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ORIGINAL PAPER

Molecular mapping of two loci that confer resistance to Asian rust in soybean

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Abstract Asian soybean rust (ASR) is caused by the fungal pathogen *Phakopsora pachyrhizi* Sydow & Sydow. It was first identified in Brazil in 2001 and quickly infected soybean areas in several countries in South America. Primary efforts to combat this disease must involve the development of resistant cultivars. Four distinct genes that confer resistance against ASR have been reported: *Rpp1*, *Rpp2*, *Rpp3*, and *Rpp4*. However, no cultivar carrying any of those resistance loci has been released. The main objective of this study was to genetically map *Rpp2* and *Rpp4* resistance genes. Two $F_{2:3}$ populations, derived from the crosses between the resistant lines PI 230970 (*Rpp2*), PI 459025 (*Rpp4*) and the susceptible cultivar BRS 184, were used in this study. The mapping populations and parental lines were inoculated with a field isolate of *P. pachyrhizi* and evaluated for lesion type as resistant (RB lesions) or susceptible (TAN lesions). The mapping populations were screened with SSR markers, using the bulk segregant analysis (BSA) to expedite the identification of linked markers. Both resistance genes showed an expected segregation ratio for a dominant trait. This study allowed mapping *Rpp2* and *Rpp4* loci on the linkage groups J and G, respectively. The associated markers will be of great value on marker assisted selection for this trait.

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Introduction

Brazil is the second largest soybean producer in the world and still has a great potential to increase production in the future given the existence of areas not cropped and the increasing world demand for soybean products. Among the problems threatening soybean production and expansion in Brazil, the fungal disease Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, is responsible for severe economic losses. Until 2000, significant yield reductions caused by *P. pachyrhizi* had been reported only in the Eastern Hemisphere, where yield losses ranged from 5 to 95% (Hartman et al. 1991, 1997). After the first identification of the disease in Paraguay, and in Paraná, Brazil, in 2001, ASR rapidly infected soybean areas all over Brazil, Paraguay, Bolivia, and some regions of Argentina, causing yield losses of up to 75% (Yorinori et al. 2005) and making this disease as the main threat affecting Latin American soybean production. With the recent detection of *P. pachyrhizi* in continental USA (Schneider et al. 2005), ASR is now present in most of the soybean growing areas of the world.

The only method used to control ASR is fungicide applications, which substantially increase production costs and

are a continuous source of environmental contamination. The use of resistant cultivars is seemed to be the most promising method to control this disease. Four distinct major genes, that confer resistance against ASR, have been reported to date: *Rpp1*, *Rpp2*, *Rpp3*, and *Rpp4* (Bromfield and Hartwig 1980; McLean and Byth 1980; Hartwig and Bromfield 1983; Hartwig 1986). Several other resistance sources have been also identified abroad (Miles et al. 2006) and in Brazil (C.A.A. Arias, personal communication). However, no commercial cultivar carrying any of these resistance genes has been released.

The usual reactions associated with this disease are the presence of reddish-brown lesions (RB lesions) on resistant reactions and the presence of tan color lesions (TAN) on the susceptible ones. In some cases, the resistance is also associated with an immune response (no visible symptoms), as is the case of *Rpp1* in the presence of certain isolates (Miles et al. 2006). To date, all known ASR resistance loci evaluated have been overcome by at least one isolate throughout the world (Miles et al. 2006; Yamaoka et al. 2002). However, *Rpp2* and *Rpp4* loci remain effective against the Brazilian isolates, whereas *Rpp1* and *Rpp3* were defeated in 2003, just 2 years after ASR detection in Brazil (José Tadashi Yorinori, personal communication). *Rpp2* and *Rpp4* loci were identified on the lines PI 230970 and PI 459025, respectively, and behave as a single dominant allele (Bromfield and Hartwig 1980; Hartwig 1986).

Monogenic rust resistance has been a volatile trait on other species. In wheat, the race-specific rust leaf resistance has a short-lived nature and it has been frequently associated to other types of resistance in order to have a more stable resistance (Kolmer 1996). Lately, efforts have been made by several breeding programs to introduce ASR resistance loci into elite soybean cultivars, and the stacking of different loci could result in more stable ASR resistance, capable of overcoming the historical failure of many monogenic resistance mechanisms in plants, especially those genes leading to a hypersensitive response (Niks and Rubiales 2002). The main difficult of this process is to select the plants carrying more than one locus by conventional methods, and techniques that could assist the introgression of multiple loci would be very useful in this manner.

DNA markers have great potential for assisting selection of resistant genotypes in breeding programs and have been used in several crops, including soybean (Concibido et al. 2004), and could facilitate stacking of different resistance loci in a single cultivar, which could provide a more sustainable resistance to *P. pachyrhizi* in the future. Microsatellites or simple sequence repeats (SSRs) are the preferred markers presently used to map host resistance to soybean diseases because of their high polymorphism, co-dominance, reproducibility, and good distribution throughout the soybean genome. Furthermore, over a thousand well-distributed SSR markers are already mapped in the soybean genetic map (Song et al. 2004). The present study describes the mapping of *Rpp2* and *Rpp4* loci on the soybean genetic map using SSR markers.

Materials and methods

Plant materials

The mapping population for *Rpp2* consisted of 130 $F_{2:3}$ families derived from a cross between the 'BRS 184', a Brazilian cultivar susceptible to soybean rust, and PI 230970, the genotype carrying the resistant dominant allele *Rpp2*. For *Rpp4* locus, the mapping population consisted of 80 $F_{2:3}$, derived from the cross between the same susceptible line as used for *Rpp4* mapping, 'BRS 184', and PI 459025, the genotype carrying the resistant dominant allele *Rpp4*. Each $F_{2:3}$ family consisted of 15 plants. The parental lines used on the crosses were obtained from Embrapa-Soybean Germplasm Collection, located at Londrina, PR, Brazil. The two $F_{2:3}$ mapping populations and the parental lines for each of *Rpp2* and *Rpp4,* were cultivated in greenhouse for evaluating their resistance to ASR, and the F_2 populations were cultivated for leaf sampling. Leaf tissues were stored at −80°C for DNA extraction.

ASR inoculation and evaluation

Inoculum consisted of a fungal population collected in commercial field in the state of Mato Grosso, and maintained on cultivar BRSMS-Bacuri in a greenhouse, for artificial inoculation. Urediniospores were collected by tapping infected leaves over a plastic tray and diluted in distilled water with 0.05% of polyoxyethylenesorbitan monolaurate (Tween 20) (v/v) to a final concentration of 80,000 spores/mL. This suspension of spores was sprayed onto the plants at V5 developmental stage. Twelve days after inoculation, the parental lines and the $F_{2:3}$ families were evaluated by classifying the plants showing reddish-brown lesions (RB) and low sporulation as resistant, whereas plants showing tan lesions (TAN) and high sporulation were classified as susceptible. The 15 plants per each family were independently evaluated by two people, and any discrepancy on the result was resolved by a third person evaluation. Families showing no segregation at F_3 generation were classified as resistant and susceptible homozygous, whereas families showing segregation were classified as resistant heterozygous. The inoculum production and $F_{2:3}$ evaluations were performed in a greenhouse with inside temperature ranging from 19 to 26°C.

The rust isolate used in this study is being maintained in a susceptible cultivar (BRMS-Bacuri) in the greenhouse. In

addition, a sample of the isolate urediniospores is stored frozen at −80°C and can be obtained upon request. The ITS (Internal Transcribed Spacer) regions were cloned from *P. pachyrhizi* urediniospores by PCR with primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′), as described by Frederick et al. (2002). The sequencing reaction was performed in the genetic analyzer ABI PRISM 3100, with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), according to manufacturer's instructions.

DNA extraction and mapping strategy

DNA of each F_2 plant was extracted using a modified CTAB protocol (Keim et al. 1988). Approximately 0.1 g of leaf tissue was ground with liquid nitrogen and mixed with CTAB extraction buffer (1% CTAB, 1.4 M NaCl, 0.1 M Tris–HCl pH 8.0, 0.5 M EDTA, 0.1% mercaptoethanol). This mixture was heated, centrifuged, and the supernatant was extracted twice with chloroform–isoamyl alcohol (24:1 v/v). The DNA was precipitated with isopropanol and treated with RNase A. DNA concentration and integrity were estimated by spectrophotometer analysis and gel electrophoresis, respectively.

The soybean SSR markers used in this study were developed by the Beltsville Agricultural Research Center—BARC/ASR (Cregan et al. 1999a; Song et al. 2004). Primer sequence information for all SSR markers is available at Soybase Web Site (http://soybase.agron.iastate.edu/resources/ssr.php; Soybase 2006). To guarantee a sufficient coverage of the soybean genome, at least one polymorphic SSR marker in each 20 cM interval was used, for each one of the 20 linkage groups. In order to quickly find SSR markers associated to the target loci, markers known for their linkage to loci for resistance to soybean diseases were preferentially tested, particularly on linkage groups A2, F, G and J. Similar strategies have been also used to map other resistance loci in soybean (Mian et al. 1999; Gordon et al. 2006). The bulked segregant analysis (BSA; Michelmore et al. 1991) strategy was used to expedite the search for linked markers to ASR resistance loci. Four bulks consisting of equal amounts of DNA from six plants were made: two bulks for the resistant phenotype and two for the susceptible. The bulks were constructed based on the $F_{2:3}$ ASR evaluations, allowing the selection of only homozygous plants for each differing phenotype. DNA samples from the two parental lines and from the four bulks, for each cross, were used for SSR analysis. In order to confirm putative linkage, markers found polymorphic among the contrasting bulks were used to amplify the full F_2 populations.

For SSR analysis, 30 ng of DNA was used as template in a 10 µl reaction containing buffer (100 mM Tris–HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 32.5 µM of each dNTP, 0.2 µM of each primer, and 1 U of Taq DNA polymerase. The cycling consisted of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C; followed by 7 min at 72°C. The amplified fragments were separated by electrophoresis in 10% polyacrylamide gels or 3% agarose-synergel, stained with ethidium bromide, and visualized under UV light.

Data analysis

All SSR markers were scored as co-dominant markers. The ASR phenotype was evaluated as a qualitative trait and also scored as co-dominant trait, according to $F_{2:3}$ progeny evaluation. Segregation ratio for SSR markers and observed ASR phenotype were tested for goodness of fit to expected ratios for segregation of a single gene using Chi-square test. A P-value greater than 0.05 in the Chi-square test reflects that the segregation fits the expected ratio. Linkage analysis and map construction were performed with the program GQMOL 9.1 (Cruz and Schuster 2006) using Kosambi function. The linkage criteria were a LOD score >3.0 and a maximum distance of 37.2 cM.

Results

Asian soybean rust evaluation

Asian soybean rust reaction was assessed following inoculation with an isolate of *P. pachyrhizi*, maintained on the cultivar BRSMS-Bacuri. This cultivar is known for its high susceptibility to the isolate of soybean rust used in this study (J.T. Yorinori, personal communication). This cultivar was also resistant to the first isolate identified in Brazil, in 2001. However, its resistance was broken by the field isolate that also overcame *Rpp1* and *Rpp3* resistance loci. The use of this cultivar to maintain the inoculum insured the use of the current isolate present in most Brazilian soybean areas. Parental lines PI 230970 (*Rpp2*) and PI 459025 (*Rpp4*) showed typical resistant reaction while 'BRS 184' showed the expected susceptible reaction to ASR. Identical susceptible reaction was found on PI 200492 (*Rpp1*) and 'Ankur' (*Rpp3*). For *Rpp2* and *Rpp4* mapping populations, a progeny of 15 plants was evaluated per F_3 family, allowing the ASR phenotype to be scored as a qualitative trait. Both genes satisfactorily fit the expected ratio for single dominant gene segregation (Table 1). Twenty-five out of 130 $F_{2:3}$ lines, evaluated for the *Rpp2* mapping population, were considered homozygous resistant, 77 were heterozygous resistant and 28 were homozygous susceptible, respectively. For *Rpp4* mapping population, 29 were homozygous resistant, 32 were heterozygous resistant, and 19 were homozygous susceptible in 80 $F_{2:3}$ lines evaluated.

Table 1 Chi-square analysis of segregation on Asian soybean rust phenotypes ($F_{2:3}$ generation) and SSR markers (F_2 generation) in *Rpp2* and *Rpp4* mapping populations

Marker/trait	Observed ratio[a]			Expected ratio[a]	$\chi^{2\ b}$	Probability[c] (%)
	A	B	C			
Rpp2 population						
Rpp2	25	77	28	1:2:1	4.57 ns	10.18
Satt406	28	73	29	1:2:1	1.99 ns	37.07
Satt280	28	72	30	1:2:1	1.57 ns	45.63
Satt596	28	73	29	1:2:1	1.99 ns	37.07
Satt456	29	71	30	1:2:1	1.12 ns	57.03
Satt529	28	72	30	1:2:1	1.57 ns	45.63
Sat_255	28	69	33	1:2:1	0.88 ns	64.50
Sct_001	28	70	32	1:2:1	1.02 ns	60.19
Sat_093	29	69	32	1:2:1	0.63 ns	72.95
Sat_366	28	71	31	1:2:1	1.25 ns	53.63
Satt620	28	71	31	1:2:1	1.25 ns	53.63
Satt547	27	62	41	1:2:1	3.29 ns	19.28
Satt431	29	58	43	1:2:1	4.52 ns	10.42
Rpp4 population						
Rpp4	29	32	19	1:2:1	5.70 ns	5.78
Satt199	22	43	15	1:2:1	1.68 ns	43.28
Satt012	21	47	12	1:2:1	4.48 ns	10.67
Satt503	21	47	12	1:2:1	4.48 ns	10.67
Satt517	22	47	11	1:2:1	5.48 ns	6.47
Satt288	28	34	18	1:2:1	4.30 ns	11.65
AF162283	26	36	18	1:2:1	2.40 ns	30.12
Satt191	29	36	15	1:2:1	5.70 ns	5.78

[a] A, B, and C mean: homozygous genotype equivalent to the resistant parental line, heterozygous genotype, and homozygous genotype equivalent to the susceptible parental line, respectively

[b] ns means: not significant at 5% significance level

[c] Probability greater than 5% means the observed segregation fit the 1:2:1 model

SSR screening and linkage analysis

In the *Rpp2* mapping population, a total of 177 SSR markers were screened and 77 were found polymorphic between the parental lines PI 230970 and ‘BRS 184’ while for *Rpp4* mapping population, 65 out of 175 SSR markers were polymorphic between the parental lines (PI 459025 and BRS 184). The four DNA bulks for each population were initially screened with the polymorphic markers. Two markers, Satt431 and Satt406, on linkage group J, were found polymorphic between the pairs of bulks for the *Rpp2* mapping. For *Rpp4* mapping, three SSR markers were polymorphic between the resistant and susceptible bulks; however, only two of them, Satt199 and Satt288, were actually linked to *Rpp4* on linkage group G. Once their linkage was confirmed by testing the full mapping populations, other SSR markers for linkage groups G and J were chosen on basis of the available integrated genetic map of soybean (Song et al. 2004). All SSR markers mapped in this work satisfactorily fit the expected ratio for co-dominant inheritance (1:2:1) on the *Rpp2* and *Rpp4* mapping populations (Table 1). The program GQMOL 9.1 was used to perform the linkage analysis and mapped *Rpp2* at 7.3 cM from Sat_255 and at 5.6 cM from Satt620, on the linkage group J, while *Rpp4* was mapped at 1.9 cM from Satt288 and at 12.8 cM from AF162283, on the linkage group G (Fig. 1).

Discussion

Asian soybean rust resistance is an important trait in most soybean breeding programs worldwide. However, no resistant cultivars have been released to date. From the four previously identified resistance loci, two were mapped in this study, *Rpp2* and *Rpp4*. This study revealed that *Rpp2* and *Rpp4* loci are located on linkage groups J and G, respectively. These resistance loci have been known for over 20 years, but only with the recent surge of ASR in South America, they have been focus of more intense molecular studies. Previous efforts, in Thailand, have also located a resistance locus in the linkage group G, in a close association to Satt012 and Satt472 (Nuntapunt et al. 2004). However, that resistance source was generated by mutagenesis, and whether it is allelic to *Rpp4* remains to be discovered. Several other resistance loci are also under investigation. Recently, a resistance loci found on the Brazilian cultivar ‘FT-2’ has been mapped on the linkage group C2 (Brogin et al. 2004). Coincidently, a resistance loci found on the

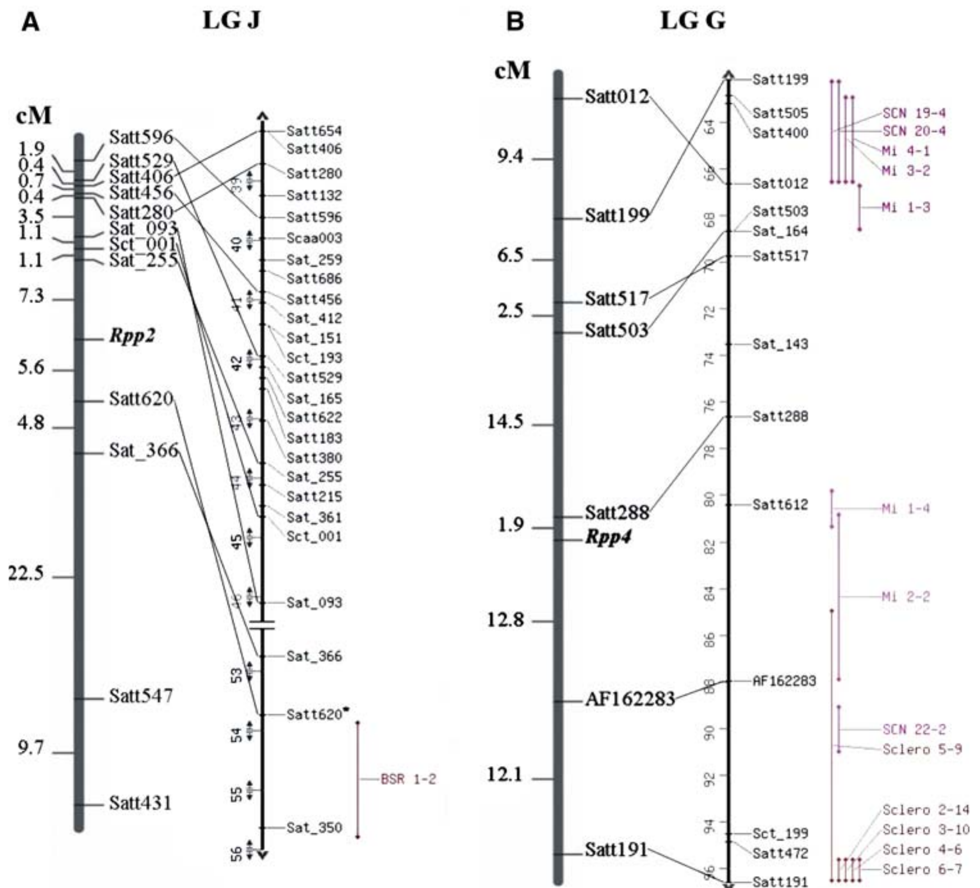

Fig. 1 Linkage maps of the *Rpp2* (**a**) and *Rpp4* (**b**) genomic regions. On the *left side* is displayed the genetic map generated by this study and on the *right side* is an inset of the consensus linkage groups J (**a**) and G (**b**), displaying the SSR markers mapped on the region with the cumulative distances in centimorgan. On the *right side* of the inset are displayed the QTLs to resistance loci mapped on these regions (BSR—brown stem rot; Mi—*M. incognita*; SCN—soybean cyst nematode; Sclero—Sclerotinia stem rot). These insets were clipped from the Soybase webpage (Williams physical map feature; Soybase 2006). *Asterisk* the marker Satt620 was not present on the Williams physical map, but its position was inferred based on the composite genetic map (Song et al. 2004), available at http://www.ars.usda.gov/SP2UserFiles/person/1190/soymap2.mht

Japanese cultivar 'Hyuuga' was also mapped on the same genomic location, between SSR markers Satt460 and Satt307 (Monteros et al. 2007). If these two loci are allelic or are independent resistance genes is still unknown. However, while 'Hyuuga' remains resistant to the ASR isolate used in this study (data not shown), the resistance of 'FT-2' has been already defeated. Recently, the *Rpp1* locus was mapped on the bottom part of linkage group G (Hyten et al. 2007), but, differently from other resistance loci, *Rpp1* confers an immune response to the India-73-1 ASR isolate, used on the mapping experiment.

Since there is no study on ASR races in Brazil yet and, the fact *Rpp2* and *Rpp4* confer resistance to most isolates characterized abroad (Yamaoka et al. 2002), this study was performed with a field isolate of the fungus, maintained on the cultivar BRSMS-Bacuri in the greenhouse. Based on ITS sequence, this isolate is probably related to the MUT Zimbabwe isolate (GenBank accession no. AF333499), showing an identity of 99.8% (data not shown). Although the ITS sequences from all 13 isolates available on GenBank are very similar (about 98% of identity), the MUT Zimbabwe isolate contains a four nucleotide deletion compared to all other isolates (Frederick et al. 2002). The isolate used in this study (GenBank accession no. EU523736) contains the same four nucleotide deletion, making it almost identical to MUT Zimbabwe (data not shown). A more detailed investigation of the genetic diversity of South American isolates is currently under way and will be reported elsewhere. No differentiation on the disease severity was found among the two resistance sources used in this study. Both resistance loci fit the expected patterns showing typical resistant reactions (RB lesions). *Rpp2* and *Rpp4* also behaved as dominant traits, confirming previous genetic studies on these two loci (Hartwig 1986; Hartwig and Bromfield 1983).

Both resistance loci, *Rpp2* and *Rpp4*, are located in regions considered hot spots for resistance genes, meaning that the strategy of using markers previously known to be linked to other resistance loci was successful. Among the markers chosen to make the initial screening, based on information of mapped resistance loci, Satt431, linked to *Rbs1* locus (Bachman et al. 2001) on linkage group J, and Satt199, linked to a QTL for Sclerotinia stem rot (Arahana et al. 2001) and also for Southern root-knot nematode (Li et al. 2001), on linkage group G, were found polymorphic between the resistant and susceptible bulks.

Actually, the chromosomal region in the vicinity of *Rpp4* contains a cluster of resistance genes. Significant quantitative trait loci (QTLs) for resistance to different diseases have been reported in this region. Within 20 cM

interval harboring the *Rpp4* locus, QTLs for resistance to the Southern root-knot nematode (*M. incognita,* Tamulonis et al. 1997; Li et al. 2001), Sclerotinia stem rot (Arahana et al. 2001), and soybean cyst nematode (Yue et al. 2001; Wang et al. 2001) can be found (Fig. 1b). In addition, the resistance genes *rhg1* (SCN; Concibido et al. 1994; Cregan et al. 1999b); *Rps4*, *Rps5*, and *Rps6* (Phytophthora root rot; Demirbas et al. 2001); *Rfs* (Sudden Death Syndrome; Chang et al. 1997); and the recently mapped *Rpp1* (Hyten et al. 2007) are also located on this linkage group.

Linkage group J is also known to contain resistance clusters, with most of the resistance loci localized at the lower part of the group. The resistance loci *Rps2* (conditions resistance to Phytophthora root rot), *Rmd* (powdery mildew) and *Rj2* (that controls nodulation by *Bradyrhizobium japonicum*) were all located within 3.8 cM interval on the bottom of this linkage group (Polzin et al. 1994). The *Rpp2* locus was mapped around 25 cM from this resistance cluster. In addition, the resistance loci *Rbs1* and *Rbs3* (Brown stem rot, Bachman et al. 2001; Patzoldt et al. 2005) and *Rcs3* (Frogeye leaf spot, Mian et al. 1999) are mapped near this region, and also a recently identified QTL for soybean cyst nematode resistance (Guo et al. 2005). Kanazin et al. (1996) and Graham et al. (2002) also demonstrated cluster of R-genes in soybean. Clustering of resistance genes is not an uncommon situation and has been reported in several plant species (Michelmore and Meyers 1998).

The linkage maps generated by this study showed that the order of the molecular markers was consistent with the soybean consensus linkage map (Song et al. 2004) with few inversions and small differences on map distance (Fig. 1). Differences on mapping position are expected since the linkage analyses were performed on different mapping populations. These maps may be useful in selecting adequate markers to be used on marker assisted selection in breeding programs that aim resistance to this pathogen. Although some of the markers mapped on these populations are not closely linked to the resistance loci, other markers located on the same region may be useful in different germplasms. The *Rpp4* locus was mapped between Satt288 and AF162283. Other two markers, Sat_143 and Satt612, located on either side of Satt288 (Fig. 1b), were not polymorphic on the population used in this study. Similar situation occurred in the *Rpp2* region. Several markers, mapped in a 2 cM interval, were located 7.3 cM upstream the *Rpp2* locus (Fig. 1a). Three additional markers on this region, Satt215, Sat_361, and Satt621 were not polymorphic on this population, and could, perhaps, reveal a closer association to *Rpp2*. No additional BARC_SSR markers are available in the interval between Sat_093/Sat_255/Sct_001 and Satt620. However, in order to have a saturated map of this region, additional mapping efforts involving different populations and other kind of molecular markers are needed. Currently, new SSR markers based on BAC-End sequencing are being developed by the Southern Illinois University (Shultz et al. 2006) and a soybean transcript map has been released (Choi et al. 2007). These may be useful to increase the marker density on these regions. The SSR markers linked to *Rpp2* and *Rpp4* described here will be of great utility in assisting the introgression of multiple loci on a single cultivar and will be of great value as a start point toward the cloning of these resistance genes.

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