OPR07_{930}$) were selected since they were consistently detected in all the repetitions performed in a single pool (Fig. 1). These polymorphisms were subsequently tested on the six individuals that formed each bulk. Out of five markers identified, three were associated in coupling phase to the resistant allele, while two were linked in repulsion phase, i.e. they were linked to the susceptible allele (Fig. 1).

All of the selected markers were then screened on the 55 F_2 individuals previously evaluated for disease resistance in the $F_{2:3}$ families. The co-segregation data between markers and resistance gene *Uvf-1* were used to construct a linkage map shown in Fig. 2. No recombinant individuals were found between the $OPI20_{900}$ marker and *Uvf-1*. Markers $OPD13_{736}$ and $OPL18_{1032}$, which were also linked in coupling phase to the resistance gene, were located 7.1 cM from *Uvf-1*. The markers linked in repulsion phase were located at 9.9 cM ($OPP02_{1172}$) and 11.5 cM ($OPR07_{930}$) from *Uvf-1*.

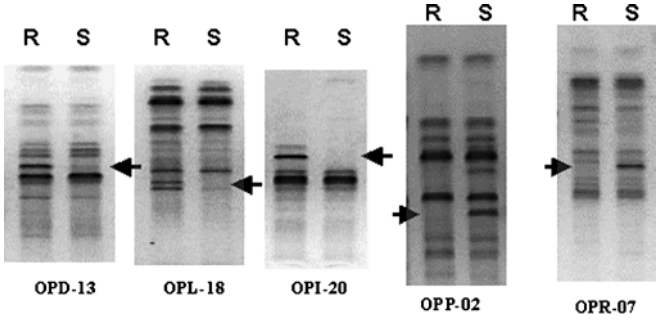

Fig. 1 Amplification products of RAPD markers OPD13, OPD18, OPI20, OPP02 and OPR07. *R* Resistant bulk, including six homozygous resistant F_2 individuals, *S* susceptible bulk, including six homozygous susceptible F_2 individuals. RAPD markers associated with resistance or susceptibility are identified with a *black arrow*. $OPD13_{736}$, $OPL18_{1032}$ and $OPI20_{900}$ were linked in coupling phase to resistant gene. $OPP02_{1172}$ and $OPR07_{930}$ were linked in repulsion phase to the resistant gene

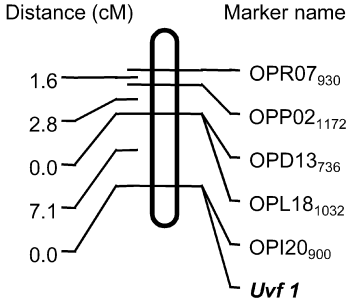

Fig. 2 Linkage map of the 2N52 × VF-176 population showing the position of molecular markers $OPD13_{736}$, $OPL18_{1032}$, $OPI20_{900}$, $OPP02_{1172}$ and $OPR07_{930}$ and the *Uvf-1* gene for resistance to rust (race 1) in faba bean. Marker names are shown on the *right* and the estimated map distances are shown on the *left*. Recombinant fractions were converted to centiMorgans using the mapping function of Kosambi (1944)

Selection efficiency

The selection efficiency was defined by Young and Kelly (1997) as the ability of a RAPD marker, used alone or in combination with another marker, to effectively distinguish between resistant and susceptible F_2 individuals based on the marker phenotype as selection criterion. Since we had identified markers linked both in coupling and repulsion phase, we determined the selection efficiency for *Uvf-1* using a single marker in coupling phase or a combination of two markers in opposite linkage phase. The selection efficiency for the closest linked markers to the resistance gene is shown in Table 2. The marker $OPI20_{900}$, which is linked in coupling phase, allows the detection of resistant individuals (RR, Rr) with 100% efficiency. However, it does not allow homozygous individuals to be distinguished from heterozygous ones. On the other hand, $OPP02_{1172}$ only showed a selection efficiency of 41% (Table 1), but the probability of selecting homozygous resistant individuals would be very high among those individuals that fail to amplify. The most interesting strategy would be to use both types of markers simultaneously because the selection efficiency for detecting resistant individuals is 100%. Moreover, practically all of the heterozygous individuals were identified with a combination of both markers (Table 2).

Table 2 Selection efficiency for resistance against race 1 of faba bean rust in each 15 plants of each of 51 $F_{2:3}$ families from cross 2N52 × VF-176 based on molecular markers

Phenotype	Observed frequency[a]	$OPI20_{900}$ (Coupling phase)	$OPP02_{1172}$ (Repulsion phase)	$OPI20_{900}/OPP02_{1172}$
Resistant plants	RR: 14 Rr: 26	R-: 40 (+)[c]	RR = 14 (–)[d]	RR:14 (+/–) Rr:23 (+/+)
Recombinants			Rr:3 (–)	Rr:3 (+/–)
Susceptible plants	rr: 11	rr: 11 (–)	r-: 34 (+)	rr: 11 (–/+)
Selection efficiency[b] (%)		100%	41%	100%

[a] Observed frequency 1:2:1. R represents the dominant allele for resistance and r the recessive allele
[b] Selection efficiency in the F_2 generation
[c] Band present
[d] Band absent

Discussion

The segregation results obtained in this work confirm the hypothesis of a single gene controlling the character under study, as previously suggested by Sillero et al. (2000). Thus, the hypersensitive resistance to rust in faba bean may be described as a monogenic and race-specific resistance. This type of resistance is attractive for breeders because the underlying genes are easy to incorporate into susceptible material through simple backcrossing, although they are usually a less durable source of resistance. Since hypersensitive resistance genes can be overcome by new virulent races of the pathogen, race-specific resistance genes have often short lifetimes when used individually. The pyramiding of resistance genes has been suggested as a strategy to provide durable resistance to pathogens through the accumulation of several resistance genes into a single line or cultivar (Duvick 1996). This process could be facilitated by the identification of molecular markers tightly linked to resistant genes that would allow the indirect selection of interesting genotypes in MAS breeding programs through a marker-assisted selection program.

The technique used to generate these markers must be simple, reliable and suitable for processing a large number of samples per unit of time. In addition to this, some factors as the inheritance type of the character under study, would determine the best method to optimize the process. In our case, due the monogenic inheritance of the *Uvf-1* gene, we have used the BSA, first described by Michelmore et al. (1991), to identify DNA markers closely linked to this resistance gene.To our knowledge, this is the first time that BSA has been used in faba bean breeding programs, although this strategy has been commonly used in other legume crops such as lentil (Eujayl et al. 1998; Ford et al. 1999; Chowdhury et al. 2001), common bean (Olaya et al. 1996; Young and Kelly 1997; Bai et al. 1997; Alzate-Marín et al. 1999; Park et al. 1999; Faleiro et al. 2000), pea (Timmerman et al. 1994; Tiwari et al. 1998) and soybean (Mian et al. 1999). In all these cases, BSA allowed rapid mapping of monogenic resistance genes using segregating populations.

As in our case, RAPD markers were usually preferred in the studies described above due to several advantages. The RAPD technique is simple, quick, relatively inexpensive and suitable to process many samples per day, making it potentially interesting for a plant breeding program (Johnson et al. 1995). The usefulness in MAS of the RAPD markers linked to the *Uvf-1* gene will depend on the distance and orientation of the markers in respect to the gene and the type of population under analysis.

High efficiencies require small distances between markers and genes. The best results are obtained when no recombination is produced between the marker and the gene of interest. Unfortunately, in most cases, some degree of recombination is observed. However, in our work, no recombinant individuals were detected between $OPI20_{900}$ and *Uvf-1* (Table 2), which determines the high selection efficiency of this marker for resistant genotypes (Table 2). On the contrary, three recombinant genotypes were detected between $OPP02_{1172}$ and *Uvf-1* (Table 2) since this marker is located at 10 cM from *Uvf-1*.

Although RAPD markers do not normally allow homozygous and heterozygous individuals to be discriminated, several strategies have been proposed to improve the selection efficiency of MAS. Thus, the use of markers linked in repulsion phase to the resistance gene would allow the discrimination of homozygous-susceptible individuals from heterozygous ones. Consequently, those individuals that lack the marker have a high probability of being homozygous for resistance, although recombination between marker and gene cannot be ruled out (Haley et al. 1994; Johnson et al. 1995). The strategy pursued in our work was to use both coupling- and repulsion-linked markers. This allows their use as a codominant marker (Haley et al. 1994; Johnson et al. 1995; Young and Kelly 1997; Alzate-Marin et al. 1999), thereby increasing the selection efficiency for resistant plants. This approach has been successfully used in other crops. Thus, for example, the selection of resistant plants to common bean mosaic virus (Haley et al. 1994), rust (Johnson et al. 1995) and anthracnose (Young and Kelly 1997; Alzate Marin et al. 1999), based on the simultaneous use of markers linked in coupling and repulsion phases to the resistance gene, has been shown to be efficient.

We obtained the same selection efficiency using only the marker linked in coupling phase, $OPI20_{900}$, or a combination of markers, $OPI20_{900}$ and $OPP02_{1172}$, asso-

ciated respectively in coupling and repulsion phase (Table 2). Only the second strategy allows genotype selection, making the process more informative since heterozygous individuals are detected. Several sources of hypersensitive resistance to rust have been reported in faba bean (Sillero et al. 2000), and it is possible that the resistance in these lines is controlled by different genes. At present, this possibility is under study by developing appropriate crosses between the different resistant lines. The line 2N52 used in this work has been characterized as resistant to another seven races of the pathogen (races 2, 4, 5, 8, 9, 10 and 13) (Emeran et al. 2001). Whether resistance to these races is controlled by the same gene or by a number of different genes is currently under study. The main goal of this research is the identification of different hypersensitive resistance genes that would allow pyramiding these genes into a single agronomically interesting *V. faba* genotype. The development of genetic markers for all these genes would avoid the tedious and time-consuming process of inoculation and evaluation that is made even more difficult by the race specificity of hypersensitive rust resistance in faba bean. This requires that the screening nurseries have to be inoculated systematically with different races of the pathogen to ensure that gene combinations are being maintained. The availability of molecular markers tightly linked to resistance genes would help identify plants carrying multiple genes without subjecting them to pathogen inoculation in early generations. The present work represents the first step towards the development of a MAS program for rust resistance in faba bean.

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