RFLP and Microsatellite Mapping of a Gene for Soybean Mosaic Virus Resistance

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ABSTRACT


Restriction fragment length polymorphisms (RFLPs) and microsatellites or simple sequence repeats (SSRs) were used as genetic markers to identify the chromosomal location of Rsv, a gene conferring resistance to soybean mosaic virus (SMV). An F2 population was constructed from a cross between soybean line PI 96983 as the resistant parent and cultivar Lee 68 as the susceptible parent. Twenty-five RFLP and three SSR loci, polymorphic between the parental lines, were analyzed in 107 F2 individuals. Genotypes of Rsv were determined by inoculating F2 progeny with the GI strain of SMV. Data also were collected for an additional soybean gene (w1/W1), which controls anthocyanin pigmentation in hypocotyls and flowers. Analyses of the data revealed that the SSR marker, SM176 (a soybean heat shock protein gene), and two RFLP markers, pA186 and pK644a, are closely linked to Rsv, with distances of 0.5, 1.5, and 2.1 centiMorgans, respectively. The close links between Rsv and the three markers were confirmed by marker analysis of three Williams near-isogenic lines (NILs) that carry Rsv alleles from PI 96983 and Marshall. Marker analysis also indicated that the SMV resistance gene in Buffalo is probably at the Rsv locus.

Additional keywords: Glycine max, potyvirus, stress protein.

Soybean mosaic virus (SMV) is one of the most common diseases in soybean production in the world, resulting in serious yield reduction and seed-quality deterioration (4,12). SMV is seedborne and transmitted by aphids in a nonpersistent manner. The use of genetic resistance appears to be the most effective and economical control strategy for SMV (4).

Host resistance to SMV has been identified in various soybean cultivars and plant introductions. Kihhl and Hartwig (21) found that SMV resistance in PI 96983 and Ogden was controlled by alleles at a single locus, designated Rsv and Rsv', respectively. Subsequent studies with cultivars York, Marshall, and Kwanggyo indicated that each contains a different allele of Rsv (5,8,27). Single-gene inheritance also has been reported for resistance in several other cultivars including: Rheidan (6), Suweon 97 (23), Columbia (7), AGS 129 (10), and Buffalo and HLS (3). SMV resistance in PI 486355 is controlled by two independent dominant genes, one of which appears to be at the Rsv locus (9). The resistance genes in Rheidan and Columbia are at different loci and are designated as Rsv2 and Rsv3, respectively (6,7). Lim (23) reported that the resistance gene in Suweon 97 is not at the Rsv locus, but he did not test for allelism with other loci. Previous studies also have indicated that the gene for SMV resistance is linked to genes conferring resistance to peanut mottle virus (PMV) (27) and peanut stripe virus (PStV) (10), suggesting a possible cluster of virus-resistance genes.

Identification of the chromosomal location of Rsv will be instrumental in clarifying the relationship among the various resistance sources and should facilitate the simultaneous transfer of SMV resistance with improvements in other agronomically important traits in soybean. Using RFLPs as genetic markers, virus-resistance genes have already been mapped in tomato (35) and maize (24). In soybean, Phytophthora-resistance genes have been mapped recently (14), but none of the reported virus-resistance genes have been mapped.

Short tandem repeats in DNA sequences termed microsatellites or simple sequence repeats (SSRs) have been described as an additional source of genetic markers (32). The repeated core sequences, usually two or three nucleotides in length, often vary in number and are flanked by conserved DNA sequences. Using primers complementary to flanking regions, SSR sequences can be amplified via the polymerase chain reaction (PCR) and analyzed for variation in the number of repeats. Although SSR variation has been exploited increasingly in genetic studies involving mammalian systems, plant microsatellites remain virtually unutilized.

The degree of polymorphism at three SSR loci was recently examined by Akkaya et al. (1) in 43 soybean accessions. Each locus studied identified six to eight allelic variants. The abundance of SSRs in rice was recently investigated by Wu and Tanksley (34) who found, on average, one (GA)n repeat every 225 kb and one (GT)n repeat every 480 kb.

Little is known about the application of SSRs as molecular markers in crop plants and no plant trait has been mapped by this new class of molecular markers. In the present study, we used RFLPs, SSRs, and a morphological marker (w1) to identify the chromosomal location of the SMV-resistance gene.

MATERIALS AND METHODS

Plant materials. A cross between PI 96983 and Lee 68 was made during 1988. PI 96983 contains the SMV-resistance gene Rsv (21), and cultivar Lee 68 is susceptible. PI 96983 has w1, which conditions white flowers and green hypocotyl, whereas Lee 68 has W1, which conditions purple flowers and purple hypocotyl. Three F1 plants were grown in the greenhouse during the following winter and were selfed to produce F2 seeds. F2 plants (107) were grown in the greenhouse during 1989. Seeds from each F2 plant were harvested to form F3 lines.

Seven near-isogenic lines (NILs) of cultivar Williams carrying SMV-resistance genes from various sources were obtained from R. L. Bernard. Among the NILs, L78-379 and L81-4420 possess
Rsv derived from PI 96983, whereas L84-2112, L83-529, L84-
431, and L29 carry SMV-resistance genes derived from Marshall, 
Buffalo, Raliken, and Hare, respectively. L85-2308 carries a 
gene (Rpv) for resistance to PMV derived from Dorman.

DNA probes. A set of soybean genomic DNA clones used as 
RFLP markers (20) were provided by R. C. Shoemaker, Iowa
State University, Ames. Insert DNA to be used as a hyridization 
probe was isolated from plasmids either by restriction digestion 
of the vector or by amplification with PCR. All probes with 
designations of "IaSU-pA" or "IaSU-pK" are described through-
out this paper as "pA" or "pK" for simplicity.

To generate additional DNA clones, a soybean genomic 
library was constructed. DNA from an experimental line, 
V85-5344, was completely digested with PstI, ligated with a dephosphorylated 
PstI-linearized pUC19 plasmid, and transformed into DH5α- 
competent cells (GIBCO-BRL, Gaithersburg, MD). Single-copy 
DNA clones were selected to serve as RFLP probes. In total, 
107 clones containing single-or low-copy DNA sequences were 
screened on the two parental lines.

RFLP analysis. Soybean DNA was extracted from individual 
F2 plants according to previously published procedures (28). 
A sample of approximately 8 μg of DNA was individually digested 
with one of 18 restriction enzymes. Restriction fragments were 
separated on 0.8% agarose gels according to standard electro-
phoresis procedures (28). DNA was transferred to nylon 
membrane via Southern blotting. Blots were hybridized with randomly 
primed 32P-labeled dCTP insert DNA (16). Preliminary screening 
of parental DNA identified polymorphic clones that consequently 
were used to collect RFLP data from the F2 progeny.

SSR analysis. A search was conducted in GenBank and EMBL 
sequences containing tandem di- or trinucleotide repeats with FASTA program in the GCG Sequence 
Analysis Software Package (13). SOYPRP1 (a proline-rich cell 
wall-protein gene [18] with [TAT]n), SOYHSP176 (a heat shock 
protein [HSP] 17.6 gene [26] with [AT]n), and SOYSCS14 (a 
lincomycinase gene [29] with [AT]n) were selected to be used as 
SSR markers (found also in literature citation 1). Primers 
were designed with sequences flanking the tandem repeats and 
synthesized by Operon Technologies (Alameda, CA).

SSR procedures were developed with the help of K. S. Wu. 
Briefly, a 20-μl PCR reaction contained 50 ng of genomic DNA, 
0.1 μM of each primer, 1X reaction buffer (10 mM Tris-HCl, 
PH 8.3, 50 mM KCl), 3 mM MgCl2, 1.5 U of AmpliTaq DNA 
polymerase (Perkin-Elmer Cetus, Norwalk, CT), 160 μM each of dGTP, dTTP, and dATP, 2 μM dCTP, and 1 μM [α-32P]dCTP. 
Samples were covered with 15 μl of light mineral oil and subjected to 
30 thermal cycles; denature (1 min, 94°C); annealing (2 min, 
55°C); and extension (1.5 min, 72°C) followed by a final extension 
step (5 min, 72°C), using a DNA thermal cycler (Perkin-Elmer 
Cetus).

PCR products were denatured for 3 min at 94°C and separated 
on 6% denaturing polyacrylamide gel with 8 M urea at 70 W 
of constant power, using a DNA sequencing unit (Model STS-
45, IB, Newhaven, CT). Gels were immediately covered with 
plastic wrap and exposed to X-ray film for 45 min.

Alternatively, SSR loci with divergent products were amplified 
via PCR without radioactive nucleotides (cold PCR) and 
separated on 3% NuSieve 3:1 (FMC Corporation, Chicago) 
agarose in 1X TAE (Tris-acetate, EDTA). PCR products were 
visualized by ethidium bromide staining (data not shown).

SMV reaction. The Rsv genotype of each F2 plant from the 
PI 96983 × Lee 68 cross was determined by progeny testing. 
At least 12 seeds from each F2 line were evaluated for response 
for SMV-G1 (strain G1, Va isolate). F3 seeds were planted in 
12-cm pots filled with soil/vermiculite/peat moss mixed in a 1:1:1 
ratio. Once the first trifoliolate leaves were partially expanded, 
unifoliate leaves were inoculated with SMV-G1/VA maintained 
on Lee 68 as described previously (19), except that inoculum 
contained 0.5% Celite 545 (Fisher Scientific Co., Pittsburgh, PA) 
as an abrasive. Local reactions appeared 3–4 days after inoculation.

One to two weeks after inoculation, mosaic symptoms 
developed fully in newly formed leaves, and reactions to SMV-
G1/VA in individual plants were recorded as resistant (symptom-
less) or susceptible (mosaic). Two subsequent observations were 
made at 1- or 2-wk intervals.

Based on the SMV reaction of the F2 lines, each F2 individual 
was categorized as homoygous resistant (RsvRsv), heterozygous 
(RsvRsv), or homoygous susceptible (rsrvs). A family size of 
12 affords a probability of 0.95 of detecting at least one susceptible 
plant in a population segregating 3 resistant/1 susceptible. Parents 
and a set of soybean differential cultivars also were inoculated to 
verify the efficiency of inoculation and the identity of the virus 
strain.

Anthocyanin pigmentation. Anthocyanin pigmentation was 
observed in both flowers and hypocotyls. Flower color (purple 
or white) was recorded for F2 plants and for their F3 progeny. 
Hypocotyl color (purple or green) was scored when seedlings were 9 days old. Hypocotyl and flower color, both of 
which are pleiotropic expressions of the same gene (17), were 
used to determine the w1 genotype.

Linkage analysis. The segregation ratios of Rsv, w1, and each 
molecular marker in the F2 population were tested for goodness 
of fit to a 1:2:1 genotypic ratio using Linkage-1, a Pascal computer

Fig. 1. F2 segregation pattern for the microsatellite or simple sequence repeat (SSR) marker SM176. Genomic sequences containing (AT)n repeats 
were amplified by polymerase chain reaction in the presence of 32P-labeled deoxyribonucleotides, run on a sequencing gel, and detected by autoradiography 
(described in text). Lanes 1 and 2 are parental lines PI 96983 and Lee 68, respectively; lanes 3–20 are a portion of the F2 population from the 
cross of the parental lines.
RESULTS

Probes (107) were screened initially with three restriction enzymes (HindIII, EcoRI, and DraI) to detect polymorphisms between the two parents. Among them, only 20 clones (19%) were polymorphic with at least one of the three restriction enzymes. Our preliminary linkage analysis located *Rsv* in soybean linkage group E (20). Based on this initial analysis, six additional clones were selected from linkage group E and tested with DNA digested by 15 additional enzymes to detect variation between the two parental lines. Three clones were polymorphic when *HaeIII*, *BstEII*, or *HpaII* were used, whereas the other three remained monomorphic after the use of 18 enzymes. In contrast, all three SSR markers examined were polymorphic between PI 96983 and Lee 68. Overall, segregation data for 30 genetic loci, including SMV resistance (*Rsv*), anthocyanin pigmentation (*w*), 25 RFLP loci (two DNA probes each detected two loci), and three SSR loci were collected from 107 F2 individuals. The F2 segregation pattern for the SSR locus SOYHSP176, hereafter referred to as SM176 (soybean microsatellite 176), is shown in Figure 1.

Both SMV resistance and hypocotyl color segregated as monogenic traits (Table 1) and did not deviate from the expected 1:2:1 ratio based on chi-square tests. RFLP and SSR markers segregated codominantly in the F2 population, and all provided good fits to the 1:2:1 ratio. Only those markers that are in the same linkage group with *Rsv* are shown in Table 1.

A linkage map for this group was constructed based on multiple linkage analyses in the Mapmaker 2.0 computer program. One SSR marker, SM176, and two RFLP markers, pA186 and pK644a, were closely linked to *Rsv*, with distances of 0.5, 1.5, and 2.1 cM, respectively. Anthocyanin pigmentation (*w*) was also mapped to this linkage group, with a distance of 48.8 cM from pK390. Associations among these molecular markers, *w*, and *Rsv* are shown in Figure 2.

Our previous genetic studies have indicated that SMV resistance in Marshall is controlled by an *Rsv* allele (8). A series of NILs carrying virus-resistance genes from PI 96983, Marshall, Buffalo, and other resistance sources, along with their recurrent parent, Williams, and corresponding donor parents were tested with pA186, pK644a, and SM176 (Fig. 3; Table 2) to confirm the close linkage detected by F2 segregation analysis. The close linkage between *Rsv* and pA186 was evident by comparing the banding patterns of L78-379 and L81-4420 with those of the recurrent parent, Williams, and the *Rsv* donor parent, PI 96983, (Fig. 3A, EcoRI; Table 2, all four enzymes). Moreover, the identical banding patterns of L84-2112 and its *Rsv*-donor parent, Marshall, for both pA186 (Fig. 3A; Table 2) and pK644a (Table 2, columns for RI, HIII, and DI) provide additional evidence of the close linkages of *Rsv* with these RFLP markers.

**Table 1. Segregation of soybean mosaic virus resistance (*Rsv*), anthocyanin pigmentation (*w*), and linked restriction fragment length polymorphism and simple sequence repeat markers in an F2 population from PI 96983 × Lee 68**

<table>
<thead>
<tr>
<th>Traits or markers</th>
<th>No. of F2 plants</th>
<th>Observed no.</th>
<th>$\chi^2$ (1:2:1)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rsv</em></td>
<td>104</td>
<td>26 49 29</td>
<td>0.52</td>
<td>0.77</td>
</tr>
<tr>
<td><em>w</em></td>
<td>102</td>
<td>26 46 28</td>
<td>0.98</td>
<td>0.61</td>
</tr>
<tr>
<td>SM176</td>
<td>100</td>
<td>28 48 24</td>
<td>0.48</td>
<td>0.79</td>
</tr>
<tr>
<td>pA186</td>
<td>106</td>
<td>27 49 30</td>
<td>0.77</td>
<td>0.68</td>
</tr>
<tr>
<td>pK644a</td>
<td>106</td>
<td>27 49 30</td>
<td>0.77</td>
<td>0.68</td>
</tr>
<tr>
<td>pK390</td>
<td>102</td>
<td>26 54 22</td>
<td>0.66</td>
<td>0.72</td>
</tr>
<tr>
<td>pK2</td>
<td>105</td>
<td>29 51 25</td>
<td>0.39</td>
<td>0.82</td>
</tr>
</tbody>
</table>

*Genotypes: A1A1 = PI 96983; A1A2 = heterozygous; and A2A2 = Lee 68.

![Fig. 2. A linkage map of *Rsv* (soybean mosaic virus-resistance gene), *w* (flower color), and linked restriction fragment length polymorphism and simple sequence repeat markers. The order and distances were computed by multiple linkage analysis with the Mapmaker 2.0 computer program. Distances in centiMorgans were computed from recombination frequencies with the Haldane function (22).*
DISCUSSION

The simple inheritance of resistance to SMV as well as knowledge from extensive genetic and virological studies makes SMV resistance an excellent model system for further genetic and molecular studies. The availability of molecular markers for Rsv is a prerequisite for the isolation of the virus-resistance gene via map-based cloning. Also, such molecular markers should provide plant breeders with a powerful tool for 1) screening for SMV resistance within advanced soybean breeding populations, 2) rapid transfer of SMV resistance to elite soybean cultivars, and 3) pyramiding multiple SMV-resistance genes.

Microsatellites or SSRs are ideal genetic markers in that they 1) are highly abundant, 2) appear to be evenly distributed throughout the genome (32), 3) are highly polymorphic (31), 4) can be typed rapidly via PCR, and 5) are disseminated easily among laboratories by publishing primer sequences. The few published plant SSR studies suggest that SSR markers are potentially as powerful in plant systems as they are in mammalian systems. In the present study, we identified an SSR marker closely linked to the SMV-resistance gene and were able to detect SSR variation with agarose gels electrophoresis without the use of radiochemicals. Such ease in screening for SSRs should further facilitate their use in practical plant-breeding settings.

NILs have been utilized previously as genetic material for rapid screening with RFLP markers in tomato (35) and recently in soybean (25). In our study, after linkages between Rsv and three molecular markers had been established based on F2 segregation, molecular marker analysis of the Rsv-carrying NILs provided supporting evidence for the existence of the observed linkage relationships. Furthermore, NIL testing with closely linked molecular markers supported our previous genetic studies (8) on the allelism between Rsv and the resistance gene in Marshall. The NIL data also suggest that the resistance gene in Buffalo (3) and possibly in Hardie (6), but not in Hardee, is a possible allele of Rsv in PI 96983.

The RFLP markers pA186 and pK644aa also are linked with a Phytophthora-resistance gene, Rps3 (14). Earlier studies established that linkage relationships exist between SMV resistance and resistance to other viruses including PMV in York (27) and PSTV in AGS 129 (10). Localization of PSTV resistance on the same chromosome as Rsv is contingent on establishing allelism between Rsv and the SMV-resistance gene in AGS 129. Nonetheless, it appears that disease-resistance genes for three viruses and one fungus are located on the same chromosome, possibly as a cluster of resistance genes. Studies are underway to construct a physical map in this region and to pursue cloning the Rsv gene based on its chromosomal location.

<table>
<thead>
<tr>
<th>Soybean lines</th>
<th>Virus-resist. allele×</th>
<th>SM176× (SSR)</th>
<th>pA186 (RFLP)</th>
<th>pK644aa (RFLP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Williams (R)</td>
<td>Rsv</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L81-4420 (N)</td>
<td>Rsv</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>L78-379 (N)</td>
<td>Rsv</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PI 96983 (D)</td>
<td>Rsv</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>L84-2112 (N)</td>
<td>Rsv-m</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Marshall (D)</td>
<td>Rsv-b</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L83-529 (N)</td>
<td>Rsv-b</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Buffalo (D)</td>
<td>Rpv</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L85-2308 (N)</td>
<td>Rpv</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dorman (D)</td>
<td>Rpv</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L88-8431 (N)</td>
<td>Rsv2</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Raiden (D)</td>
<td>Rsv2</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L29 (N)</td>
<td>Rsv2</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

×R = the recurrent parent (Williams) for all NILs; N = NILs; and D = donor parents of virus-resistance genes.

×Virus-resistance genes: Rsv = Rsv allele of Marshall; Rsv-m = the Rsv allele of Marshall; Rsv-b = the Rsv allele of Buffalo; Rsv2 = non-Rsv-allelic soybean mosaic virus (SMV)-resistance gene; and Rpv = peanut mottle virus (PMV)-resistance gene.

×1, 2, and 3 within each probe/enzyme combination designate different sizes of restriction fragments.

×Restriction endonucleases: RI = EcoRI; HIII = HindIII; DI = DraI; and RV = EcoRV.

×L88-8431 has a band that is different from both the recurrent parent and Raiden, the donor parent. It appears that Raiden used in this study may not be the original donor parent used in developing L88-8431.

A close linkage was detected between Rsv and SM176, a low molecular weight (LMW) HSP gene. LMW HSPs, encoded by a multigene family, are among the most abundant stress proteins identified in soybean and other plant species (11,26). During heat

Fig. 3. Allelic comparison at Rsv-linked marker loci among Williams, resistant near-isogenic lines (NILs), and their corresponding donor parents. Lane 1 is the recurrent parent Williams; lanes 2 and 3 are L81-4420 and L78-379 (both of which carry Rsv [soybean mosaic virus (SMV)-resistance gene] from the donor parent, PI 96983 [lane 4]; lanes 5 and 7 are L84-2112 and L83-529 (whose SMV-resistance donors are Marshall [lane 5] and Buffalo [lane 7], respectively); lane 9 is L85-2308 (a NIL with Rpv [peanut mottle virus-resistance gene] from Dorman [lane 9]); lanes 10 and 12 are L88-8431 and its Rsv2 (a SMV-resistance gene independent of Rsv) donor parent; lane 13 is L29, a NIL with SMV resistance from Hardee (not shown). A, Autoradiograph of the EcoRI-digested DNA samples separated on an agarose gel and probed with the restriction fragment length polymorphism marker pA186. DNA size standards (MW) are indicated as kilobase pairs. B, Autoradiograph of the polymerase chain reaction-amplified soybean DNA with the simple sequence repeat marker SM176 separated on an acrylamide sequencing gel. Two banding patterns are observed: lanes 1–6, 9, and 13 display the shorter product, and the remaining lanes show the longer fragment.
shock or other stressful conditions, the accumulation of denatured or abnormally folded proteins in cells initiates a stress response, elevating the concentration of HSPs in the cell. HSPs have been described as molecular chaperones functioning to facilitate the removal of abnormal proteins (15). In humans, increased levels of HSPs have been found in response to infection and autoimmune disease (33). The observed chromosomal relationships of Rev with genes controlling resistance to other diseases warrant further investigation of the soybean LMW HSP multigene family, which is presently underway in our laboratory.

LITERATURE CITED