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ARTICLE

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Inheritance and genetic mapping of resistance to Asian soybean rust in cultivar TMG 803

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Abstract – *This study analyzed the inheritance and identified microsatellite markers linked to the resistance gene to* Phakopsora pachyrhizi *in soybean cultivar TMG 803. Hybridization between the cultivars TMG 803 and BRS Valiosa RR was performed to obtain* F_1 *progenies and the* F_2 *population. The response of the parents 'TMG 803' and 'BRS Valiosa RR' to* P. pachyrhizi *was, respectively, resistant and susceptible, and among the 116* F_2 *plants, 93 were resistant and 23 susceptible, under natural infection and field conditions. It was found that the resistance of cultivar TMG 803 is controlled by one gene with complete dominance, mapped as resistance locus Rpp4 of linkage group G. Of the 16 tested, one microsatellite marker, sc21_3420, was completely linked to the resistance gene (distance 0.0cM) and the favorable allelic form was present in cultivar TMG 803, which may therefore be useful in assisted selection in segregating populations.*

Key words: Glycine max*, breeding,* Phakopsora pachyrhizi*, molecular marker, SSR marker.*

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is the chief oilseed grown and consumed worldwide. Brazil, with an estimated production of 86,273,200 tons in the 2013/14 harvest, ranks as the second largest producer of the grain (CONAB 2014), but has the potential to become the largest, according to Sediyama et al. (2009).

However, a major negative influence on the national soybean production is soybean rust (SR), a disease caused by *Phakopsora pachyrhizi* Sydow & Sydow (Dhingra et al. 2009), first reported in Brazil in the 2000/01 growing season (Yorinori et al. 2005). According to Lima et al. (2012), the cost of the mainly fungicide-based control of this crop disease, must have exceeded US$ 16 billion by the 2010/11 growing season.

Thus, Morales et al. (2012) emphasized that the best way to avoid significant production losses is disease prevention by planting resistant cultivars. This is directly related to knowledge about the pathogen and plant defense mechanisms as well as the development of resistant cultivars and effective control strategies.

With regard to SR, five loci (Rpp1, Rpp2, Rpp3, Rpp4, and Rpp5) were reported that allocate dominant alleles: *Rpp1* identified in PI200492 (McLean and Byth 1980), *Rpp2* of PI230970 (Bromfield and Hartwig 1980), *Rpp3* in PI462312 (Bromfield and Melching 1982), *Rpp4* in PI459025 (Hartwig 1986), and *Rpp5* in PI 200526 (Garcia et al 2008); as well as two alleles of alternative resistance (*Rpp1?* And *Rpp1-b*) at locus Rpp1 (Chakraborty et al. 2009, Ray et al. 2009). Since then, the pyramiding of these genes has been studied with a view to soybean breeding. According to Yamanaka et al. (2013), lines containing the pyramided alleles *Rpp2, Rpp4* and *Rpp5* are promising for breeding for SR-resistance and may be effective against a population of several *P. pachyrhizi* isolates.

However, for the success of a breeding program resistant pathogen genotypes in segregating populations must be identified in the early and intermediate breeding stages. This can be done with DNA marker-assisted selection. In many crops, DNA markers have been successfully used, of which

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microsatellites and SNPs (Single Nucleotide Polymorphism) are the most commonly used for the selection and mapping of genomic regions associated with genes and QTLs, as in soybean (Fuganti et al. 2004, Morceli et al. 2008, Dias et al. 2009), rice (Pinheiro et al. 2012) and maize (Teixeira et al. 2013). Certainly, microsatellite markers have been used due to their high polymorphism, codominance, reproducibility and good distribution throughout the genome (Ferreira and Grattapaglia 1998, Song et al. 2004).

In this context, the segregation of an F_2 population originated from the cross 'TMG 803' x 'BRS Valiosa RR' was investigated to identify microsatellite markers linked to the resistance gene against Asian soybean rust in 'TMG 803'.

MATERIAL AND METHODS

The cultivars TMG 803 and BRS Valiosa RR were hybridized to obtain F_1 progenies and the F_2 population, in a greenhouse of the soybean breeding program of the Crop Science Department, Universidade Federal de Viçosa (UFV). After harvesting the seeds from artificial pollination, the F_1 generation was planted and grown in the same environment where hybridizations had been performed, as recommended for the crop (EMBRAPA 2008).

The F_2 population and parents were grown on the experimental field Diogo Alves de Mello of the UFV (lat 20º 46' 03'' S, long 42º 52' 10'' W, alt 650m asl), according to the technical recommendations for the crop (EMBRAPA 2008). In this experiment, a F_2 population of 116 plants and 38 plants of each parent were used. The plants were grown in 2-m long rows, spaced 0.80m apart, with five plants per meter. Additionally, cultivar BRS Valiosa RR was planted around the borders and additional sprinkler irrigation was provided, according to crop requirements. For an undisturbed development of the pathogen (*P. pachyrhizi*) on the plant leaves, no disease control was applied.

After identifying the pathogen on the leaves, the plants were discriminated for the typical SR reactions (RB: Red-Brown lesion, indicating resistance and TAN: Tan lesion, indicating susceptibility). The data of the phenotypic evaluation were tested for fitting the segregation model 3: 1, in relation to the control provided by a dominant gene, and 13: 3, in relation to the control provided by two genes with epistatic interaction, by the chi-square test, at the 5% level of significance.

Molecular analyses were performed at the Laboratory of Genomics, Department of Plant Pathology/Bioagro, UFV. The DNA was extracted from the plant leaves by the CTAB method (Doyle and Doyle 1987), with modifications. The DNA integrity and concentration were determined on ethidium bromide-stained agarose gel (1%). Based on the estimated concentration, the samples were diluted in autoclaved Milli-Q $H_2 0$ to a final concentration of 5 ng μL^{-1}, to be used in the work solution. Part of each sample was stored undiluted at -20 °C for later use.

Since there are no reports in the literature about which resistant gene is present in TMG 803, we selected microsatellite markers linked to the five SR- resistance loci of soybean. Thus, 16 microsatellite markers were tested (locus Rpp1: Satt191, Sct_187 and Sat_372; locus Rpp2: Satt620, Sat_255, Sat_165 and Satt622; locus Rpp3: Satt460 and Satt708; locus Rpp4: Satt612, sc21_3360, sc21_3420, sc21_4058 and sc21_4808; locus Rpp5: Sat_166 and Sat_275) to detect polymorphism between the parents (TMG 803 and BRS Valiosa RR). Information on the marker sequence for all microsatellite markers can be found at the site Soybase (http://soybase.agron.iastate.edu/resources/ssr.php).

After identifying polymorphisms, 10 resistant and 10 susceptible plants were analyzed by the BSA (Bulked Segregant Analysis) developed by Michelmore et al. (1991). It is worth emphasizing that the plants were analyzed separately without mixing DNA for bulk formation. The SSR markers with polymorphism between the parents and the bulks (resistant and susceptible) were considered candidates for the evaluation of F_2 plants.

Amplification reactions were performed in a Gene Amp PCR System 9700 Thermal Cycler in a final volume of 20 µL, containing 6.0 µL DNA at 5 ng μL^{-1} genomic DNA of soybean, 2.0 µL of 10X buffer, 1.2 µL $MgCl_2$ at 1.25 mM μL^{-1}, 1.6 µL dNTPs at 2. 5 mM μL^{-1}, 0.8 µL primer at 10 µM (Foward and Reverse mixture) and 0.1 µL Taq (5 U μL^{-1}). The program used for DNA amplification consisted of an initial denaturation at 94 ºC/5min followed by 40 denaturation cycles at 94 ºC/40s. At this stage, the annealing temperature of the markers varied from 50 °C to 61.3 ºC (Table 1) and 72 ºC/40s for DNA amplification. The final program consisted of polymerization at 72 °C/25min.

The PCR product was first analyzed on (1%) agarose gel stained with ethidium bromide and visualized on an ultraviolet light transilluminator. Once successful, the PCR product was diluted in autoclaved Milli-Q $H_2 0$ and analyzed on an automated DNA sequencer model ABI3100 or, when possible, directly on the agarose gel. The size of the amplified base pairs analyzed by an automated sequencer was estimated with software GeneMapper, version 3.5.

The segregations of the amplified microsatellite markers in all F_2 plants were tested for fitting to a codominant gene

Table 1. Annealing temperature of the markers used in the study

Markers	Annealing temperature (ºC)	Markers	Annealing temperature (ºC)
Satt191	55.0	Satt708	50.0
Sct_187	60.0	Satt612	55.0
Sat_372	52.0	sc21_3360	50.0
Satt620	60.0	sc21_3420	50.0
Sat_255	50.0	sc21_4058	55.0
Sat_165	55.0	sc21_4808	55.0
Satt622	50.0	Sat_166	55.0
Satt460	61.3	Sat_275	59.5

Table 2. Chi-square test (χ^2) of the phenotypic segregation of the F_2 population of ('TMG 803' x 'BRS Valiosa RR') and of polymorphic microsatellite markers[1]

Trait	Reaction classes			Hypothesis			X^2	Probability (%)	
	RB		TAN						
Phenotypic evaluation	93		23	3		1	1.65	19.8	ns
Phenotypic evaluation	93		23	13		3	0.08	76.6	ns
	R	H	S						
Satt191	33	59	24	1	2	1	1.43	48.8	ns
sc21_3420	30	63	23	1	2	1	1.70	42.5	ns

[1] RB: red-brown lesion – resistant. TAN: tan lesion – susceptible. R: homozygous resistant. H: heterozygous. S: homozygous susceptible. ns: non-significant at the 5% significance level, by the chi-square test.

model, by the chi-square test at the 5% level of significance. For linkage analysis and map construction software GQMOL (Schuster and Cruz 2008) was used with the Kosambi mapping function. The linkage criterion was a LOD score > 3.0 and the maximum distance was 30.0 cM.

The selection efficiency (SE%) of microsatellite markers, identified in this study, was calculated as described by Silva et al. (2007). Similar studies were performed by Morceli et al. (2008).

$$SE_{\%} = \left(\frac{MFMF + mfmf}{TP}\right) x100$$

Where

MFMF = Plants selected correctly as resistant based on marker analysis and phenotypic evaluation;

mfmf = Plants selected correctly as susceptible, based on markers and phenotypic evaluation;

TP = Total plants analyzed.

RESULTS AND DISCUSSION

On the plants of the cultivars TMG 803 and BRS Valiosa RR, we observed RB lesions (resistant) and TAN lesions (susceptible), respectively, indicating that the quantity and quality of the *P. pachyrizi* inoculum in this area and the environmental conditions were favorable for the pathogen development on the plant leaves.

Among the 116 F_2 plants evaluated, 93 behaved as resistant and 23 as susceptible to SR. Segregation was tested for the agreement with the expected Mendelian ratio of 3:1 (3 resistant plants and 1 susceptible plant) and 13:3 (13 resistant and 3 susceptible plants).

Of the 16 SSR markers tested, six (Satt191, Sat_372, Satt460, Satt708, sc21_3420, and sc21_4808) indicated polymorphism between the cultivars TMG 803 and BRS Valiosa RR and after analyzing the resistant and susceptible bulks, two (Satt191 and sc21_3420) were used to analyze the adequacy of segregation, which satisfactorily fit the expected rate of codominant inheritance (1:2:1) by the chi-square test (Table 2).

In the analysis of phenotypic data (RB and TAN lesions) of 116 plants, the chi-square values were 1.65 ($p = 0.198$) and 0.08 ($p = 0.766$) for the ratios 3:1 and 13:3, respectively. Thus, considering ($p = 0.05$), both hypotheses of segregation were considered true. This confirms results reported in other studies, where monogenic inheritance with complete dominance was observed in PI 200492 (Rpp1) (Hyten et al. 2007), in PI 230970 (Rpp2) (Silva et al. 2008) and in PI 459025 (Rpp4) (Silva et al. 2008, Costa et al. 2008). Similarly, a 13:3 ratio was observed in several segregating F_2 soybean populations resistant to SR, considering the segregation pattern of the RB and TAN reactions (Pierozzi et al. 2008). This ratio is a modification of the proportions 9:3:3:1 and indicates digenic inheritance (two unlinked

genes). The resistant cultivar would have the genotypes A_B_, A_bb, aabb, and the susceptible genotype, genotype aaB_, i.e. 13 resistant and 3 susceptible (Viana et al. 2003).

Between the phenotypic evaluation of F_2 plants and the DNA amplification pattern with microsatellite marker sc21_3420 an agreement of 100% was also observed. That is, the phenotypically resistant plants (lesion RB) had a similar amplification pattern to 'TMG 803' or were heterozygous with marker sc21_3420. The susceptible plants had an amplification pattern similar to that of cultivar BRS Valiosa RR. Thus, it can be stated that the resistance of cultivar TMG 803, for lesion RB and TAN, is controlled by one gene with complete dominance.

It is noteworthy that if a greater number of F_2 plants of this cross is evaluated, the values obtained by the chi-square test and probabilities could be changed. In addition, the agreement between the phenotypic and molecular evaluation may be less than 100%. For the acceptance of the ratio of 13: 3 in this study, at least one phenotypically resistant (lesion RB) plant (1/13) had to have the amplification pattern of BRS Valiosa RR for marker sc21_3420.

The estimates of the distances between each marker and the SR-resistance gene and LOD score values are shown in Table 3. The distance between the marker sc21_3420 score was equal to 0.0cM and the LOD score was 25.4 at a ratio of 3:1 for the lesion type (RB and TAN) of the F_2 population. For 13:3, the LOD value was 22.1. The other estimates, of the distance and LOD, were also similar between the two ratios (3:1 and 13:3).

The LOD is a statistic associated with an acceptable test to evaluate the hypothesis of linkage or independent segregation between locus pairs, since it is directly related to the likelihood ratio considering the probability of two genes being linked or not. Loci are linked when the LOD score is greater than 3 (probability of 1:1000), i.e., LOD = 3 means that the likelihood considering the r value is 1000

Table 3. Estimates of the distances between each marker and the resistance gene (Gene R) and LOD *scores* from the analysis of 116 F_2 plants considering a Proportion 3:1 (3 resistant and 1 susceptible) and 13:3 (13 resistant and 3 susceptible)

Marker 1	Marker 2	Distance (cM)		LOD
		Morgan	Kosambi	
--------------------- Proportion F_2 (3:1 – 3 Resistant : 1 Susceptible)) ---------------------				
Gene R[1]	Satt191	26.6	29.6	3.3
Gene R[1]	sc21_3420	0.0	0.0	25.4
Satt191	sc21_3420	19.4	20.5	10.0
-------------------- Proportion F_2 (13:3 – 13 Resistant : 3 Susceptible)) --------------------				
R[2] Gene	Satt191	25.8	28.5	2.9
R[2] Gene	sc21_3420	0.0	0.0	22.1
Satt191	sc21_3420	19.4	20.5	10.0

[1] Phenotypic evaluations (RB or TAN lesion): one gene with 3:1 segregation,
[2] Indicates one of the two independent genes with 13:3 segregation.

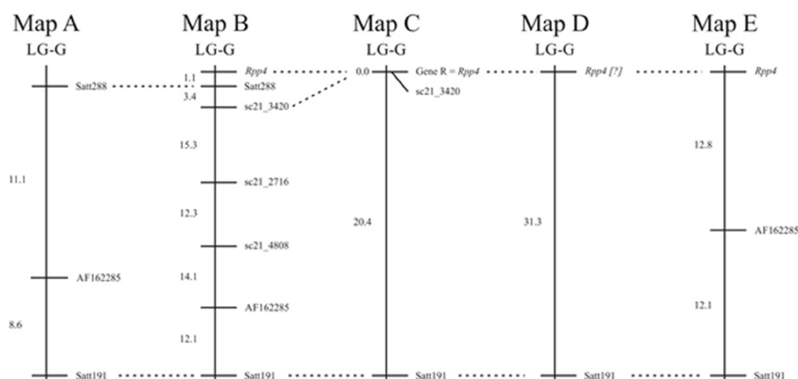

Figure 1. Linkage map of locus Rpp4 of linkage group G, showing the position of the resistance gene of cultivar TMG 803 to Asian soybean rust. Map A: Consensus linkage map of soybean for the region between Satt191 and Satt288 in linkage group G with the distance (in cM) adapted from Song et al. (2004), Map B: Linkage map presented by Meyer et al. (2009), C Map: Linkage map obtained in this study from the analysis of 116 F_2 plants derived from a cross between TMG 803 x BRS Valiosa RR, D Map: Linkage map presented by Garcia et al. (2008), Map E: Linkage map presented by Silva et al. (2008). Distance between the identified markers (in cM) on the left side of each map.

Table 4. Phenotypic segregation of an F_2 population with two independent genes and complete dominance

	Microsatellite markers	
	+ (*B_*)	– (*bb*)
Phenotypically resistant (RB) (*A_*)	9	3
Phenotypically susceptible (TAN) (*aa*)	3	1

times greater than the likelihood in the case of no linkage (Schuster and Cruz 2008).

Considering the 3:1 segregation (accepted in this study), the resistance gene of cultivar TMG 803 was mapped at a distance of 0.0cM from marker sc21_3420 and these markers (sc21_3420) were 20.4cM from marker Satt191 (Figure 1). The results indicate that the resistance gene in cultivar TMG 803 is intimately linked to the resistance locus Rpp4, in linkage group G.

Marker Satt612, located at a distance of 3.61cM from Satt288 (Song et al. 2004), showed no polymorphism between the cultivars TMG 803 and BRS Valiosa RR. With this marker (Satt612), Silva et al. (2008) identified no polymorphism either between BRS 184 and PI 459025 (carrier of the dominant resistance allele - Rpp4). The microsatellite marker Satt288 was mapped at locus Rpp4 by Garcia et al. (2008), Silva et al. (2008) and Meyer et al. (2009). In this study, this marker was not used to identify polymorphism between the cultivars TMG 803 and BRS Valiosa RR because the genomic DNA could not be amplified.

The selection efficiency of the resistant plants with microsatellite markers Satt191 and sc21_3420 was 80.2% and 100.0%, respectively.

The phenotypic segregation of an F_2 population with two independent genes and with complete dominance each is 9:3:3:1 (Table 4). Considering that MFMF (plants selected correctly as resistant, based on marker analysis and phenotypic evaluation) = 9; mfmf (plants selected correctly as susceptible, based on markers and phenotypic evaluation) = 1; and TP (Total number of studied plants) = 16, the selection efficiency is 62.5%. The value of 62.5% would result from the use of microsatellite markers unlinked to the resistance gene. Thus, the $SE_{\%}$ limits would be 62.5% for unlinked genes, or microsatellite markers unlinked to the resistance gene, and 100.0% for completely linked genes, i.e., for microsatellite markers completely linked to the resistance gene. It is worth emphasizing that the value of 62.5% is a result of the F_2 segregating population analysis with complete dominance of two independent genes. The marker combination Satt309 + Satt356 in the selection of soybean genotypes resistant to race 14 cyst nematode resulted in 100% selection efficiency (Silva et al. 2007). By combining the markers Sat_275 + Sat_280 flanking the resistance gene Rpp5, the resulting $SE_{\%}$ was also 100% (Morceli et al. 2008). According to this author, the use of these markers for marker-assisted selection results in total selection efficiency and is valid, because it identifies homozygous genotypes and the fixation of the resistance gene can be completed in a few selection cycles.

Microsatellite marker sc21_3420 enabled the correct identification of the dominant homozygote and heterozygote genotypes, and is therefore suitable for marker-assisted selection for the development of SR resistant genotypes, from the resistance source TMG 803.

Thus, the resistance to soybean rust in cultivar TMG 803 is controlled by one gene with complete dominance; the resistance gene in cultivar TMG 803 is at the Rpp4 resistance locus in linkage group G; and microsatellite sc21_3420 may be indicated for marker-assisted selection of soybean genotypes, to incorporate *Phakopsora pachyrhizi* resistance of TMG 803 in other genotypes.

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Herança e mapeamento genético da resistência da cultivar TMG 803 à Ferrugem Asiática da Soja

Resumo – *Objetivou-se analisar a herança e identificar marcadores microssatélites ligados ao gene de resistência da 'TMG 803' à* Phakopsora pachyrhizi. *Foi realizada hibridação entre as cultivares TMG 803 e BRS Valiosa RR para obtenção da progênie F_1 e população F_2. Os genitores 'TMG 803' e 'BRS Valiosa RR' comportaram-se, respectivamente, como resistentes e suscetíveis e entre as 116 plantas F_2, 93 comportaram-se como resistentes e 23 como suscetíveis à* P. pachyrhizi*, submetidas à infecção natural, em con-*

dições de campo. Verificou-se que a resistência da cultivar TMG 803 é governada por um gene com dominância completa, mapeado como o loco de resistência Rpp4, do grupo de ligação G. Dentre os 16 marcadores microssatélites testados, um marcador, sc21_3420, encontrou-se completamente ligado ao gene de resistência (distância de 0,0cM) estando a forma alélica favorável presente na cultivar TMG 803, podendo, portanto, ser útil na seleção assistida em populações segregantes.

Palavras-chave: Glycine max*, melhoramento,* Phakopsora pachyrhizi*, marcador molecular, marcador SSR.*

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