Purple seed stain (PSS) of soybean caused by Cercospora kikuchii is an important disease that reduces market grade and can affect seed germination and vigor. A single dominant gene was shown to confer PSS resistance in PI 80837. The objective of this research was to map the PSS resistance gene in PI 80837 using simple sequence repeat (SSR) markers. A cross was made between the PSS-susceptible cultivar Agripro 350 (AP 350) and PI 80837. The F2 population and parents were grown in the field, and the resistance or susceptibility of individual plants was determined by assaying the seed for infection by C. kikuchii. DNA of parent and F2 plants was extracted for SSR analysis and mapping. Segregation ratios for seed infection and for SSR markers showed that a single dominant gene conditions resistance to PSS in PI 80837. The candidate resistance gene was mapped between Sat_308 (6.6 cM) and Satt594 (11.6 cM) on molecular linkage group G. These markers may be useful in marker-assisted selection for utilizing PSS resistance from PI 80837 in a breeding program.

Purple seed stain (PSS) caused by Cercospora kikuchii (Matsumoto & Tomoyasu) M.W. Gardner reduces market grade, processing qualities, and vigor of soybean [Glycine max (L.) Merr.] seed grown worldwide (Wilcox and Abney 1973; Pathan et al. 1989; Schuh 1999). Economic losses due to PSS have been estimated at 25.25 metric tons (Doupnik 1999). Recent genetic studies with several F2 populations and F2,3 lines showed that resistance to C. kikuchii seed infection in PI 80837 is conditioned by a single dominant gene (Jackson et al. 2006).

Simple sequence repeat (SSR) markers located on all 20 soybean molecular linkage groups (MLGs) provide tools for genetic mapping and marker-assisted selection (MAS) (Gregan et al. 1999; Song et al. 2004). SSR markers have been used to map numerous qualitative and quantitative trait loci that control resistance to diseases and nematodes (see e.g., Meksem et al. 1999; Iqbal et al. 2001; Orf et al. 2004). Results reported here confirm that the PSS-resistant phenotype in PI 80837 is conferred by a single dominant gene, and it is shown by SSR mapping that this gene is located on MLG G.

Materials and Methods

F1 seed were obtained from a cross between PSS-susceptible cultivar Agripro 350 (AP 350) and PI 80837, and the F2 population was raised in the greenhouse. Field screening was done at Kibler, AR, from May to September 2003. Seed were sown approximately 8 cm apart in rows, with 1.8-m-long rows of parent seed (about 36 seed) distributed every 25–30 F2 seed. Rows were spaced 0.91 m apart, and borders were planted to help maintain a uniform environment.
Twenty-eight days after sowing, all 148 F2 plants were labeled and newly emerging leaf tissue was collected, frozen at -80 °C, lyophilized, and stored at -20 °C. About 10 days after maturity (R8) (Fehr et al. 1971), seed were harvested from the lower 65 cm of 17 randomly selected plants of both parents and all F2 plants using a single-plant thresher.

Because many seeds infected by C. kikuchii do not show PSS symptoms, infection was determined by plating seed. A random sample of 30 seed from each parent and F2 plant was surface disinfested for 4 min with 0.5% NaOCl amended with 5 drops of Tween 20/L and rinsed twice with sterile deionized water. Seed were plated onto potato dextrose agar (Difco®, YWR International cat no. 90005-300) amended after autoclaving with about 75 μg/mL streptomycin sulfate and 1 μg/mL fenpropathrin (Valent USA Corp., Walnut Creek, CA) and adjusted to pH 4.8 with lactic acid. Seed were incubated at 22–24 °C under fluorescent light with a 14-h photoperiod. After 10 days incubation, the percentage of seed infected by C. kikuchii was recorded.

Percent Cercospora seed infection of parent plants was analyzed by ANOVA (P = 0.05; JMP, SAS Institute Inc., Cary, NC). Arcsine transformation of percentage data did not affect statistical significance; therefore, analysis was done using the percentage data. Because the range of Cercospora seed infection of PI 80837 (0.0–17.5%) did not overlap that of AP 350 (25.0–40.0%), F2 plants were rated as resistant if their percent seed infection was less than 17.5%, the highest level for PI 80837. Chi-square was used to test the goodness-of-fit of F2 phenotypic data and segregation of SSR markers their percent seed infection was less than 17.5%, the highest level for PI 80837. Chi-square was used to test the goodness-of-fit of F2 phenotypic data and segregation of SSR markers in F2 plants to ratios expected for a single dominant gene.

Lyophilized leaves were pulverized in liquid N2, and DNA was extracted using acetyl trimethyl ammonium bromide (CTAB) method. In brief, 500 μL of DNA extraction buffer containing 0.140 M sorbitol, 0.22 mM Tris–HCl, 0.022 M EDTA, 0.8 M NaCl, 0.8% CTAB, and 1.0% Sarcosine was added to each tube and incubated at 65 °C for 25 min. After incubation, 300 μL of chloroform:isoamyl alcohol (24:1) was added, the solution was mixed and centrifuged at 6000 g for 25 min; and the supernatant was collected. An equal volume (1:1) of chilled 70% isopropyl alcohol was added to supernatant, and the precipitated DNA was removed with a sterile pipette tip. RNAse was used to remove RNA from each sample, the DNA was washed with 70% ethanol, dried overnight, and resuspended in TRIS/EDTA (ED). DNA concentration and quality was determined by A260 nm/A280 nm and diluted to 20 ng/μL for polymerase chain reaction (PCR). Bulked segregant analysis (Michelmore et al. 1991) was done with 160 SSR primer pairs selected from all 20 MLGs. The bulks contained an equal amount of DNA from 10 resistant F2 or 10 susceptible F2 plants, respectively.

PCR amplifications were run for 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s in a total of 25 μL containing 16.6 μL of deionized H2O, 2.5 μL of 10× buffer (Promega, Madison, WI cat. no. M1901), 2 μL of 25 mM MgCl2, 0.8 μL of 10 mM dNTPs, 0.5 μL of each forward and reverse SSR primer (5 μM), 0.1 μL of 5 U/μL Taq DNA polymerase (Promega, Madison, WI, cat. no. M2668), and 2 μL of 20 ng/μL DNA template. PCR products were separated on 3% agarose gels (SFR/AC, Amresco, Solom, OH) run in 1× TAE buffer at 90 V for 3 h. Gels were stained with ethidium bromide, and the images were recorded with a Bio-Rad Image System (Bio-Rad, Hercules, CA). Primer pairs showing polymorphisms between the 2 parents and the resistant and susceptible bulks were used to determine the genotype of all F2 plants. MAPMAKER 3.0 software was used to calculate the genetic distances between the SSR markers and the Cercospora-resistant phenotype and to draw a linkage map (Lander et al. 1987).

### Results

Cercospora seed infection of PI 80837 (6.5%) was significantly less (P = 0.05) than that of AP 350 (27.5%). Of the 148 F2 plants from AP 350 × PI 80837, 107 were rated resistant and 41 were rated susceptible to C. kikuchii seed infection.

The ratio of resistant to susceptible plants fit a 3:1 model (χ2 = 0.577, P = 0.50–0.25), indicating that resistance is conferred by a single dominant gene (Table 1).

Fifteen of 160 primer pairs tested showed polymorphisms between AP 350 and PI 80837 and were used to screen the resistant and susceptible bulks for polymorphisms. Only Sat_308, located on MLG G, cosegregated with parents and resistant and susceptible bulks; therefore, it was used to amplify DNA from 146 of the 148 F2 plants.

Sat_308 primers produced a band of approximately 310 bp in PI 80837 and the resistant bulk and a band of approximately 240 bp in AP 350 and the susceptible bulk (Figure 1). With Sat_308 primers, 42 resistant plants produced the 310-bp band and 34 susceptible plants produced the 240-bp band. Sixty-five resistant plants and 2 susceptible plants produced both bands, whereas 1 susceptible plant produced the 310-bp band and 2 resistant plants produced the 240-bp band. In this population, 43 plants had the 310-bp band, 36 plants had the 240-bp band, 15 plants were intermediate, and 13 plants were null.

<table>
<thead>
<tr>
<th>Parents/Genotype</th>
<th>No. of plants</th>
<th>Chi-square for 3:1 or 1:2:1 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI 80837 (R)</td>
<td>17</td>
<td>0.577</td>
</tr>
<tr>
<td>AP 350 × PI 80837</td>
<td>107</td>
<td>0.50–0.25</td>
</tr>
<tr>
<td>AP 350 (S)</td>
<td>0</td>
<td>0.247</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.90–0.75</td>
</tr>
</tbody>
</table>

* S = susceptible, 25.0–40.0% (mean = 27.5%); R = resistant, 0–17.5% (mean = 6.5%), seed infection (P < 0.05), N = 17.

* R = resistant, H = heterogeneous, S = susceptible.
and 67 plants had both bands. The ratio of resistant:heterozygous:susceptible genotypes fit a 1:2:1 model ($\chi^2 = 0.1658, P = 0.50–0.25$) (Table 1). Based on these data, the PSS-resistant phenotype (candidate gene Rpss1) was mapped 6.6 cM from Sat_308 on MLG G.

Sixteen SSR primer pairs, covering a 28-cM region around Sat_308, were used to screen the parents and bulks from the F2 population. Only Satt594 was polymorphic and used to amplify DNA from 146 of the 148 F2 plants. Satt594 produced a band of approximately 120 bp in PI 80837 and the resistant bulk and a band of approximately 150 bp in AP 350 and the susceptible bulk (Figure 1). With Satt594 primers, 37 resistant plants produced the 120-bp band and 36 susceptible plants produced the 150-bp band. Sixty-seven resistant plants and 3 susceptible plants produced both bands, whereas 1 susceptible plant produced the 120-bp band and 2 resistant plants produced the 150-bp band. In this population, 38 plants had the 120-bp band, 38 plants had the 150-bp band, and 70 plants had both bands. The ratio of resistant:heterozygous:susceptible genotypes fit a 1:2:1 model ($\chi^2 = 0.247, P = 0.95–0.75$) (Table 1). Based on these data, candidate resistance gene Rpss1 was found to be 11.6 cM from Satt594 and located between Satt594 and Sat_308 on MLG G (Figure 2).

Discussion

The inheritance data and the segregation ratios for SSR markers in the AP 350 × PI 80837 F2 population show clearly that a single dominant gene conditions resistance to PSS in PI 80837 soybean. Single dominant gene resistance explains the conclusion of Wilcox et al. (1975) from heritability data that resistance in this PI is under strong genetic control.

This candidate resistance gene (Rpss1) was mapped to MLG G at a location between Sat_308 and Satt594. In our (AP 350 × PI 80837) F2 mapping population, Sat_308 was mapped 18.2 cM from Satt594, whereas on the current integrated genetic linkage map for soybean, these 2 markers are separated by 9.8 cM in the same orientation (Cregan et al. 1999; Song et al. 2004). Several factors can affect the determination of genetic distance between loci including the type of genetic markers used for mapping, the specific pedigree of the mapping population, and the population size. Further development of genetic markers mapping in this region of MLG G should clarify this discrepancy in map distances.

Many disease resistance genes in soybean have been found to be closely linked in clusters (e.g., Polzin et al. 1994; Ashfield et al. 1998; Meksem et al. 1999; Iqbal et al. 2001). In our study, Rpss1 mapped to a region on MLG G that...
contained a quantitative trait loci conferring resistance to race 3 of soybean cyst nematode \((\text{rpg}1; \text{Wang et al. 2001})\). Additionally, a quantitative trait loci (QTL) conferring resistance to soybean cyst nematode (SCN) races 1, 2, 3, 5 was identified by Yue et al. (2001) 20 cM from \(R_{\text{ps}3}\), whereas a QTL for sudden death syndrome \((\text{rfs}1; \text{Meksem et al. 1999})\) was identified 27.5 cM from the gene mapped in this study. Overall, these data indicate that \(R_{\text{ps}3}\) could be part of a resistance gene cluster on the G linkage group.

The identification of 2 SSR markers linked to a candidate gene that confers resistance to PSS in PI 80837 could facilitate the future use of MAS to incorporate this candidate gene in breeding resistant lines and varieties. This could be difficult, however, because of the considerable genetic distance (6.6 cM) between \(R_{\text{ps}1}\) and the closest marker (Sat_308). More precise mapping of this gene and other disease resistance genes in this region of MLG G will require further development of SSR and other molecular markers that map to current gaps in this MLG G (Iqbal et al. 2001; Song et al. 2004).

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Polzin KM, Lohnes DG, Nickell CD, Shoemaker RC. 1994. Integration of \(R_{\text{ps}3}\), \(R_{\text{ps}2}\), and \(R_{\text{ps}1}\) into linkage group J of the soybean molecular map. J Hered. 85:300–303.


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