



## Identification of SSR and RAPD markers linked to a resistance allele for angular leaf spot in the common bean (*Phaseolus vulgaris*) line ESAL 550

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### Abstract

The objective of this study was to identify RAPD and SSR markers associated with a resistant allele for angular leaf spot (*Phaeoisariopsis griseola*) from the line 'ESAL 550', derived from the Andean 'Jalo EEP 558' cultivar, to assist selection of resistant genotypes. The resistant line 'ESAL 550' and the susceptible cultivar 'Carioca MG' were crossed to generate F<sub>1</sub> and F<sub>2</sub> populations. One hundred and twenty F<sub>2:3</sub> families were evaluated. The DNA of the 12 most resistant families was bulked and the same was done with the DNA of the 10 most susceptible, generating two contrasting bulks. One RAPD and one SSR marker was found to be linked in coupling phase to the resistant allele. The SSR marker was amplified by the primer PV-atct001<sub>282C</sub>, and its distance from the resistant allele was 7.6 cM. This is the most useful marker for indirect selection of resistant plants in segregating populations. The RAPD marker was amplified by the primer OPP07<sub>857C</sub> linked in coupling phase to the resistant allele, and distant 24.4 cM. Therefore, this RAPD marker is not so useful in assisting selection because it is too far from the resistant allele.

*Key words:* *Phaseolus vulgaris*, *Phaeoisariopsis griseola*, DNA markers, resistance, bulk segregant analysis.

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### Introduction

The consumption of common bean in Brazil has decreased more than 22% in the past 25 years, mainly due to irregular supplies and higher prices (Teixeira and Thung, 1994; Santos and Braga, 1998). Increasing grain yield may reverse this trend. The bean crop faces several threats such as diseases, among which angular leaf spot, caused by the fungus *Phaeoisariopsis griseola*, is one of the most important (Paula-Jr and Zambolim, 1998).

The most efficient method of disease control is the use of resistant cultivars (Sartorato and Rava, 1994; Paula-Jr and Zambolim, 1998). Although some information on resistance sources and genetic control is already available, the procedures for artificial inoculation are not yet accurate resulting in low selection gain (Sartorato and Rava, 1994). An alternative is to select under natural incidence of the disease, which generally occurs only in the dry season, when the inoculum pressure is enough to assure efficient selection. The use of molecular markers for identifying vertical resistance alleles stands as an important contribution for bean breeding, mainly because they enable selection of genotypes with one or more resistance alleles at any time.

Large variability has been found in the population of the pathogen (Sartorato and Rava, 1994; Paula-Jr and

Zambolim, 1998; Nietzsche *et al.*, 2000; Sartorato, 2002). However, most cultivars currently used in Brazil, and consequently the disease-causing races, are of Mesoamerican origin. Thus, resistance alleles of Andean origin, such as the one from 'Jalo EEP 558', should contribute to improving resistant cultivars in a more efficient and durable way. Like 'Jalo EEP 558' cultivar, the 'ESAL 550' line, selected as a pure line from it, share the same resistant allele. The objective of this study was to identify RAPD and SSR markers linked to the resistant allele of the line 'ESAL 550' to help selection of plants resistant to angular leaf spot.

### Material and Methods

'ESAL 550' and 'Carioca MG' were crossed and the F<sub>1</sub> and F<sub>2</sub> generations were obtained. The 'ESAL 550' is a pure line selected within the 'Jalo EEP 558' cultivar, and possesses large, yellow seeds (about 50 g per 100 seeds), resistance to angular leaf spot and is incompatible to most small seeded cultivars of Mesoamerican origin. The 'Carioca MG' possesses seeds similar to those of the 'Carioca' cultivar, slightly smaller and darker (about 20 g per 100 seeds); and is highly susceptible to angular leaf spot, but is compatible to cross with 'ESAL 550'. One hundred and twenty F<sub>2:3</sub> families were used, whose phenotypes were evaluated from February to May in a field experiment with three replications under conditions of natural disease incidence (Bruzzi *et al.*, 2002).

## DNA extraction

Total DNA was extracted from all the F<sub>2:3</sub> families using the usual CTAB procedure (Rogers and Bendich, 1988; Hagiwara *et al.* 2001). About 2 g of young leaves were taken from 12 plants of each family, being one young leaflet of about 1 cm<sup>2</sup> from each plant. The DNA of the 12 most resistant and of the 10 most susceptible families were bulked to make up the two contrasting bulks, one resistant and the other susceptible (Michelmore *et al.*, 1991; Mackay and Caligari, 2000).

## RAPD and SSR analysis

Each RAPD reaction contained 30 ng of DNA; 100 µM of each dNTPs; 0.4 µM primer; 0.6 U Taq DNA polymerase; 2.5 mM MgCl<sub>2</sub>; 20 mM KCl; 50 mM Tris pH 8.3; 250 µg bovine serum albumin/mL; 1.0% Ficoll 400; 1 mM tartrazine and pure water up to final volume of 16 µL (Hagiwara *et al.* 2001). One thousand and eighty 10-mer primers from Operon Technologies were tested. The amplification reaction was conducted in a Gradient 5331 Eppendorf Master Cycler thermocycler, programmed for 39 cycles. In the first two cycles, the denaturation was conducted at 94 °C for 2 min, the annealing at 37 °C for 15 s, and the elongation at 72 °C for 60 s, followed by an additional 37 cycles, which differed for denaturation by 15 s. A final extension was done for 2 min at 72 °C. The DNA fragments were analyzed in 1% agarose gel electrophoresis (75 V 4-5 h) and photographed under UV light.

Thirty-two pairs of SSR primers for *Phaseolus vulgaris* were also tested (Yu *et al.*, 2000). The reaction was performed in the same thermocycler, but the reactions consisted of 30 ng DNA; 200 µM of each dNTPs; 0.6 U Taq DNA polymerase; 0.2 µM of each primer; 50 mM Tris pH 8.3; 2.0 mM MgCl<sub>2</sub>; 20 mM KCl; 250 µg/mL BSA; 1% Ficoll 400; 1mM tartrazine, and pure water to a final volume of 18.5 µL. The PCR reaction included an initial denaturation at 95 °C for two min, followed by 9 cycles with denaturation at 94 °C, annealing at 68 °C and extension at 72 °C for 20 s each, plus 25 cycles with the annealing temperature at 60 °C, and a final extension step for 10 min at 72 °C. The DNA fragments were resolved in 3% agarose gel electrophoresis (80 V and 4-5 h) for bulk analysis, and in 2.5% agarose gel for the 120 F<sub>2:3</sub> families with the primers that detected polymorphism in the bulks.

## Data analysis

The F<sub>2:3</sub> family segregation based on disease reaction, RAPD and SSR markers were analyzed by the  $\chi^2$  test. Recombination frequencies between the resistance gene and one marker or between two markers were estimated according to Allard (1956), using the maximum likelihood procedure and the GQMOL software (Cruz and Schuster 2001). The standard error, confidence interval and LOD score were also estimated for each recombination frequency. The

recombination frequencies estimated were also transformed in map units (cM) according to Haldane's function (Schuster, 2002) in which r stands for recombination frequency:  $cM = [-1n(1-2r)]/2$ .

## Results and Discussion

The phenotypic analysis of the 120 F<sub>2:3</sub> families from the cross 'ESAL 550' x 'Carioca MG' presented a segregation of 90 resistant and 30 susceptible families, therefore, exactly three resistant to one susceptible ( $\chi^2 = 0$ ,  $p = 100\%$ ), indicating a monogenic inheritance, with resistance being due to the dominant allele.

Similar results have been observed from other Mesoamerican resistance sources, such as cultivars 'Cornell 49-242' and 'MAR-2' (Niestche *et al.*, 2000; Ferreira *et al.*, 2000), and the line 'BAT 332' (Caixeta, 2002). 'Jalo EEP 558' is one of the few Andean beans used in Brazil and it has shown almost complete resistance for more than 30 years. Therefore, its resistance should be more durable because most cultivars grown in Brazil are derived from a Mesoamerican origin and the predominant races of the pathogen are surely adapted to overcome the resistance alleles of that origin (Vanderplank, 1963). Similar monogenic resistance was also observed in the Andean cultivar AND 277 to the 63-23 race of *P. griseola*, and this resistant allele was also tagged with a RAPD marker (Carvalho *et al.*, 1998).

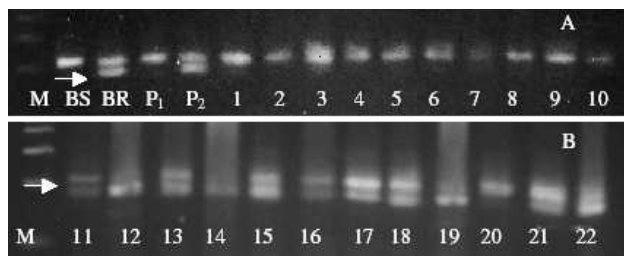
## Identification of RAPD and SSR markers

One RAPD and one SSR marker presented segregation in F<sub>2:3</sub> families that confirmed the dominant simple inheritance (Table 1). These results are important because they show that these DNA fragments are inheritable, confirming them as genetic markers.

Based on co-segregation analysis, the RAPD marker amplified by the primer OPP07 showed to be in coupling phase, and the DNA fragment has around 857 bp. The second marker identified was a DNA fragment of 282 bp (Figure 1) amplified by the following SSR pair of primers (PV-atct 001): 5'CAATTAAACTCAACCAACCCAAATA3' and 5'TTTCCCGCCATAGAATATGTGAGA3', and it is in coupling phase with the resistant allele. Typically most of the SSR markers exhibit a co-dominant inheritance (Ferreira and Grattapaglia, 1998). However, the marker amplified by the primer PV-atct 001, linked to the dominant allele for angular leaf spot resistance, showed dominant inheritance (Table 1). Liu *et al.* (2001) also iden-

**Table 1** - Segregation of the RAPD OPP07 and the SSR PV- atct 001 markers among F<sub>2:3</sub> families from 'ESAL 550' x 'Carioca MG'.

| Locus                        | Observed frequencies | Expected rate | $\chi^2$ | Probability (%) |
|------------------------------|----------------------|---------------|----------|-----------------|
| OPP07 <sub>857C</sub>        | 96:24                | 3:1           | 1.6      | 20.5903         |
| PV- atct 001 <sub>282C</sub> | 94:26                | 3:1           | 0.711    | 39.9075         |



**Figure 1** - DNA band pattern amplified by the primer SSR PV-atct 001. BR is the resistance bulk. BS is the susceptible bulk. P<sub>2</sub> is the ‘ESAL 550’ resistant parent. P<sub>1</sub> is the ‘Carioca MG’; In A. from 1-10 are the susceptible F<sub>2,3</sub> families; In B. from 11-22 are the resistant F<sub>2,3</sub> families; M is the 100 pb Promega DNA ladder. The arrow indicates the polymorphic band of 282bp.

tified dominant SSR markers and stated that this might happen due to point mutation at primer site in the susceptible parent, or insertion or deletion between the primer sites, preventing amplification or identification in the gel. One or more of these events might have happened with the PV-atct 001<sub>282C</sub> marker.

A number of RAPD markers have been identified associated to the common bean alleles resistant to *P. griseola*. For example, Nietsche *et al.* (2000) found two RAPD markers, OPN02<sub>890</sub> and OPE04<sub>650</sub>, linked to resistance to angular leaf spot in the cultivar ‘Cornell 49-242’, located at 3.2 and 12.5 cM from the locus, respectively. Ferreira *et al.* (2000) found a marker amplified by the primer OPE04 at 5.8 cM away from the resistance allele in the cultivar ‘MAR-2’. The resistance allele of ‘BAT 332’ was also identified by two RAPD markers (OPAA07<sub>950</sub> and OPAO12<sub>950</sub>), linked in coupling phase to that allele at a distance of 5.10 and 5.83 cM, respectively (Caixeta, 2002). The resistant allele of the Andean cultivar ‘AND 277’ was also identified by the primer OPH13<sub>490C</sub> (Carvalho *et al.* 1998). However, none of them showed amplification in the cross ‘ESAL 550’ x ‘Carioca MG’ utilized in the present study, which suggests that the angular leaf spot resistance allele of the line ‘ESAL 550’ is different from the reaction genes from other common bean cultivars. There might also have been a repeatability problem in the amplification by those reported RAPD primers.

In the co-segregation analysis among the markers and the resistance allele, it was found that OPP07<sub>857C</sub> and the microsatellite PV-act001<sub>282C</sub> were linked to the resistance allele and linked between them (Table 2).

The genetic distances and respective LOD scores, their standard errors and confidence intervals are presented in Table 3. Considering that 5% error is the cutting point of the LOD score to be 0.83, as suggested by Lander and Botstein (1989), when only a genetic marker is tested, it was indeed ascertained that the two markers were linked to the resistance allele. However, they are located only on one of its sides as illustrated in Figure 2.

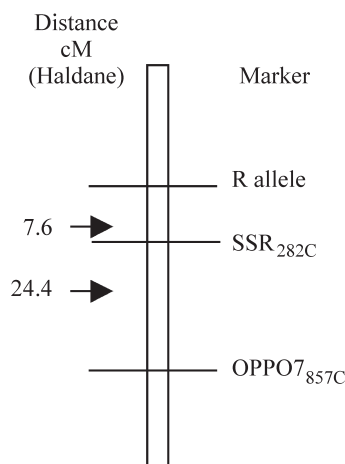
**Table 2** -  $\chi^2$  test of the segregation observed among the F<sub>2,3</sub> families, considering independent assortment of the markers and the reaction gene loci pairwise.

| Loci                  | Observed frequencies<br>AB:Ab:aB:ab <sup>a</sup> | Expected frequencies<br>AB:Ab:aB:ab <sup>a</sup> | $\chi^2$ | Prob. (%) |
|-----------------------|--|--|----------|-----------|
| OPP07/ R allele       | 83:7:13:17                                       | 9: 3: 3: 1                                       | 30.2**   | 0.00      |
| PV-atct 001/ R allele | 88:2:6:24  | 9: 3: 3: 1                                       | 73.3**   | 0.00      |
| OPP07/ PV-atct 001    | 86:8:10:16                                       | 9: 3: 3: 1                                       | 30.9**   | 0.00      |

<sup>a</sup>AB presence of the both dominant alleles; Ab presence of dominant and susceptible allele; aB presence of susceptible and dominant allele; ab presence of the both susceptible alleles.

**Table 3** - Recombination frequencies (r) between the reaction gene and the markers, considering the pairwise co-segregation, and the respective Haldane distance (cM), LOD score, standard error (SE), and inferior (CI<sub>l</sub>) and superior (CI<sub>s</sub>) confidence intervals.

| Loci                  | Distance (cM) | r (%) | LOD score | SE     | CI <sub>l</sub> | CI <sub>s</sub> |
|-----------------------|---------------|-------|-----------|--------|-----------------|-----------------|
| OPP07/ R allele       | 24.4          | 19.20 | 6.33      | 0.04   | 0.11            | 0.27            |
| PV-atct 001/ R allele | 7.6           | 7.08  | 16.21     | 0.0005 | 0.03            | 0.11            |
| OPP07/ PV-atct 001    | 21.9          | 17.71 | 6.82      | 0.03   | 0.10            | 0.25            |



**Figure 2** - Partial genetic map showing the gene of reaction to angular leaf spot and the two markers identified in the F<sub>2,3</sub> population of ‘ESAL 550’ x ‘Carioca MG’ cross.

The SSR marker is the most useful of the two for selection because it is closer to the resistance allele (Table 3). The relatively low recombination frequency between the SSR and the locus can be characterized as a useful marker for indirect selection. For example, the expected frequency of susceptible plants selected as resistant in a F<sub>2</sub> population is 4.5% (Table 4). In addition, the SSR marker, when ob-

**Table 4** - Expected frequency estimates of susceptible plants (EFESP) selected as resistant in the F<sub>2</sub> through the RAPD (OPP07) and SSR (PV-atct 001) markers.

| Primer      | Sequence  | r(%)  | EFESP (%) |
|-------------|---|-------|-----------|
| OPP07       | 5'GTCCATGCCA3'  | 19.20 | 11.56     |
| PV-atct 001 | 5'CAATTAATACTCAACCAACCCAAATA3'<br>5'TTCCCGCCATAGAATATGTGAGA3' | 7.02  | 4.5       |

tained by a PCR reaction with a larger and specific primer, is more reproducible than the RAPD marker.

The RAPD marker amplified by the primer OPP07<sub>857C</sub>, which recombines with the resistant allele at a frequency of 19.2% would result in the selection of 11.56% of susceptible plants carrying the marker, as if they were resistant in a F<sub>2</sub> generation (Table 4), almost three times less efficient than the SSR marker. Considering the worse repeatability and the larger distance between the marker and the resistance locus, it would be better to use the SSR to help selection.

Finally, it is important to stress the great help that the SSR marker should offer for indirect selection, because in most breeding programs for resistance to angular leaf spot, direct selection under natural incidence of the disease is efficient only in the dry season, once a year. That procedure has been generally adopted because there is great difficulty in causing the disease artificially. With the use of that marker, it would be possible to practice indirect selection at any time of the year.

## Conclusion

The allele of the 'ESAL 550' line resistant to *Phaeoisariopsis griseola* was tagged by one RAPD marker amplified by the primer OPP07 and one SSR marker amplified by the primer PV-act 001. The SSR is the most useful marker to be used in breeding programs, because it is closer to the resistance allele. The relatively low recombination frequency among them (7.02%) characterizes it as a useful marker for indirect selection, due to the small percentage of plants with a marker, erroneously selected in a segregating population.

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