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Validation of microsatellite markers for assisted selection of soybean genotypes resistant to powdery mildew

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ABSTRACT – *Powdery mildew is one of the most serious diseases of soybean and is found in all producing countries. The purpose of this study was to validate microsatellite markers previously identified as associated with resistance to powdery mildew in soybean. The study was conducted in two F_2 parent populations with contrasting resistance to powdery mildew. In the analysis 10 SSR primers were used for the populations, and four polymorphic markers were identified for cross 1 (MGBR95-20937 x IAC-Foscarin 31) and three for cross 2 (MGBR-46 x EMBRAPA 48). The Chi-square analysis of the phenotypic evaluation confirmed the expected segregation (3:1) of a dominant gene related to resistance. The polymorphic markers also segregated as expected (1:2:1). The markers Sat_366 and Sat_393 in the crosses 1 and 2, respectively, located at 9.41 and 12.45 cM from the gene, were considered promising for marker-assisted selection for resistance to powdery mildew in soybean, at a selection efficiency of 92.7% and 60.3% respectively.*

Key words: *Glycine max, Erysiphe diffusa*, Marker-Assisted Selection, Bulked Segregant Analysis.

INTRODUCTION

Since soybean was first planted in Brazil, major technological advances were obtained in research, especially during the last decades. There was progress in environmental improvement for the crop, as well as in the genetic potential of cultivars. Soybean is the most important oilseed grown on a global scale. Brazil is the second largest world producer. In the growing season of 2006/07, the crop was planted on an area of 20,687 million hectares, with a total yield of 58.4 million tons (Conab 2008).

Considering that the yield increase and expansion of the crop are mainly due to the genetic improvement by the development of cultivars adapted to Brazilian conditions, the important role improvement programs have played to obtain gains in yield and quality is quite clear (Kiihl and Almeida 2000).

As one of the essential diseases of soybean powdery mildew can be mentioned, which caused losses of 30 to 40% in the production of several cultivars in the 1996/97 growing season, and has been counted among the major diseases of soybean since then. The existence of cultivars with genetic resistance to powdery mildew provides greater security and, consequently, lower risks for soybean producers (Embrapa 1998).

Soybean powdery mildew is caused by the fungus *Erysiphe diffusa* (Cooke and Peck) U. Braun and S.

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Takamatsu (Takamatsu et al. 1998), and is found in all soybean-producing countries. The disease was first reported in Germany in 1921 (Grau 1985).

Powdery mildew occurred on a surprisingly large area in the growing season 1996/97, favored by rainy weather and mild temperatures. The epidemic affected susceptible cultivars, involving all areas of soybean production from the Cerrado in central Brazil to Rio Grande do Sul in the South. Average losses were estimated at 15 to 20%, with peaks ranging from 50 to 60% (Yorinori and Hiromoto 1998). The symptoms are characterized by the presence of a thin layer of powdery mycelium and spores (conidia) of the fungus, which can evolve from small white spots to a complete coverage of the affected parts.

Several studies have tried to identify SSR markers linked to diseases: resistance to sudden death, cyst nematode, brown spot, and Asian soybean rust (Fronza et al. 2004, Silva et al. 2007, Brogin 2005); resistance to witches' broom in cacao (Santos et al. 2007), angular leaf spot and powdery mildew in common bean (Teixeira et al. 2005); and powdery mildew in wheat (Liu et al. 2001).

Prior knowledge on the resistance mechanism that acts in the trait inheritance under study is of paramount importance. In a study conducted by Buzzel and Haas (1978), we observed the segregation of a dominant gene in crosses of the resistant cultivar Blackhawk with the susceptible cultivars Harosoy 63 and IP 65.388, and proposed the symbol of the dominant allele (RMD) as resistance of the adult plants and recessive allele (RMD) as susceptibility. Studies conducted by Unêda-Trevisoli et al. (2002), to determine the inheritance of resistance to powdery mildew in segregating soybean populations, also indicated that the disease control is ruled by a dominant gene with two alleles.

The objective of this study was to validate microsatellite markers that are polymorphic and discriminate the resistance reaction to powdery mildew in soybean.

MATERIAL AND METHODS

The tests were conducted at the Laboratory of Applied Biotechnology for Plant Breeding Department of Plant Production, Faculdade de Ciências Agrárias e Veterinárias da UNESP, campus of Jaboticabal - SP.

For the study, F_2 populations derived from crosses between powdery mildew- resistant (R) and susceptible parentals (S) were used. For cross 1, we used the cultivars MGBR 95-20937 (R) x-Foscarini IAC-31 (S), and for cross 2 the cultivars MGBR 46 (R) x EMBRAPA 48 (S).

The F_1 seeds from these crosses in a greenhouse were sown to obtain F_2 seeds. The F_2 seeds were sown in pots on benches and left there for natural pathogen infection aiming to classify the plants according to their reaction as susceptible or resistant to powdery mildew, based on the scale of Yorinori (1997).

Young leaf samples were collected from the pots of all F_2 plants before symptoms appeared, and were stored in an ultrafreezer at –80 °C for later DNA extraction. Seventy-three plants of cross 1 and 70 plants of cross 2 were evaluated. After collecting the trifoliate leaves, we proceeded to the extraction of genomic DNA from all plants by the CTAB method (cetyltrimetilammonium bromide), proposed by Doyle and Doyle, as described in a protocol of Ferreira and Grattapaglia (1998), with modifications. The amount of DNA present in the DNA samples was quantified in a biophotometer, and the quality determined by the relationship between the readings of 260 and 280nm, where DNA of good quality was indicated by values between 1.8 and 2.0 (Sambrook et al. 1998). Based on the quantification DNA, working solutions of all plants were established at a concentration of 30 ng iL^{-1}.

Compositions of resistant and susceptible bulks were made for each F_2 population, according to the Bulked Segregant Analysis (BSA) methodology (Michelmore et al. 1991). The methodology known as BSA (Arnheim et al. 1985; Michelmore et al. 1991) is normally used to detect genes that control traits with simple Mendelian inheritance and is considered the most direct way to detect a marker associated with a locus of interest.

To compose the bulks, the most contrasting plants were chosen, 10 plants for each bulk. Only those with rates of 0-10% infected leaf area were established as resistant and those with at least 90% infected leaf area as susceptible plants, for greater certainty in the classification. In the case of polymorphism between the two bulks, the probability of an association with the allele(s) that control(s) the trait of interest is high (Alzate-Marin et al. 2005).

For the PCR reactions with SSRs primers a MJ thermocycler, PTC model 100 was used, programmed for: 2 min at 94 °C; 25 s at 94 °C (denaturation); 25 s at 47 °C (primer annealing), 2 min at 68 °C (extension by Taq

polymerase). This cycle was repeated 40 times and ended with a step of 7 min at 68 °C and was finally stabilized at 4 °C, until the microtubes were removed from the device. The amplification reactions were performed in a final volume of 25 mL, which consisted of: 1X PCR buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl), 1.5 mM MgCl2, 0.15 mM dNTP, 0.15 ðmol of each primer (foward and reverse), 500 ng genomic DNA, 1 unit Taq polymerase and autoclaved and filtered milli-Q water. The amplified fragments were analyzed by high-resolution agarose gel electrophoresis (agarose 1000) at a concentration of 3% (w / v), stained with ethidium bromide, at a concentration of 10 mg mL^{-1}. Electrophoresis was run at 80V for about two hours, using TAE buffer pH 8.0 (2M Tris, 1M acetic acid and 100mM EDTA). The gels were visualized under UV light and photographed with the Kodak EDAS 290 imaging system.

The 10 pairs of SSR primers or microsatellites used in the amplification reactions were previously selected by the location in group J, presumably RMD flanking the *Rmd* gene for resistance to powdery mildew (CREGAN 1999). The following SSR markers were used: Sat_366, Satt244, Satt547, Sat_224, Satt431, Sat_395, Satt712, Sat_393, Sat_394, Sat_350.

The chi-square (÷2) test for the analysis of marker segregation and phenotypic evaluation of resistance was performed for both crosses using software Genes (Cruz 2001). In turn, the distances between the identified markers and the gene for resistance were estimated by GQMOL (Cruz and Schuster 2008), based on the distance of Kosambi, LOD score > 3.0 at a maximum distance of 30 cm, as proposed in the methodology of Schuster and Cruz (2004). The efficiency of selection was calculated as follows: EF = 100 [(MFMF + mfmf) / (MM + mm)]; MFMF is the number of plants selected as resistant, based on the marker and the phenotype; mfmf is the number of plants selected as susceptible, based on the marker and phenotype; MM + mm is the total number of resistant and susceptible plants of the population, based on the marker only (Silva et al. 2007).

RESULTS AND DISCUSSION

After the phenotypic evaluations, the hypothesis of a 3:1 segregation in the two populations was tested (Table 1). The results for both populations confirmed the hypothesis of segregation of a dominant gene, with odds of 83.94 and 67.88% respectively. The result of the test of heterogeneity (Table 1) indicates, at a probability of 66.16%, that the results of the Chi-square (÷2) test were consistent for both populations, confirming the expected segregation, with the presence of one dominant gene associated to resistance. These results are consistent with those of Gonçalves et al. (2002), Grau and Laurence (1975), Mignucci and Lim (1980) and Unêda- Trevisoli et al. (2002).

Table 1. Number of resistant (RP) and susceptible plants (SP) and results of the c^2 test and the heterogeneity in the two crosses evaluated for powdery mildew resistance in soybean, in the F_2 generation for the hypothesis of 2:1 segregation

Crosses	RP	SP	DF	χ^2	P (1)
1	54	19	1	0.041^{ns}	83.94
2	54	16	1	0.171^{ns}	67.88
Total			2	0.212^{ns}	89.92
Deviation			1	0.02^{ns}	88.48
Heterogeneity			1	0.191^{ns}	66.16

For the molecular evaluations, the initial reactions with SSR markers were performed with the 10 primers using the DNA of the resistant and susceptible parentals, to verify any polymorphism between them in both crosses. No polymorphism was detected in the primers Sat_394, Sat_350 and Satt244 for the parents of cross 1 and the primers Sat_394, Satt547, Satt244 and Sat_350 were not polymorphic for cross 2, and therefore excluded from the later stages of molecular analysis.

After this step, polymorphic primers were identified among the resistant and susceptible bulks. For cross 1, the primers that remained polymorphic were Sat_366, Sat_224, Satt712 and Sat_395 and for cross 2, Satt431, Sat_393 and Sat_366. After this confirmation, the primers were tested for the whole population. These primers were also discriminatory in the complete population analysis, as shown by the respective markers (Figures 1a and 1b).

The segregation of polymorphic primers at a ratio of 1:2:1 (homozygous resistant: heterozygote: homozygous susceptible) was confirmed by the chi-square (X^2) test as expected for codominant markers (Table 2). For cross 2, the hypothesis was only refuted for primer Satt431.

The distance between each marker and the resistance gene was estimated by the program GQMOL (Cruz and Schuster 2008), as well as the standard deviation and the LOD score value for the populations studied. For cross 1, the distance between the marker

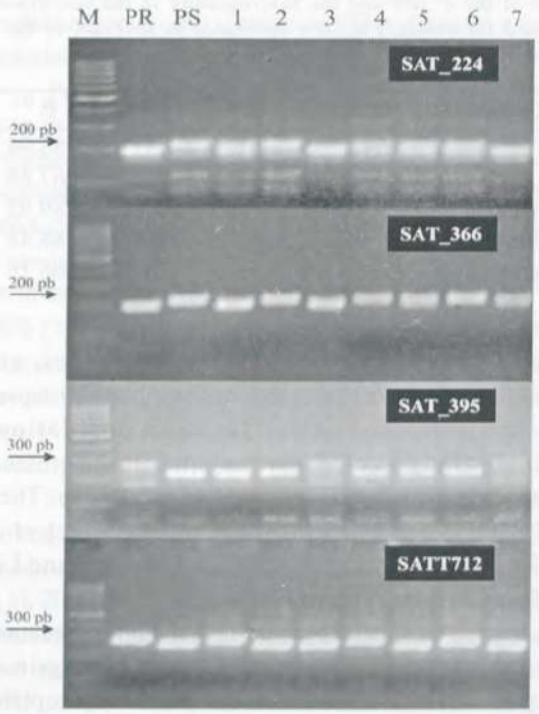

Figure 1a. SSR markers in cross 1 with polymorphic fragments for resistance to powdery mildew in soybean. The sequence shows the molecular weight marker (M) and the reactions of the resistant (RP) and the susceptible parental (SP) and some plants of cross 1, numbered 1 to 7, for each marker

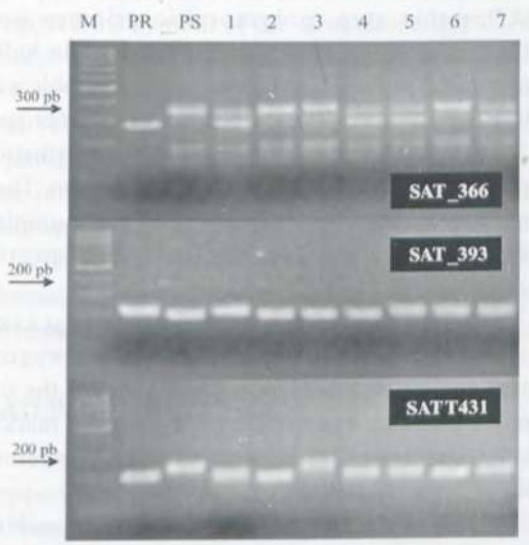

Figura 1b. SSR markers in cross 2 with polymorphic fragments for resistance to powdery mildew in soybean. The sequence shows the molecular weight marker (M) and the reactions to the resistant (RP) and the susceptible parental (SP) and some plants of cross 1, numbered 1 to 7, for each marker

Table 2. Number of plants classified as homozygous resistant (HR), heterozygous (HE) and homozygous susceptible (HS) and results of the χ^2 test for the hypothesis of 1:2:1 segregation of the markers, in both evaluated crosses

SSR markers	HR	HE	HS	DF	χ^2	P [1]
Cross 1						
Sat_366	15	35	23	2	1.87^{ns}	39.13
Satt224	18	37	17	2	0.08^{ns}	95.92
Sat_712	16	38	18	2	0.33^{ns}	84.65
Sat_395	22	30	21	2	2.34^{ns}	30.99
Cross 2						
Sat_393	21	30	19	2	1.54^{ns}	46.24
Satt431	27	26	16	2	7.69^{*}	2.13
Sat_366	15	31	22	2	1.97^{ns}	37.33

^{ns} = non-significant at 5% probability. [1] P = probability (%) in the χ^2 test

and gene of Sat_366 was 9.3 cM and the LOD score 9.45. For cross 2, only Sat_393 was considered to be associated to the resistance gene, with a distance of 12.45 cM and a LOD score of 7.24. A LOD score of over 3.0 indicated the reliability of the hypothesis of linkage between the marker and the gene.

With the estimates of the distances between the markers and the gene in both crosses, a map of hypothetical linkage to each of the intersections was generated, with the best distance estimates (Figures 2a and 2b).

The efficiency of selection was also calculated. For marker Sat_366, in cross 1, the efficiency was estimated at 60.3% and for Sat_393, in cross 2, the estimated efficiency was 92.7%. These results confirm the possibility of an efficient use of these two markers in marker-assisted selection.

Several studies have been conducted to identify microsatellite markers related to the major soybean diseases. In one, Silva et al. (2007) identified several microsatellite markers associated with resistance to races 3 and 14 of soybean cyst nematode (*Heterodera glycines* Ichinohe) for programs of marker-assisted selection. Fuganti et al. (2004) identified SSR markers linked to the resistance gene to the Root-Knot Nematode (*Meloidogyne javanica*) in soybean, particularly Satt114 and Satt423. For powdery mildew, Muniz (2007) used the markers SSRs Sat_366, Satt431, Sat_224 and Satt547 to analyze the genetic variability by crossovers in soybean crosses for powdery mildew resistance.

The results of this study indicate the possibility of using the SSR markers Sat_366 and Sat_393 for

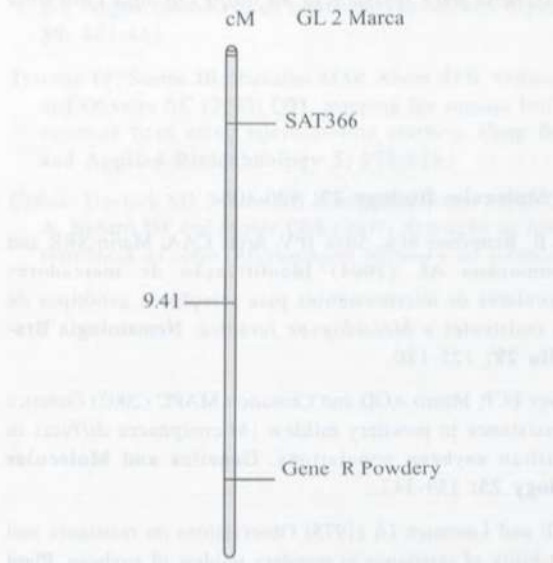

Figure 2a. Linkage map with the estimated distances (in cM) between the SSR marker Sat_366 and the gene of resistance to powdery mildew (Gene R Powdery mildew) in cross 1 (MGBR 95-20937 x IAC-Foscarin-31)

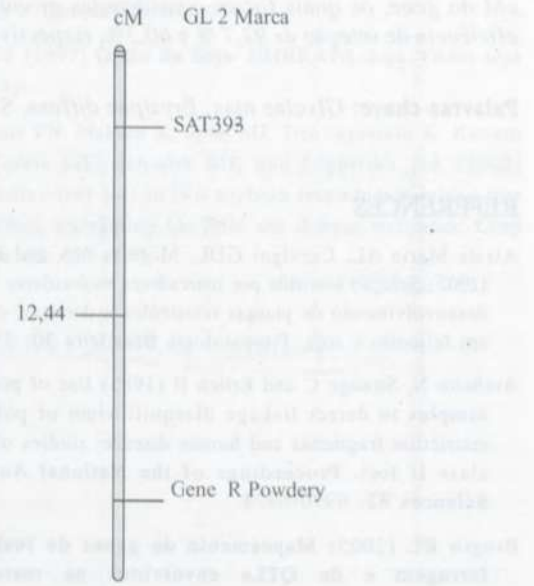

Figure 2b. Linkage map with the estimated distances (in cM) between the SSR marker Sat_366 and the gene of resistance to powdery mildew (Gene R Powdery mildew) in cross 2 (MGBR 46 x EMBRAPA 48)

assisted selection of genotypes resistant to powdery mildew, carrying the same resistance source as in the evaluated crosses, which should be validated for crosses with other resistance sources than used here.

The acquisition of polymorphic and discriminatory microsatellite markers for the resistance reaction to soybean powdery mildew is of great importance, allowing the use in marker-assisted selection, to identify resistant and susceptible genotypes to the pathogen in segregating populations of soybean breeding programs.

CONCLUSIONS

Markers SSRs Sat_366 and Sat_393 are indicated for assisted selection in segregating soybean populations that have the same resistance source as in this study.

Validação de marcadores moleculares microssatélites para seleção assistida de genótipos de soja resistentes ao oídio

RESUMO - *O oídio da soja constitui-se em uma das principais doenças desta cultura, estando presente em todos os países produtores. Assim, o objetivo deste estudo foi validar marcadores microssatélites previamente identificados como sendo associados à resistência ao oídio em soja. O estudo foi realizado em duas populações F_2, de parentais contrastantes quanto à resistência ao oídio. Para a análise foram utilizados dez iniciadores SSR para as populações, sendo identificados quatro marcadores polimórficos para o cruzamento 1 (MGBR95-20937 x IAC-Foscarin 31) e três para o cruzamento 2 (MGBR-46 x EMBRAPA 48). Pela análise de Qui-quadrado da avaliação fenotípica, confirmou-se a segregação esperada (3:1) de um gene dominante condicionando a resistência. Os marcadores polimórficos também segregaram conforme o esperado (1:2:1).*

Para os cruzamentos 1 e 2, destacaram-se os marcadores Sat_366 e Sat_393 respectivamente, localizando-se a 9,41 e 12,45 cM do gene, os quais foram considerados promissores na seleção assistida para resistência ao oídio em soja com uma eficiência de seleção de 92,7 % e 60,3%, respectivamente.

Palavras-chave: *Glycine max*, *Erysiphe diffusa*, SAM, BSA.

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