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ORIGINAL PAPER

Genetics and mapping of adult plant rust resistance in soybean PI 587886 and PI 587880A

Jeffery D. Ray · Wilfrido Morel · James R. Smith · Reid D. Frederick · Monte R. Miles

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Abstract Two soybean accessions, PI 587886 and PI 587880A, previously identified as having resistance to *Phakospora pachyrhizi* Syd. (soybean rust, SBR) were used to create two populations (POP-1 and POP-2) segregating for SBR resistance. F_2-derived F_3 ($F_{2:3}$) families from each population were grown in a naturally SBR-infected field in Paraguay to determine inheritance and map resistance genes. Over 6,000 plants from 178 families in POP-1 and over 5,000 plants from 160 families in POP-2 were evaluated at R5 for lesion type: immune reaction (IR), reddish-brown (RB), or tan (TAN) colored lesions. Based on the lesion type present, each $F_{2:3}$ family was rated as resistant, segregating or susceptible and this classification was used to infer the F_2-phenotype and genotype. For both populations, the F_2 segregation ratios fit a 1:2:1 (resistant:segregating:susceptible) ratio expected for a single gene ($P > 0.05$). The RB lesions occurred almost exclusively in the heterozygous class, indicating incomplete dominance under the conditions of this study. Molecular markers flanking the locations of the known resistance genes were used to map the resistance gene in both populations to the *Rpp1* locus. However, evaluation of PI 587886 and PI 587880A against eight *P. pachyrhizi* isolates indicated that the resistance allele in these two accessions was different from *Rpp1*. This test also demonstrated that these accessions were resistant to at least one *P. pachyrhizi* isolate collected in the southern US. This is the first report of using an adult plant field-screen with natural rust pressure to map SBR resistance.

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J. D. Ray (✉) · J. R. Smith
USDA-ARS, Crop Genetics and Production Research Unit,
Stoneville, MS 38776, USA
e-mail: jeff.ray@ars.usda.gov

W. Morel
Centro Regional de Investigación Agrícola,
Capitán Miranda, Itapúa, Paraguay

R. D. Frederick
USDA-ARS, Foreign Disease-Weed Science Research Unit,
Ft. Detrick, MD 21702, USA

M. R. Miles
USDA-ARS, National Soybean Research Center,
University of Illinois, Urbana, IL 61801, USA

Introduction

At least five dominant genes with resistance to soybean rust (SBR, also known as Asian soybean rust) caused by *Phakospora pachyrhizi* Syd. have been identified. Four have been known since at least the mid-1980s; *Rpp1* (McLean and Byth 1980), *Rpp2* and *Rpp3* (Hartwig and Bromfield 1983) and *Rpp4* (Hartwig 1986). More recently, a new locus, *Rpp5*, was reported by Garcia et al. (2008). Other recent research has identified recessive genes controlling SBR resistance (Calvo et al. 2008) and a new allele (*Rpp1b*, Chakraborty et al. 2009) of a known gene. Considerable research is underway worldwide to identify new resistance genes, alternative alleles, and new sources of resistance.

The renewed interest in SBR resistance began with the discovery of *P. pachyrhizi* in South America in 2001 (Morel et al. 2004), and its subsequent devastating effects on Brazilian soybean production (Yorinori et al. 2005). Even before SBR was discovered in the continental US in 2004 (Schneider et al. 2005), USDA-ARS scientists at Urbana, IL and Ft. Detrick, MD had begun screening the entire USDA-ARS Soybean Germplasm Collection for

potential sources of resistance to SBR (Miles et al. 2006). Of the over 16,000 soybean accessions screened in a seedling assay at Ft. Detrick, MD, approximately 805 had low severity or the reddish-brown (RB) lesion type and were considered potential sources of resistance. A major question from this research was whether these putatively resistant accessions represented sources of new genes or were alternate sources (with potentially alternative alleles) of the same four-genes known at that time. This still remains an important question for the majority of these putatively resistant accessions. Answering this question has been made much easier with the recent identification of the genomic locations of the known resistance genes. Hyten et al. (2007) mapped *Rpp1* to molecular linkage group (MLG) G, Silva et al. (2008) mapped *Rpp2* to MLG J and *Rpp4* to MLG G, Monteros et al. (2007) and Hyten et al. (2009) mapped *Rpp3* to MLG C2, and finally, Garcia et al. (2008) mapped *Rpp5* to MLG N.

Miles et al. (2008) grew 530 of the putatively resistant accessions identified in the seedling screen at Ft. Detrick, MD (Miles et al. 2006) in an adult plant field-screen in Paraguay. From this screen, PI 587886 and PI 587880A were identified as among the ten accessions with the highest level of resistance to the natural SBR infection occurring those years in Paraguay. As detailed in this report, we used these two accessions to create separate segregating populations that were screened in Paraguay during the 2007/2008 growing season in a field environment with heavy SBR pressure. We then used molecular markers flanking the genomic locations of the five known SBR resistance genes to determine the genetic relationship of the resistance identified in each accession to the known SBR resistance genes. This is the first report of using an adult plant field-screen to determine SBR inheritance in over 30 y (Sing and Thapliyal 1977) and the first to use an adult plant field-screen to map SBR resistance.

Materials and methods

Population development

Soybean accessions PI 587886 and PI 587880A were reported as resistant based on field and greenhouse screenings in 2005/2006 and 2006/2007 in Paraguay (Miles et al. 2008). PI 587886 is a maturity group VI accession and was used in the first population (POP-1) as the male parent in a cross with LG01-5087-101. LG01-5087-101 was selected as the female parent because it is high yielding and adapted to the early soybean production system of the mid-southern USA. It is an F_8-derived maturity group IV breeding line developed by Dr. Randall Nelson (USDA-ARS, Urbana, IL, USA). The second population (POP-2) was created from a cross between JTN5503 (Arelli et al. 2006) and PI 587880A. PI 587880A was used as the male parent and is characterized as a maturity group VI accession. JTN5503 was used as the female parent in the second cross because of its high-yield potential and resistance to soybean cyst nematode (*Heterodora glycines* Ichinohe). It is characterized as a maturity group V cultivar. Both crosses (LG01-5087-101 × PI 587886 and JTN5503 × PI 587880A) were made at the USDA-ARS facility at Stoneville, MS in 2006. The F_1 plants were grown from December 2006 to April 2007 at the USDA-ARS Tropical Agriculture Research Station at Isabela, Puerto Rico. The resulting F_2 seed were planted in the field at Stoneville, MS in May of 2007. F_2 plants were harvested at maturity and the seed from each individual plant was bulked to form F_2-derived F_3 ($F_{2:3}$) families. For both populations, seed from each family was divided and packaged into three replications of 15 seed each, shipped to Paraguay, planted and then the families evaluated for SBR response.

Population screening

On 11 December 2007, 186 $F_{2:3}$ families for POP-1 and 164 $F_{2:3}$ families for POP-2 were planted in the field at the Centro Regional de Investigación Agrícola (CRIA) research station (Latitude 27°11′60″S and Longitude 55°47′60″W) of the Ministerio de Agricultura y Ganadería, in Capitán Miranda, Itapúa, Paraguay. The $F_{2:3}$ families were hand planted in 1 m rows spaced 45 cm apart with 15 seed per row and a 1 m alley between ranges. The soil was an Oxisol of Basaltic origin. During the growing season, plants were sprayed with the insecticides cipermetrin, acephate and clorpirifos as needed for control of *Anticarsia gemmatalis*, *Euchistus heros*, *Poligotarsonemus latus*, and *Bemisia tabaci*. The experimental design was a randomized complete block with three replications for each population. Three rows of each parental line were randomly planted within each replication. At every ten rows within a range, two susceptible spreader rows (cultivars "A 4910" and "A 8000") were maintained (i.e. replanted and inoculated as necessary) year-round for the prior 3 years. In previous years, the spreader rows were inoculated to maintain the SBR epidemic. However, in this experiment the natural SBR infection was severe enough that no artificial inoculation was required. The spreader rows provided a continuous, naturally occurring source of *P. pachyrhizi* throughout the course of the experiment.

In the field, each plant within each row was rated for lesion type: tan (TAN), reddish-brown (RB), or immune reaction (IR) (Bromfield 1984). Plants were primarily evaluated based on the bottom third of the canopy, but lesions appearing anywhere on the plant were recorded. The ratings were made on 6 February, 22 February, and 25 February

2008, which corresponded to the R5 growth stage (Fehr and Caviness 1977). $F_{2:3}$ families were classified as immune, susceptible, or segregating based on the proportion of TAN, IR, or RB reactions present (individual family data not shown, but Table 1 presents a summary across all families). The phenotype of each F_2 plant was inferred from analysis of the summarized F_3 phenotypes. Families showing discrepancies between replications were reexamined. For POP-1, eight families out of the original 186 could not be properly classified, and for POP-2, four families out of the original 164 could not be properly classified. Unclassified families were dropped from the analysis.

DNA isolation and molecular markers

Leaf samples were collected from individual F_2 plants from both populations (POP-1 and POP-2) grown at Stoneville, MS. The samples were freeze dried in a Model 2400 freeze dryer (The Freeze Dry Company, Nisswa, MN 56468, USA) and ground to a fine powder using a tissue pulverizer (Garcia Manufacturing, Visalia, CA 93292, USA). DNA was isolated from the samples using a Maxwell 16™ automated DNA isolation machine (Promega, Madison, WI 53711, USA) following manufacturer's protocols. DNA was also isolated from individual plants of selected F_3 families grown in Paraguay using the PowerPlant™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA 92010, USA). Approximately ten 9.5 mm diameter leaf punches were taken from each individual F_3 plant and placed in 2 ml screw-cap tubes from the PowerPlant™ DNA Isolation Kit and frozen at −80°C. DNA was extracted following manufacturer's instructions using a vortex adapter (MoBio, cat # 13000-V1-24) attached to a Vortex Genie (Scientific Industries, Inc., Bohemia, NY 11716, USA).

Simple sequence repeat (SSR) molecular markers were selected based on the reported genomic locations of the known *Rpp* genes. SSR primer sequences were obtained from SoyBase (http://soybase.org/resources/ssr.php). Primers were manufactured with either a hexachlorofluorescein (HEX) or 6-carboxyfluorescein (FAM) 5′-fluorescent label (Integrated DNA Technologies, Coralville, IA 52241, USA). PCR amplification was performed on a MJ Research PTC 225 (Biorad, Hercules, CA 94547, USA) using conditions of 95°C for 120 s; 30 cycles of 94°C for 30 s, 46°C for 30 s, 72°C for 30 s; and one cycle of 72°C for 300 s followed by maintenance at 4°C until detection. Amplicons were detected on an ABI 3730 (Applied Biosystems, Foster City, CA 94404, USA) at the USDA-ARS Midsouth Area Genomics Facility at Stoneville, MS. The products were analyzed using GeneMapper 3.7 (Applied Biosystems, Foster City, CA 94404, USA). All polymorphic SSR markers identified in this study were co-dominant.

P. pachyrhizi isolate response evaluation

The resistant parents used in this study (PI 587886 and PI 587880A) along with three other soybean genotypes, 'Williams 82' (Bernard and Cremeens 1988) (*rpp1*), PI 200492 (*Rpp1*) and PI 594538A (*Rpp1b*) were inoculated with eight *P. pachyrhizi* isolates (for isolate details see Table 4) at the USDA-ARS Foreign Disease-Weed Science Research Unit (FDWSRU) Biological Safety Level-3 Plant Pathogen Containment Facility at Ft. Detrick, MD

Table 1 The percentage of each lesion type (TAN, RB, or IR) observed across all plants in each $F_{2:3}$ family classification (resistant, susceptible, or heterozygous) for the 178 $F_{2:3}$ families from the LG01-5087-101 × PI 587886 population and the 160 $F_{2:3}$ families from the JTN5503 × PI 587880A population grown in Paraguay in the 2007/2008 season

$F_{2:3}$ family rating	No. of families	No. of plants evaluated	Percentage of plants with each soybean rust lesion phenotype		
			TAN (%)	RB (%)	IR (%)
POP-1 (LG01-5087-101 × PI 587886)					
Immune	45	1,512	1.32	1.92	96.76
Susceptible	46	1,639	97.68	1.71	0.61
Segregating	87	2,970	32.76	41.25	25.99
POP-2 (JTN5503 × PI 587880A)					
Immune	31	994	0.91	3.92	95.17
Susceptible	38	1,293	97.91	2.01	0.08
Segregating	91	3,037	21.63	58.87	19.49

45 $F_{2:3}$ seed from each family were divided into three replications arranged in a randomized complete block. In both populations, poor seed germination lowered the number of plants screened from the targeted number of 45 plants to an average of 34.4 (POP-1) and 33.3 (POP-2) plants per family

TAN tan colored lesions, *RB* reddish-brown colored lesions, *IR* an immune reaction (no lesions)

(Melching et al. 1983) under appropriate APHIS permit. On 16 July 2008, seed of each genotype were planted in two replications per isolate in 9 cm square × 8 cm deep plastic pots (catalog # 400-SVT-500; T.O. Plastics, Inc., Clearwater, MN 55320, USA) containing Sunshine LC1 mix (Sun Gro Horticulture Products, Belleview, WA 98008, USA). The seeding rate was two seed per pot except for PI 594538A for which one seed per pot was sown (due to a lack of seed). All plants that emerged were inoculated. The first replication was inoculated on 5 August 2008 and the second replication on 6 August 2008. Plants had reached the second fully expanded trifoliolate by these dates.

Urediniospores of the *P. pachyrhizi* isolates had been previously collected using a mechanical harvester (Cherry and Peet 1966) and maintained under liquid nitrogen. Prior to inoculation, stored urediniospores were removed from liquid nitrogen, heat shocked at 40°C for 5 min, and maintained in a humid environment overnight as described in Bonde et al. (2006). Spores were suspended in 0.01% (v/v) Tween20/water and adjusted to a concentration of approximately 60,000 spores ml^{-1} with a hemacytometer.

Approximately 8 ml of inoculum were applied to each pot using an atomizer at 20 psi, after which the plants were incubated inside dew chambers at 20°C for 18 h. Pots that were inoculated with a particular isolate were grouped together in the dew chamber and then placed together in a greenhouse maintained at 25°C. Supplemental lighting was provided by 1,000 w metalarc lights (Sylvania, Danvers, MA 01923, USA) spaced 0.6 m apart above the bench. Pots were placed in metal trays and bottom watered. The reaction phenotypes were visually evaluated 14 days after inoculation and recorded as IR, RB or TAN.

Over all isolates and both replications, the average spore inoculation rate was 58,900 spores ml^{-1} (STD 8,994) and ranged from a low of 46,500 spores ml^{-1} for isolate TW72-1 to a high of 76,000 spores ml^{-1} for isolate AL04-1. Urediniospore viability was determined by spraying inoculum of each isolate onto the surface of sterile water agar in Petri dishes and determining the percent germination after 18 h incubation at 20°C. The average spore germination rate was 74% (STD 5.5) and ranged from 65.3% for isolate AL04-1 to 83.6% for isolate HW94-1.

Statistical analysis

By evaluating $F_{2:3}$ families, the phenotype of the respective F_2 source plant was inferred (resistant, segregating, or susceptible). The expected segregation ratios of the F_2 plants and F_3 families were compared to observed ratios using a χ^2 Goodness of Fit test (Sokal and Rohlf 1995) with the Yates' correction for sample size (Yates 1934) as appropriate. Once the qualitative single gene nature of the observed SBR resistance in this study had been confirmed, SSR markers and SBR phenotypes were analyzed for putative linkage using a two-way contingency table to analyze allele frequencies. The probabilities of deviations from expected values were made using the Fisher's Exact Test (Sokal and Rohlf 1995) as computed using SAS ver. 9.2 (SAS Institute, Cary, NC, USA). JoinMap 4.0 (Van Ooijen 2006) was used to further evaluate linkage, order molecular markers and integrate the linkage groups produced for each population.

Non-parametric correlation was used to evaluate the association between lesion type (TAN, RB, or IR) and SSR marker alleles in 33 F_3 families (16 from POP-1 and 17 from POP-2). The 33 families consisted of 20 segregating and 13 non-segregating families chosen based on completeness and consistency of data. The number of plants subjected to DNA analysis was limited by the facilities in Paraguay. Even so, DNA from 358 plants was obtained and data from individual plants from both populations were combined for analysis. Lesion type and marker allele were coded in an ordinal array where TAN lesions = 1, RB lesions = 2, and no lesions (IR) = 3. Marker alleles were coded as homozygous susceptible parental allele = 1, presence of both alleles (heterozygotes) = 2, and homozygous resistant parental allele = 3. The Γ (gamma) statistic was used as the measure of ordinal association between lesion type and marker allele. The range of the test statistic is $-1 \leq \Gamma \leq 1$ where values close to zero indicate independence. Γ was computed using the PROC FREQ procedure of SAS ver. 9.2 (SAS Institute, Cary, NC, USA).

Results

In POP-1 the parents (LG01-5087-101 and PI 587886) differed in pubescence color. LG01-5087-101 was gray (*t*) and PI 587886 was tawny (*T*). In a cross between these parents, pubescence was expected to segregate in a 1:2:1 (*TT*/*Tt*/*tt*) genotypic ratio in the F_2 generation (as summarized by Palmer et al. 2004). Respective F_2 plant pubescence color was inferred from the pubescence color (i.e. tawny, gray, or both for each family) summarized across three replications for the 186 $F_{2:3}$ families evaluated in Paraguay. Subsequent analysis indicated that F_2 pubescence segregated in the expected genotypic ratio of 1:2:1 (*TT*/*Tt*/*tt*; $\chi^2 = 4.04$, $P = 0.13$). These results confirmed the ability to derive the F_2 plant phenotype/genotype of plants grown in Stoneville, MS from the composite F_3 phenotype of plants grown in Paraguay. The results also indicated that the population was segregating normally. Similarly, in POP-2, the parents (JTN5503 and PI 587880A) differed in pod color (brown or tan). JTN5503 had tan (*l2*) colored pods and PI 587880A had brown (*L2*) colored pods. Data from the 160 $F_{2:3}$ families evaluated in Paraguay were used to infer the F_2

phenotype/genotype. Pod color segregation in the F_2 fit a 1:2:1 genotypic ratio (*L2L2*/*L2l2*/*l2l2*; $\chi^2 = 1.82$, $P = 0.40$) as expected (Bernard 1967) and also indicated this population was segregating normally.

For POP-1, out of 186 $F_{2:3}$ families evaluated, eight families were eliminated from the analysis because of phenotypic discrepancies in SBR lesion type that could not be resolved between replications. Of the remaining 178 families, an average of 34.4 plants per family were individually scored for SBR lesion type (TAN, RB, or IR) for a total of 6,121 plants evaluated. Based upon a composite rating across all three replications, the phenotype (resistant:segregating:susceptible) of the F_2 plant, from which the $F_{2:3}$ family was derived, was inferred. The results indicated that the population segregated 45:87:46 (resistant:segregating:susceptible) in the F_2 generation, which very closely matched the expected 1:2:1 ratio (44.5:89:44.5) for a single gene ($\chi^2 = 0.048$, $P = 0.98$).

The results for POP-2 were very similar to that of POP-1. Out of 164 $F_{2:3}$ families planted in Paraguay, four families were dropped because of phenotypic discrepancies in SBR lesion type that could not be resolved between replications. In the remaining 160 families, a total of 5,324 individual plants (average 33.3 per family) were evaluated for lesion type (IR, RB, or TAN) and that data was used to infer the phenotype (resistant:segregating:susceptible) of the F_2 source plant. As in POP-1, the data indicated that the F_2 generation segregated as a single gene in a 1:2:1 ratio (resistant:segregating:susceptible; 31:91:38, $\chi^2 = 3.24$, $P = 0.20$) for POP-2.

Table 1 shows the percentage of TAN, RB, and IR plants for each SBR phenotypic classification (resistant, susceptible, and segregating) for the $F_{2:3}$ families evaluated in POP-1 (6,121 plants in 178 families) and POP-2 (5,324 plants in 160 families). Across both populations, for those $F_{2:3}$ families rated as susceptible, greater than 97% of the individual plants had TAN lesions (Table 1). In POP-1, across all $F_{2:3}$ families rated as resistant, 96.76% of the individual plants evaluated were IR and did not produce visible lesions. Similar results were observed for POP-2, where 95.17% of the individual plants were rated as IR (no visible lesions) in $F_{2:3}$ families rated as resistant. That such a high percentage of the plants in the susceptible families had TAN lesions (>97%), is indicative of the uniformity and high degree of SBR pressure across the field and supports the conclusion that plants rated as IR were actually immune rather than escapes.

In both POP-1 and POP-2, $F_{2:3}$ families rated as segregating had plants with no lesions (i.e. IR), plants with TAN lesions, and plants with RB lesions (Table 1). Across all segregating families in POP-1, 32.76% of the plants had TAN lesions, 41.25% had RB lesions and 25.99% had an IR. Similar results were found for POP-2 for segregating $F_{2:3}$ families, where 21.63% of the plants had TAN lesions, 58.87% had RB lesions, and 19.49% of the plants had no lesions (i.e. IR). In no segregating family did the RB lesion type occur on more than 71% of the plants. Because the RB lesion phenotype indicated a level of resistance, the F_2 generation resistant and segregating classes were combined and tested against a 3:1 (resistant:susceptible) ratio. Both populations fit a 3:1 ratio very well (POP-1, 132:46, $\chi^2 = 0.03$, $P = 0.86$ and in POP-2, 122:38, $\chi^2 = 0.08$, $P = 0.78$). If there were only two classes, these results would indicate a single dominant gene in both populations. However, as three lesion-type classes were evident (IR, RB, and TAN) in $F_{2:3}$ families, incomplete dominance was indicated.

Incomplete dominance was supported by the analysis of lesion types observed on the parents of the two populations. The parental accessions were randomly interspersed among the $F_{2:3}$ families in three plots for each parent in each replication for both populations. In POP-1, 102 plants of LG01-5087-101 were individually evaluated and all produced TAN lesions. Of the 92 plants evaluated for the resistant parent (PI 587886) all were immune (i.e. IR). No RB lesions were observed on either parent. In POP-2, all of the 104 individual plants evaluated for the susceptible parent (JTN5503) had the TAN lesion type. However, for the resistant parent (PI 587880A), five out of 101 plants evaluated produced TAN lesions, three produced a RB lesion type and the rest had no lesions (i.e. IR). It is likely that the off-types observed for PI 587880A were rogue plants either from seed mixtures or volunteer plants. Taken together, the appearance of three lesion-types (IR, TAN and RB) in $F_{2:3}$ families, coupled with the lack of (or very few) RB lesions observed on either parent, indicated that the inheritance of SBR resistance in both PI 587886 and PI 587880A was a single gene acting with incomplete dominance under the conditions of this study.

Currently, five different SBR resistance genes have been identified and each has been molecularly mapped. Using the published location of each SBR resistance locus, we used the molecular map of Song et al. (2004) to identify potential SSR markers for use in our populations. For each of our populations (POP-1 and POP-2) we identified at least one polymorphic marker on either side of each of the five known *Rpp* loci (Table 2). At each locus, these flanking markers were determined to be linked (LOD > 3.0), indicating that the polymorphisms we identified matched the loci as reported by Song et al. (2004). Individual markers flanking the loci for *Rpp2*, *Rpp3*, and *Rpp5* showed no linkage to the F_2-inferred SBR phenotypic data for either POP-1 or POP-2 (Table 2). Since no significant linkage was found between flanking SSR markers and our F_2-inferred SBR phenotypic data for *Rpp2*, *Rpp3*, and *Rpp5*, we concluded that the genes at these loci are not involved in the resistance exhibited by our populations.

Table 2 Molecular marker associations between polymorphic SSR markers at each of the five major known resistance loci (*Rpp1*, *Rpp2*, *Rpp3*, *Rpp4*, and *Rpp5*) and the F_2-inferred resistance SBR phenotypic data generated in Paraguay for the two segregating populations

SSR marker and *Rpp* locus	Molecular Linkage Group	Map Position[a] cM	Fisher's Exact Test[b]	
			POP 1[c]	POP 2[d]
Satt191	G	96.57	<0.0001	<0.0001
Rpp1	G			
Sat_372	G	107.75	<0.0001	<0.0001
Sat_064	G	108.70	<0.0001	NP[e]
Sat_165	J	42.20	NP	0.1141
Satt622	J	42.25	0.7414	NP
Rpp2	J			
Satt620	J	53.71	0.7859	0.7628
Satt708	C2	115.49	NP	0.2325
Satt460	C2	117.77	0.9697	NP
Rpp3	C2			
Staga001	C2	119.85	NP	0.5219
Sat_307	C2	121.27	0.8440	NP
Sat_164	G	68.67	0.2441	NP
Satt288	G	76.77	NP	< 0.0001
Rpp4	G			
Satt612	G	80.38	NP	< 0.0001
AF162283	G	87.94	< 0.0001	NP
Sat_166	N	38.39	0.2841	NP
Rpp5	N			
Sat_280	N	43.45	0.6171	0.7722

At least one polymorphic SSR marker was found on either side of the reported position of each of the five SBR resistance loci. At a locus within a linkage group, all markers were linked to each other (LOD > 3.0, data not shown)

[a] Molecular linkage group and map position as reported by Song et al. (2004)

[b] Markers with Fisher's Exact Test values less than 0.05 were considered linked to the *Rpp* gene within that group

[c] Population 1, LG01-5087-101 × PI 587886

[d] Population 2, JTN5503 × PI 587880A

[e] NP = not polymorphic in the respective population

On MLG G, five polymorphic SSR markers (Sat_164, AF162283, Satt191, Sat_372, and Sat_064) were identified in POP-1. These markers surround both the *Rpp1* and *Rpp4* locus on MLG G. Independently, four of the five SSR markers exhibited significant linkage to our F_2-inferred SBR phenotypic data from POP-1 (Table 2). A molecular linkage group was constructed using our phenotypic data from POP-1 and the five SSR markers from MLG G (Fig. 1). This linkage group very closely matched the marker order for MLG G of the soybean consensus map published by Song et al. (2004), with the exception of switching the order of Sat_064 and Sat_372 (Fig. 1). This inversion was also reported by Hyten et al. (2007) in the population they used to map *Rpp1*. From their subsequent analysis they concluded that the order of Sat_064 and Sat_372 shown in Fig. 1 is most likely correct. The reported location of *Rpp1* is between Satt191 and Sat_064 (Hyten et al. 2007), while *Rpp4* is located between Sat_164 and AF162283 (Silva et al. 2008). For POP-1, our F_2-inferred SBR phenotypic data mapped resistance to a locus between Satt191 and Sat_064 (Fig. 1), which is the location reported for *Rpp1*. Note that the marker on MLG G furthest away from *Rpp1* (Sat_164) did not show linkage to *Rpp1*(Table 2), although, as shown in Fig. 1, it was linked to the other markers around *Rpp4* on MLG G.

As shown in Table 2, for POP-2 we identified two markers flanking *Rpp1* (Satt191 and Sat_372) and two markers flanking *Rpp4* (Satt288 and Satt612). All four of these markers independently showed linkage to our F_2-inferred phenotypic data. Using the four markers and the phenotypic data for this population we constructed a linkage group and, as with POP-1, the resistance gene mapped to the location of *Rpp1* on MLG G (Fig. 1). Comparatively, this linkage group matched that generated for POP-1, and the two linkage groups were integrated into a combined linkage group as shown in Fig. 1.

Markers Satt191 and Sat_372 were polymorphic in both populations and were determined to be linked to *Rpp1*. Associations between the parental alleles of these two markers (i.e. the resistant parental allele, the susceptible parental allele, or both alleles) present in plants from selected F_3 families and the individual lesion type of each plant (TAN, RB or IR) were measured using an asymptotic statistical test (Table 3). The association was determined for 358 plants from 33 F_3 families combined across both populations. The 33 families consisted of 13 homozygous families (6 families wherein all plants had TAN lesions and seven families wherein all plants had no lesions) and 20 segregating families wherein there were plants with TAN lesions, plants with RB lesions and plants with no lesions (IR). The test statistic (Γ, gamma) for the association has a range of $-1 \leq \Gamma \leq 1$ where values close to zero indicate independence. Results of the analysis indicated a high and significant ($P < 0.0001$) association across all plants between the parental alleles and lesion type ($\Gamma = 0.89$ for Satt191 and 0.92 for Sat_372). The associations were even stronger for plants from families that contained only plants with TAN lesions or only plants that had an IR (0.99 for Satt191 and 1.0 for Sat_372). The associations were somewhat weaker but still highly significant ($P < 0.0001$) for plants from families segregating for lesion type (0.77 for Satt191 and 0.83 for Sat_372). These data indicated that for the two linked SSR markers analyzed, the marker alleles from the susceptible parent were highly associated with plants with TAN lesions, the molecular marker alleles from the resistant parent were highly associated with plants with

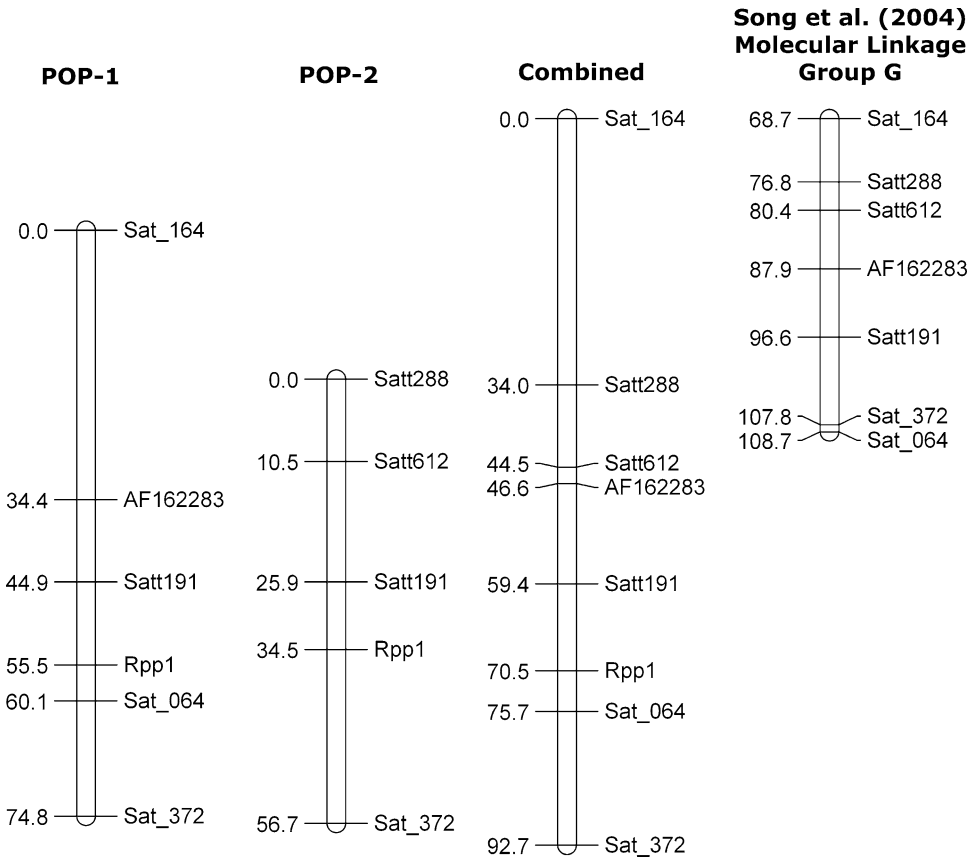

Fig. 1 Map location putatively identified as *Rpp1* from the F_2-inferred SBR phenotypic data from POP-1 (LG01-5087-101 × PI 587886) and POP-2 (JTN5503 × PI 587880A) evaluated in Paraguay. The "Combined" linkage group is the integrated map of POP-1 and POP-2. The consensus map of MLG G of Song et al. (2004) is included for reference. Note that Sat_064 and Sat_372 are reversed from that of the Song et al. (2004) map based on the linkage analysis of this study and that reported by Hyten et al. (2007). Linkage calculations and integration of maps were calculated using JoinMap 4.0 (Van Ooijen 2006)

an IR lesion type, and plants with alleles from both parents (heterozygotes) were highly associated with plants with RB lesions (Table 3). These F_3 molecular data coupled with the field and mapping data, strongly support a single gene with incomplete dominance located at or very near the *Rpp1* locus in both populations.

To provide further insight into the genes controlling rust resistance in PI 587886 and PI 587880A, we compared the lesion-type response of these two accessions to the responses of three other accessions including a susceptible Williams 82 with *rpp1*, PI 200492 (the original source of *Rpp1*), and PI 594538A, which has an alternative allele to *Rpp1* (*Rpp1b*, Chakraborty et al. 2009). Each of the five accessions was challenged in independent inoculations with eight different *P. pachy*rhizyi isolates (Table 4). Chakraborty et al. (2009) concluded that PI 594538A had an alternative allele for *Rpp1*, in large part due to the differing response of this line to *P. pachy*rhizyi isolates TW72-1 and ZM01-1 compared to the original source of *Rpp1* (PI 200492). Our test comparing the lesion-type response to these isolates produced very similar results to those of Chakraborty et al. (2009). As shown in Table 4 for both isolates TW72-1 and ZM01-1, PI 594538A had a RB lesion type, whereas PI 200492 had a TAN lesion-type. Both of the resistant lines used in our study (PI 587886 and PI 587880A) also had RB lesions in response to these two isolates, indicating more similarity to PI 594538A (*Rpp1b*) than to PI 200492 (the original source of *Rpp1*). However, the somewhat different response of PI 587886 and PI 587880A to the Australian *P. pachyrhizyi* isolate AU79-1 compared to PI 594538A (*Rpp1b*) indicated that the gene or allele in PI 587886 and PI 587880A might be different from either *Rpp1* or *Rpp1b* (Table 4). The possibility of another alternative allele for *Rpp1* needs additional investigation and confirmation. PI 587886 and PI 587880A responded similarly to isolates from Louisiana (TAN lesions) and Alabama (RB lesions) as shown in Table 4. These results also demonstrated that *P. pachyrhizyi* isolates in the southern US are not homogeneous and that PI 587886 and PI 587880A are resistant to at least one isolate found in the southern US.

Discussion

On the basis of the parental responses to SBR infection in Paraguay, we expected plants with TAN lesions and plants with an IR in the segregating class of $F_{2:3}$ families. We did not expect plants with the RB lesion type. However, the RB lesion type was observed primarily in segregating $F_{2:3}$ families, indicating incomplete dominance. Overall, we concluded incomplete dominant gene action in both popu-

Table 3 Measure of association between individual F_3 plants within families and SSR markers flanking the *Rpp1* locus on molecular linkage group G

Individual F_3 Plants From:	No. of Families	Satt191[a] Γ[d]	Prob.[b]	*n*[c]	Sat_372 Γ	Prob.	n
All Families	33	0.89	<0.0001	357	0.92	<0.0001	358
Heterozygous Families	20	0.77	<0.0001	242	0.83	<0.0001	243
Homozygous Families[e]	13	0.99	<0.0001	115	1.00	<0.0001	115

Plants were rated as having tan colored lesions, reddish-brown colored lesions or no lesions (immune reaction). The marker data consisted of the homozygous allele from each parent and the heterozygous state (one allele from each parent). Plants from POP-1 and POP-2 were combined for analysis

[a] SSR molecular marker

[b] Significance level

[c] Number of individual F_3 plants evaluated

[d] The range of the test statistic is $-1 \leq \Gamma \leq 1$ where values close to zero indicate independence

[e] The 13 families consisted of six F_3 families (44 plants) with all TAN lesions and seven F_3 families (71 plants) with all IR (no visible lesions) response

lations because (1) three classes of SBR lesion types were evident (TAN lesions, RB lesions and immunity) in segregating families, (2) F_3 data for two molecular markers flanking the resistance locus indicated an association between lesion type and parental allele source, with the RB lesion type significantly associated with the presence of an allele from each parent (i.e. heterozygotes), (3) very few plants with RB lesions were observed outside of segregating families, and 4) no RB lesions were observed on any plants of PI 587886 and only on 3 of 101 plants for PI 587880A, while all plants of both susceptible parents had only TAN lesions. The occurrence of the RB reaction type predominately in the heterozygous state for both POP-1 and POP-2 is supported by Hartwig and Bromfield (1983). They reported that plants with a genotype of *Rpp1/Rpp1* had an immune reaction to IN73-1, whereas plants with a genotype of *Rpp1/rpp1* gave a resistant reaction (i.e. RB lesions).

Of the five known resistance genes, only *Rpp1* has been reported to have an IR (no lesions) when challenged with *P. pachyrhizi* isolate IN73-1 (Hyten et al. 2007; Hartwig and Bromfield 1983). Chakraborty et al. (2009) proposed a putative new allele (*Rpp1b*) for *Rpp1,* which showed a dominant RB reaction when challenged by *P. pachyrhizi* isolate ZM01-1 (rather than TAN lesions as in the original *Rpp1* source). For *Rpp4*, Hartwig (1986) reported that his resistance class contained both IR and RB reactions when challenged with three *P. pachyrhizi* isolates (IN73-1, TW72-1, and TW80-2), although he did not separate the two reaction types or specify which reactions could be attributed to which SBR isolate. Silva et al. (2008), in mapping *Rpp4*, reported only dominant RB reactions when challenged by an unknown SBR isolate. Resistance reactions exhibited by the other three genes (*Rpp2*, *Rpp3*, and *Rpp5*) have all been characterized as having a dominant RB lesion type (Garcia et al. 2008; Silva et al. 2008; Hartwig and Bromfield 1983). In our study, we concluded that the RB lesion was expressed co-dominantly when challenged by natural infection in an adult plant field-screen in Paraguay. How the expression of incomplete dominance denoted by the RB reactions observed in this study relates to the dominant RB reactions observed for *Rpp2–Rpp5* is not readily apparent. Additionally, except for perhaps Hartwig and Bromfield (1983), no other study of SBR segregating populations evaluated IR/RB segregation. Data presented by Hartwig and Bromfield (1983) indicated a dosage effect for resistance alleles as does the data from our study.

Our data indicate that the SBR resistance genes found in PI 587886 (POP-1) and in PI 587880A (POP-2) both map to close proximity to the location of that reported for *Rpp1* (Hyten et al. 2007). Additionally, as shown in Table 4, the reaction of PI 587886 and PI 587880A to inoculation with various *P. pachyrhizi* isolates was very similar to that observed by Chakraborty et al. (2009) on PI 594538A, which led them to conclude that it had an alternative allele of *Rpp1* (i.e. *Rpp1b*). Whether the resistance genes identified in PI 587886 and PI 587880A represent *Rpp1b* or a closely linked gene cannot be unequivocally ascertained from the current study. Based on the responses to the various *P. pachyrhizi* isolates presented in Table 4, they are clearly more like *Rpp1b* than *Rpp1*. Although, the somewhat different responses to inoculation with an Australian isolate (AU79-1, Table 4), compared to that of PI 594538A (*Rpp1b*), may suggest an additional alternative allele.

The resistance locus identified in PI 587886 and PI 587880A mapped to the location of *Rpp1* and *Rpp1b*, although the phenotypic response of these lines to SBR infection was different from previous reports for these loci. Hyten et al. (2007) for *Rpp1* did not report any RB reactions (only IR or TAN lesions) using PI 200492 and the *P. pachyrhizi* isolate IN73-1. Chakraborty et al. (2009)

Table 4 Lesion-type reactions of eight different *P. pachyrhizyi* isolates applied to five soybean genotypes, including the two resistant parents of the segregating populations evaluated in this study, PI 587886 (POP-1) and PI 587880A (POP-2)

P. pachyrhizi Isolate	Source Location	Year Acquired	Williams 82 (*rpp1*)	PI 200492 (*Rpp1*)[a]	PI 594538A (*Rpp1b*)[b]	PI 587886	PI 587880A
TW72-1	Taiwan	1972	TAN	TAN	RB	RB	RB
ZM01-1	Zimbabwe	2001	TAN	TAN	RB	RB	RB
IN73-1	India	1973	TAN	RB[c]	RB	RB	RB
HW94-1	Hawaii, USA	1994	TAN	IR	RB	RB	RB
HW98-1	Hawaii, USA	1998	TAN	IR	RB	RB	RB
AU79-1	Australia	1979	TAN	RB[c]	RB	IR/RB	IR/RB
LA04-1	Louisiana, USA	2004	TAN	RB[c,d]	TAN	TAN	TAN
AL04-3	Alabama, USA	2004	TAN	TAN	RB	RB	RB

Seedling assays were conducted at the USDA-ARS, Foreign Disease-Weed Science Research Unit Biological Safety Level 3 Plant Pathogen Containment Facility at Ft. Detrick, MD in 2008

TAN tan lesion color, *RB* reddish-brown lesion color, *IR* immune reaction (no visible lesion)

[a] Original source of *Rpp1*

[b] *Rpp1b* reported by Chakraborty et al. (2008)

[c] A few RB lesions were observed, but this isolate-genotype combination usually produces an immune reaction

[d] Pham et al. (2009) also reported a weak RB reaction for this isolate-genotype combination

inoculated PI 594538A with SBR isolate ZM01-1, and reported only RB or TAN lesions for *Rpp1b*. In our study using PI 587886 and PI 587880A, we observed all three reaction types (TAN, RB, and IR) and the SBR isolate or isolates were unknown. The combination of different soybean accessions and *P. pachyrhizi* isolates may account for the phenotypic differences observed between our study and those of Hyten et al. (2007) and Chakraborty et al. (2009).

Nonetheless, there were also major cultural differences between our study and those of Hyten et al. (2007) and Chakraborty et al. (2009) that may have affected the observed results. In both of the above cited studies, 13 or fewer plants were evaluated per family, whereas we evaluated an average of about 34 plants per family. Also, Hyten et al. (2007) and Chakraborty et al. (2009) inoculated seedlings 14–15 days after planting with specific *P. pachyrhizi* isolates (IN73-1 and ZM01-1, respectively) in a controlled environment and evaluated the plants 32–35 days after planting. However, the plants in our study were evaluated as adult plants beginning about 57 days after planting and were exposed in the field to a naturally occurring infection that was of unknown and possibly mixed *P. pachyrhizi* isolates. Further, the field grown plants in our study were under continuous re-inoculation and variable environmental conditions. The expression of RB lesions observed in our study could have resulted from a reaction to an unknown pathogen isolate, a mixture of pathogen isolates or possibly the age of the plants when evaluated. However, no RB lesions (or very few) were observed on any plants of the four parents over the course of the experiment. Similarly, the IR lesion type observed in our study could be a result of different *P. pachyrhizi* isolate(s) in Paraguay or that the IR was overcome in the other studies when the seedlings were inoculated at higher urediniospore concentrations under environmental conditions that were more favorable for infection than occurred naturally in Paraguay. It is also possible that the resistance observed in PI 587886 and PI 587880A represent alternative alleles for *Rpp1* similar to that found by Chakraborty et al. (2009) for *Rpp1b*, as hinted at by the response to the Australian isolate AU79-1 (Table 4). Fine mapping, allelism tests and/or single pathogen evaluations in controlled conditions will be required to determine if the locus identified in this study for PI587886 and PI587880A represents an additional alternative allele of *Rpp1*.

Conclusions

The results of our study indicate that the resistance observed in PI 587886 and PI 587880A is most likely controlled by an alternative allele to *Rpp1*. Under the conditions of our study, the allele (*Rpp1?*) exhibited incomplete dominance, demonstrated by the presence of RB lesions in the heterozygous state (*Rpp1?*/*rpp1?*), immunity in the homozygous state of one allele (*Rpp1?*/*Rpp1?*) and TAN lesions in the homozygous state of the other allele (*rpp1?*/*rpp1?*). Additionally, unlike the adult plant field screen used in our study, all previous mapping efforts for soybean rust resistance genes have been based on seedling assays in controlled environments. In this study, we report two new sources of resistance (PI 587886 and PI 587880A) that map to the location of *Rpp1* on the basis of adult plant field evaluations under natural rust pressure. These accessions were also shown to be resistant to at least one *P. pachyrhizi* isolate found in the southern US.

This research is the product of an effort begun in 2002 by USDA-ARS scientists (Miles et al. 2006) to identify SBR resistant lines in the USDA-ARS Soybean Germplasm Collection and represents two of the most resistant accessions reported by Miles et al. (2008) from field evaluations in Paraguay. The nature of the resistance in the remaining resistant accessions from that study has yet to be determined. However, whether they are sources of new resistance genes or alternate sources of known genes, they are a valuable asset that needs to be characterized and exploited.

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