Genetics

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

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ABSTRACT

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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 F_2 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 F_2 individuals. Genotypes of *Rsv* were determined by inoculating $F_{2:3}$ progeny with the G1 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. Kiihl and Hartwig (21) found that SMV resistance in PI 96983 and Ogden was controlled by alleles at a single locus, designated *Rsv* and rsv^t, 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: Raiden (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 Raiden 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 conditioning 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 F_1 plants were grown in the greenhouse during the following winter and were selfed to produce F_2 seeds. F_2 plants (107) were grown in the greenhouse during 1989. Seeds from each F_2 plant were harvested to form $F_{2:3}$ 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

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Rsv derived from PI 96983, whereas L84-2112, L83-529, L84-8431, and L29 carry SMV-resistance genes derived from Marshall, Buffalo, Raiden, and Hardee, 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 hybridization probe was isolated from plasmids either by restriction digestion of the vectors or by amplification with PCR. All probes with designations of "IaSU-pA" or "IaSU-pK" are described throughout 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 *Pst*I, ligated with a dephosphorylated *Pst*I-linearized pUC19 plasmid, and transformed into DH5-α 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 F_2 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 electrophoresis procedures (28). DNA was transferred to nylon membrane via Southern blotting. Blots were hybridized with randomly primed ^{32}P-labeled dCTP insert DNA (16). Preliminary screening of parental DNA identified polymorphic clones that consequently were used to collect RFLP data from the F_2 progeny.

SSR analysis. A search was conducted in GenBank and EMBL databases for soybean sequences containing tandem di- or tri-nucleotide repeats with FASTA program in the GCG Sequence Analysis Software Package (13). SOYPRP1 (a proline-rich cell wall-protein gene [18] with $(TAT)_{19}$), SOYHSP176 (a heat shock protein [HSP] 17.6 gene [26] with $(AT)_{15}$), and SOYSC514 (a lipoxygenase gene [29] with $(AT)_{14}$) 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, 1× reaction buffer (10mM Tris-HCl, pH 8.3, 50 mM KCl), 3 mM $MgCl_2$, 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-^{32}P]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, IBI, 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 1× TAE (Tris-acetate, EDTA). PCR products were visualized by ethidium bromide staining (data not shown).

SMV reaction. The *Rsv* genotype of each F_2 plant from the PI 96983 × Lee 68 cross was determined by progeny testing. At least 12 seeds from each $F_{2:3}$ line were evaluated for response to SMV-G1 (strain G1, Va isolate). F_3 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 (symptomless) or susceptible (mosaic). Two subsequent observations were made at 1- or 2-wk intervals.

Based on the SMV reaction of the $F_{2:3}$ lines, each F_2 individual was categorized as homozygous resistant (*RsvRsv*), heterozygous (*Rsvrsv*), or homozygous susceptible (*rsvrsv*). 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 F_2 plants and for their F_3 progeny. Hypocotyl color was observed in the same F_3 progeny used for SMV testing. 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* genotypes.

Linkage analysis. The segregation ratios of *Rsv*, *w1*, and each molecular marker in the F_2 population were tested for goodness of fit to a 1:2:1 genotypic ratio using Linkage-1, a Pascal computer

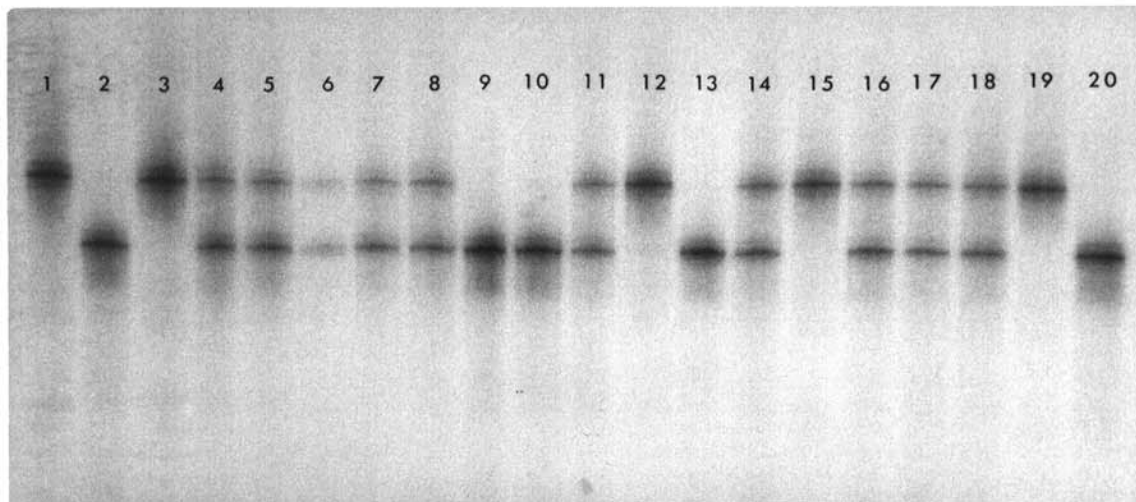

Fig. 1. F_2 segregation pattern for the microsatellite or simple sequence repeat (SSR) marker SM176. Genomic sequences containing $(AT)_n$ repeats were amplified by polymerase chain reaction in the presence of ^{32}P-labeled deoxynucleotides, run on a sequencing gel, and detected by autoradiography (described in text). Lanes 1 and 2 are parental lines PI 96983 and Lee 68, respectively; lanes 3–20 are a portion of the F_2 population from the cross of the parental lines.

program developed by Suiter et al (30). The most probable order and map distances were determined by multiple linkage analysis with the computer program Mapmaker 2.0 (22; Whitehead Institute, Cambridge, MA) at LOD = 3.0 and a maximum Haldane distance of 50 centiMorgans (cM).

RESULTS

Probes (107) were screened initially with three restriction enzymes (*Hin*dIII, *Eco*RI, and *Dra*I) 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 *Hae*III, *Bcl*I, or *Hpa*II 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 (*w1*), 25 RFLP loci (two DNA probes each detected two loci), and three SSR loci were collected from 107 F_2 individuals. The F_2 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 F_2 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 (*w1*) was also mapped to this linkage group, with a distance of 48.8 cM from pK390. Associations among these molecular markers, *w1*, 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 F_2 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, *Eco*RI; Table 2, all four enzymes). Moreover, the identical banding patterns of L84-2112 and its *Rsv*-m 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 (*w1*), and linked restriction fragment length polymorphism and simple sequence repeat markers in an F_2 population from PI 96983 × Lee 68

Traits or markers	No. of F_2 plants	Observed no.			χ^2 (1:2:1)	Probability
		A_1A_1[z]	A_1A_2	A_2A_2		
Rsv	104	26	49	29	0.52	0.77
w1	102	28	46	28	0.98	0.61
SM176	100	28	48	24	0.48	0.79
pA186	106	27	49	30	0.77	0.68
pK644a	106	27	49	30	0.77	0.68
pK390	102	26	54	22	0.66	0.72
pK2	105	29	51	25	0.39	0.82

[z] Genotypes: A_1A_1 = PI 96983; A_1A_2 = heterozygous; and A_2A_2 = Lee 68.

The closely linked SSR marker, SM176, is not polymorphic among Williams, PI 96983, or Marshall (Fig. 3B, lanes 1, 4, and 6); thus, comparison of the corresponding NILs is not informative. However, Williams and Buffalo are polymorphic for SM176 (Fig. 3B, lanes 1 and 8), and the identical banding patterns of the latter with NIL L83-529 provides evidence for the possible linkage of the SMV-resistance gene in Buffalo with SM176 (Fig. 3B, lanes 7 and 8). This linkage is further supported by a similar observation with RFLP marker pA186 and the enzyme *Eco*RV (Table 2). Allelism between the SMV-resistance gene in Buffalo and *Rsv* had not been established previously (3). This observation indicates that the SMV resistance of Buffalo also may be controlled by an *Rsv* allele. No analogous association was found with L85-2308, L88-8431, and L29 (Fig. 3; Table 2), which carry virus-resistance genes other than *Rsv*.

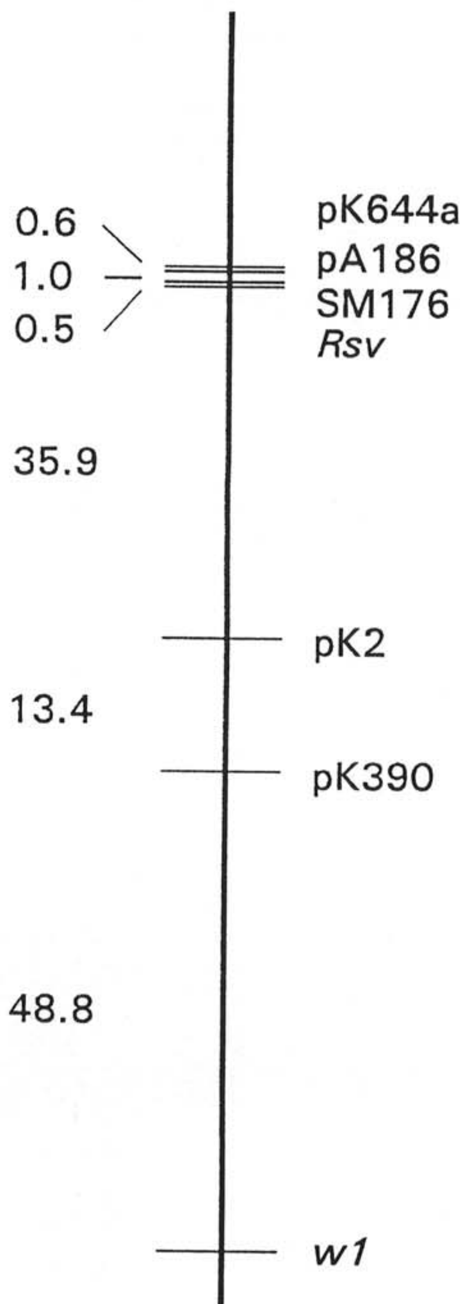

Fig. 2. A linkage map of *Rsv* (soybean mosaic virus-resistance gene), *w1* (flower color), and linked restriction fragment length polymorphism and simple sequence repeat markers. The order and distances were computed by multiple linkage analysis with the Mapmaker 2.0 computer program. Distances in centiMorgans 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 gel 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 F_2 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 Raiden (6), but not in Hardee, is a possible allele of *Rsv* in PI 96983.

The RFLP markers pA186 and pK644a 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.

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

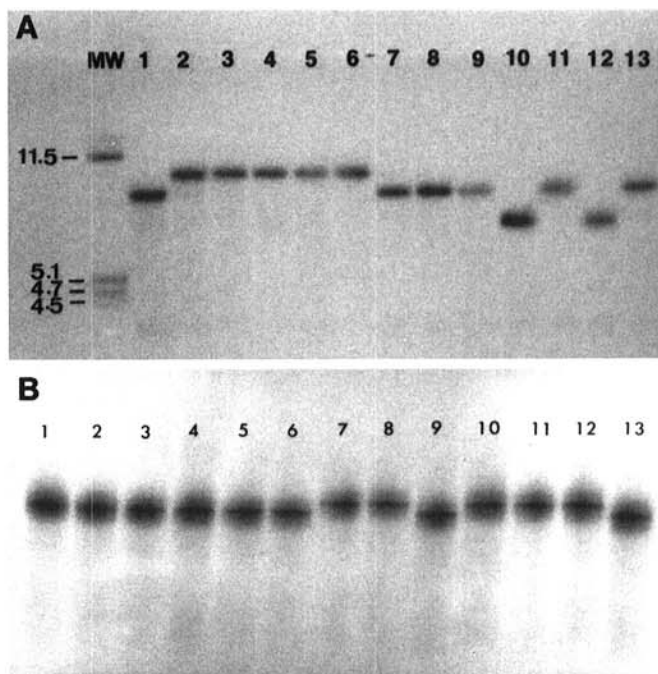

Fig. 3. Allelic comparison at *Rsv*-linked marker loci among Williams, resistant near-isogenic lines (NILs), and their corresponding donor parents. Lane 1 is the recurrent parent Williams; lanes 2 and 3 are L81-4420 and L78-379 (both of which carry *Rsv* [soybean mosaic virus (SMV)-resistance gene] from the donor parent, PI 96983 [lane 4]); lanes 5 and 7 are L84-2112 and L83-529 (whose SMV-resistance donors are Marshall [lane 6] and Buffalo [lane 8], respectively); lane 9 is L85-2308 (a NIL with *Rpv* [peanut mottle virus-resistance gene] from Dorman [lane 10]); lanes 11 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 *Eco*RI-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.

TABLE 2. Molecular marker phenotypes of the recurrent parent Williams, various donor lines, and their corresponding near-isogenic lines (NILs) for linked simple sequence repeats (SSR) and restriction fragment length polymorphisms (RFLPs)

Soybean lines[v]	Virus-resist. allele[w]	SM176[x] (SSR)	pA186 (RFLP)[y]				pK644a (RFLP)			
			RI	HIII	DI	RV	RI	HIII	DI	RV
Williams (R)		1	1	1	1	1	1	1	1	1
L81-4420 (N)	*Rsv*	1	2	2	2	2	1	1	1	1
L78-379 (N)	*Rsv*	1	2	2	2	2	1	1	1	1
PI 96983 (D)	*Rsv*	1	2	2	2	2	1	1	1	1
L84-2112 (N)	*Rsv*-m	1	2	2	2	2	2	2	2	1
Marshall (D)	*Rsv*-m	1	2	2	2	2	2	2	2	1
L83-529 (N)	*Rsv*-b	2	1	1	1	2	1	1	1	1
Buffalo (D)	*Rsv*-b	2	1	1	1	2	1	1	1	1
L85-2308 (N)	*Rpv*	1	1	1	1	1	1	1	1	1
Dorman (D)	*Rpv*	2	3	1	1	1	1	1	1	1
L88-8431 (N)	Rsv_2	2	1	1	1	2	1	1	1	1
Raiden (D)	Rsv_2	2	3	3	1	1[z]	1	1	1	1
L29 (N)	$Rsv_?$	1	1	1	1	1	1	1	1	1

[v] R = the recurrent parent (Williams) for all NILs; N = NILs; and D = donor parents of virus-resistance genes.
[w] Virus-resistance genes: *Rsv*-m = the *Rsv* allele of Marshall; *Rsv*-b = the *Rsv* allele of Buffalo; Rsv_2 and $Rsv_?$ = non–*Rsv*-allelic soybean mosaic virus (SMV)-resistance genes; and *Rpv* = peanut mottle virus (PMV)-resistance gene.
[x] 1, 2, and 3 within each probe/enzyme combination designate different sizes of restriction fragments.
[y] Restriction endonucleases: RI = *Eco*RI; HIII = *Hin*dIII; DI = *Dra*I; and RV = *Eco*RV.
[z] 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.

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 chaperons 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 *Rsv* with genes controlling resistance to other diseases warrant further investigation of the soybean LMW HSP multigene family, which is presently underway in our laboratory.

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