

**MOLECULAR GENETIC ANALYSIS OF
HOST RESISTANCE TO SOYBEAN MOSAIC VIRUS**

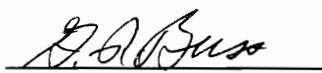
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(ABSTRACT)

Soybean mosaic virus (SMV), a potyvirus detected worldwide, can cause serious diseases in soybean (*Glycine max* L. Merr.). Host resistance to SMV conferred by a single dominant gene, Rsv1, was studied as a model to gain insights of plant virus resistance genes, and to facilitate the breeding of resistant cultivars. DNA restriction fragment length polymorphisms (RFLPs) and microsatellites (or simple sequence repeats, SSRs) were used as genetic markers to identify the chromosomal location of Rsv1 in a cross between PI 96983 (resistant) and a susceptible cultivar. Twenty five RFLP and three SSR loci polymorphic between the parental lines were analyzed in 107 F₂ individuals. Genotypes of Rsv1 were determined by inoculating F_{2:3} progeny with SMV-G1. Genetic analysis revealed that one SSR (HSP176L) and two RFLP (pA186 and pK644a) markers are closely linked to Rsv1, with a distance of 0.5, 1.5, and 2.1 cM, respectively. The tight linkages of the three markers to Rsv1 were confirmed by SSR and RFLP analysis of three near-isogenic lines (NILs) of Rsv1 derived from PI 96983 or Marshall.

The three Rsv1-linked markers were then used to screen 67 diverse soybean types. These marker loci showed a remarkably high level of polymorphism, indicating a possible association between disease resistance and rapid sequence divergence. At

each Rsv1-linked marker locus, one SSR allele or RFLP haplotype is highly correlated with SMV resistance. These resistance markers, especially the SSR allele at HSP176L which can be detected by the polymerase chain reaction (PCR), may be useful for germplasm screening. The grouping of the 67 accessions according to their Rsv1-linked multilocus marker haplotypes agrees with available pedigree information. A set of differential cultivars known to contain Rsv1 clustered into putative Rsv1-carrying groups. Based on molecular marker analysis and previous inheritance studies, 37 of the 45 resistance accessions probably derive their resistance from Rsv1. The remaining eight accessions include Columbia (Rsv3), and the other potentially diverse resistance sources.

A heat shock protein (HSP) multigene family, HSP176L included, was analyzed for its positional proximity to the Rsv1 gene cluster. A technique termed amplified sequence length polymorphism (ASLP) was developed to convert known DNA sequences to PCR-based genetic markers. Among six pairs of HSP primers used, two (HSP175E and 185C) detected ASLPs between the parents, and segregated in the F₂ population with a size of 174. HSP175E was found to be closely-linked (0.7 cM) to HSP176L, both of which are Class I small HSP genes. HSP185C, however, was mapped to a different linkage group, suggesting that it may belong to another family. ADR11, a member of auxin down-regulated (ADR) multigene family, is known to be linked to HSP173B, also a Class I gene but not mappable in this population. ASLP analysis of ADR11 in a set of Rsv1 NILs indicates that it is linked to Rsv1, and ADR11 co-segregates with HSP175E in the F₂ population. Thus, the Class I small HSP multigene family including HSP176L, 175E, and 173B, and possibly a family of ADR genes, is located near the Rsv1 resistance gene cluster.

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CHAPTER I.

Introduction

Viruses cause plant diseases ranging from latent infection to death, the most common symptoms being mosaics or mottles. Of various plant pathogens, viruses are second only to fungi with respect to the disease loss they cause (Matthews 1991). Breeding cultivars with host resistance is the most desirable, and sometimes the only effective way of virus control. Knowledge of the inheritance of virus resistance and of the host-pathogen interactions is essential to the development of durable virus-resistant cultivars. Yet we know little about the molecular mechanism of host resistance, and no plant virus resistance gene has been cloned (Wilson, 1993).

Soybean Mosaic Virus

Soybeans are susceptible to at least 111 viruses or virus strains, which belong to 10 of the 27 plant virus taxonomic groups (Demski et al., 1989, Ford et al., 1989). Viral infection in soybean may induce stunting, rugosity, mosaic or mottle patterns, yellowing or reddening of foliage, and necrosis. Twenty viruses that are known to occur naturally in soybeans cause significant economic damage. The potyvirus group includes the largest number of soybean viruses, among which soybean mosaic, peanut mottle, peanut stripe, and bean yellow mosaic viruses are economically important in soybean (Demski et al., 1989).

Soybean mosaic virus (SMV) is a potyvirus that causes one of the most prevalent soybean viral diseases worldwide. The disease symptoms caused by SMV on soybeans range from mild mosaic to lethal necrosis (Ross, 1969; Tu and Buzzell, 1987; and Buss et al., 1989a). Yields may be reduced by 50% in any one field when

infected by SMV, which also causes seed coat mottling and results in lower grade classification at marketing (Demski and Kuhn, 1989). We have observed yield losses at 14% in natural infestations in Virginia (G. R. Buss, unpublished data). The primary cause for the worldwide distribution of SMV is its seed transmission (Bowers and Goodman, 1991). SMV is also transmitted by aphids in a non-persistent manner (Demski and Kuhn, 1989).

Cho and Goodman (1979) classified 98 SMV isolates into seven strain groups (G1-G7) based on the reactions they cause (resistance, necrosis, or mosaic) in a set of differential soybean cultivars including two susceptible and six resistant. Jayaram et al. (1992) determined the complete 9588-nucleotide sequences of two SMV strains, and predicted that the positive ssRNA genome encodes a polyprotein of 3066 amino acids, which is presumably processed to produce nine mature proteins. Differences in amino acid sequences of these proteins were found between two SMV strains, G2 and G7, which elicit different responses in soybean line PI 96983.

Inheritance studies found that host resistance to SMV in PI 96983 is controlled by a single dominant resistance gene designated as Rsv (renamed later as Rsv1), and in the cultivar Ogden by a recessive allele (rsv') (Kiihl and Hartwig, 1979). Roane et al. (1983) demonstrated that a single dominant gene in York conditions resistance to SMV strain G1. Buss et al. (1989b) also reported that the resistant cultivars Marshall and Kwanggyo carry single allelic resistance genes. In an allelism test of the genes in PI 96983, Ogden, York, Marshall, and Kwanggyo, Chen et al. (1991) concluded that resistance in each cultivar is controlled by a single dominant gene, and

that the genes in these cultivars are alleles at a common locus, Rsv1.

Buzzel and Tu (1984) reported that SMV resistance in Raiden is controlled by a single gene at a locus different from Rsv1, designated as Rsv2. They (1989) also proposed that a dominant resistance gene at a third locus (Rsv3) produces a necrotic reaction to SMV in Columbia and its derivatives. Lim (1985) found that PI 483084 and PI 486355 each had single non-allelic genes, both of which were not at the Rsv1 locus. Bowers et al. (1992) reported that the single dominant genes in Buffalo and the line HLS are located at two different loci. Using an SMV strain different from that of Lim (1985), Chen et al. (1993) concluded that resistance in PI 486355 is controlled by two independent dominant genes, one of which is allelic to Rsv. No gene symbol has been assigned to the resistance genes in PI 483084 and HLS, and the non-Rsv allelic gene in PI 486385, since their allelic relationships with Rsv₂ and Rsv₃ have not been determined.

Molecular Mechanisms of Plant Virus Resistance

Fraser (1987) classified plant virus resistance into three types: non-host resistance which separates the majority of plant species from the host range of a virus, host resistance that is constitutive and occurs among cultivars of a host species, and induced resistance on susceptible plants by a prior inoculation. Whether a virus can infect and replicate in protoplasts was suggested as the distinction between a host ("infectible") and non-host ("immune") species (Matthews, 1991).

Host or cultivar resistance is the most important type of virus resistance to

plant breeders. A typical of host resistance gene functions by targeting an essential step of virus replication. For instance, the tomato Tm-1 gene confers resistance to tobacco mosaic virus (TMV) by disrupting the virus replication via virus-coded replicase (Watanabe et al., 1987; Meshi et al., 1988). A single dominant gene controlling resistance to cowpea mosaic virus in the cowpea cultivar 'Arlington' encodes a protease inhibitor which terminates the processing of virus proteins (Ponz et al., 1988). Nonetheless, many host resistance genes do allow virus replication in a small number of initially infected cells, which subsequently elicit a hypersensitive reaction and restrict further spread. Examples of hypersensitive resistance genes include the tomato Tm-2 and Tm-2² genes (Meshi et al., 1989) and the N' gene in Nicotiana glutinosa (Culver and Dawson, 1991).

The distinction between host and non-host resistance, and between different levels of host resistance are provisional due to a lack of molecular biology of plant-virus interactions. The Rx gene in the potato line USDA 41956 confers "extreme resistance" or "immunity" to all except one strain of potato virus X, in both whole plants or protoplasts (Cockerham, 1970). A recent study, however, indicated that Rx-mediated resistance is induced when the virus coat protein is produced in the infected cells (Kohm et al., 1993). Further studies on resistance genes at the molecular level are needed to uncover the mechanism of resistance, and to provide more effective means of breeding for high and durable resistance.

The potyvirus group is the largest and economically most important group of plant viruses (Matthews, 1991). In addition to SMV resistance, a number of potyvirus

resistance genes have been identified for bean common mosaic (I, Ali, 1950), maize dwarf mosaic (Mdm1, McMullen and Louie, 1989; Rmd1, Roane et al., 1989), and potato Y (Ry, Cockerham, 1970) viruses. Mansky et al. (1992) studied that effect of protein extract from a resistant cultivar, Davis, on the *in vitro* translation profile of SMV G2 strain, and found no evidence the involvement of proteinase inhibitor in resistance. Rsv1-mediated resistance may involve the inhibition of virus replication, since no virus was recovered from inoculated leaves of resistant soybean cultivars following SMV inoculation (Chen et al., 1991; S. A. Tolin, personal comm.). A variety of resistance reactions ranging from hypersensitivity, systemic necrosis, to symptomless resistance has been reported for potyviruses in different crops (Matthews, 1991). Studies of tobacco vein mottling virus in tobacco (Gibb et al., 1989), and MDMV in maize (Law et al., 1989), indicate that these potyviruses can replicate locally in the inoculated leaves of the resistant cultivars studied, and the resistance may rely on the control of systemic spread.

A non-conventional type of virus resistance, termed coat-protein mediated protection (CPMP), was developed in the mid-1980s by introducing a TMV viral coat protein gene into the tobacco genome (Powell-Abel et al., 1986), and was subsequently reported for over 20 viruses in at least 10 groups (Wilson, 1993). The scope of CPMP was broadened such that a new term, pathogen-derived resistance (PDR), is used to describe resistance derived from antisense RNA, ribozymes, satellites, defective interfering molecules, and non-structural sequences (Hull and Davies, 1992). Nonetheless, the use of host resistance genes that occur naturally is

the more desirable and durable in the long term than PDR because 1) host resistance genes do not have the possible risks of PDR, eg. in producing recombinant viruses (Schoelz and Wintermantel, 1993); 2) PDR is limited to positive stranded RNA viruses with the exception of a few ambisense RNA viruses; 3) there is considerable difficulty in obtaining PDR in monocots, and none is yet available (Wilson, 1993); and 4) with many different host resistance mechanisms existing in germ plasm collections, it is possible to produce stable resistance by pyramiding and diversification.

Molecular Breeding Schemes for Host Resistance

Despite their obvious benefits, the use of host virus resistance genes in breeding is limited by the lack of genetic knowledge of the available resistance sources, and the time required for backcrossing into cultivars. Pyramiding multiple resistance genes into the same cultivar, for instance, can be accomplished only if these genes are not allelic. Also, selecting for two or more resistance genes in the same plants is impossible unless each of the genes can be recognized individually by strain-specific responses or by other means. The diversification of resistance genes by breeding requires a knowledge of divergence among resistance genes. In many cases, resistance sources are found only in wild germ plasms in which resistance genes are often linked to undesirable traits.

The center stage of a breeding program is the selection of desirable individuals, usually based on their phenotypes. Molecular markers that detect

variation at the DNA level allow direct selection for the genotypes of the individuals. Numerous types of molecular markers are now available, among which the most important types are restriction fragment length polymorphism (RFLP) (Botstein et al., 1980; Saghai Maroof et al., 1984), simple sequence repeat (SSR) or microsatellites (Weber, 1990; Akkaya et al., 1992), and randomly amplified DNA polymorphism (RAPD) (Williams et al., 1990; Martin et al., 1991).

The most common molecular breeding schemes involve the use of closely-linked markers in selecting desirable individuals from a cross or backcross (Tanksley et al., 1989). Other types of application include marker-based pedigree analysis (Shoemaker et al., 1992) and the assessment of divergence of resistance genes. Host genes controlling resistance to different viruses, even to different pathogens, are frequently found as clusters (Kesseli et al., 1993). The close linkages pose a difficulty in recombining multiple resistance genes of the same cluster from different sources, each of which may be resistant to one pathogen but not to the others, in individual breeding programs. The close linkages between resistance genes, on the other hand, makes it possible to construct "resistance gene blocks" in which multiple resistance genes from various sources are recombined into a small chromosomal segment, and available for backcrossing and other breeding purposes.

The ultimate stage of molecular breeding involves the cloning of resistance genes, and the transfer of resistance by producing transgenic plants. This type of breeding can make use of resistance sources from wild germ plasms or even across different species. No plant virus resistance gene has been cloned to date, but several

groups are closing in by transposon tagging and positional cloning. The cloning of the maize Hm1 gene for race-specific resistance to Cochliobolus carbonum was accomplished by transposon tagging (Johal and Briggs, 1992). A tomato resistance gene, Pto, was recently isolated by positional cloning (Martin et al., 1993), proving that positional cloning is a feasible approach for cloning resistance genes.

Objectives of This Study

Resistance breeding is the only practical method of protecting soybeans from potential SMV epidemics that have caused severe losses in other countries. SMV resistance has been one of the important aspects of soybean breeding programs in the United States and over the world. At Virginia Polytechnic Institute and State University, numerous studies have been conducted on the inheritance of strain-specific host reactions (eg. Buss et al., 1985, 1989a; Chen et al., 1991, 1993), and several SMV resistant soybean cultivars have been released (eg. Buss et al., 1987, 1988). Nonetheless, none of the SMV resistance genes including Rsv1 has been mapped. The long term goal of this study is to develop a molecular breeding approach for SMV resistance.

The specific objectives of this study were:

- i) to determine the chromosomal location of the Rsv1 gene with molecular markers, particularly RFLP and microsatellite markers;
- ii) to explore a potential use of molecular markers in assessing the level of divergence, and in classifying SMV resistance sources;

- iii) to saturate the Rsv1 region with closely-linked multigene families, and to investigate their possible connection with SMV resistance.

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CHAPTER II.

**RFLP and Microsatellite Mapping of a Gene
for Soybean Mosaic Virus Resistance¹**

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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 Rsv1, a gene conferring resistance to soybean mosaic virus (SMV). An F₂ population was constructed from a cross between the soybean line PI 96983 as the resistant parent and the 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 Rsv1 were determined by inoculating F_{2,3} progeny with the G1 strain of SMV. Data were also collected for an additional soybean gene (w1), which controls anthocyanin pigmentation in hypocotyls and flowers. Analyses of the data revealed that the SSR marker, HSP176L (a soybean heat shock protein gene), and two RFLP markers, pA186 and pK644a, are closely linked to Rsv1, with distances of 0.5 cM, 1.5 cM and 2.1 cM, respectively. The close linkages between Rsv1, and the three markers were confirmed by marker analysis of three Williams near-isogenic lines (NILs) which carry Rsv1 alleles from PI 96983 and Marshall. Marker analysis also indicated that the SMV resistance gene in Buffalo is probably at the Rsv1 locus.

Additional Keywords: Glycine max, potyvirus, stress protein.

INTRODUCTION

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 (Buss et al. 1989a, Demski and Kuhn 1989). SMV is seed-borne and transmitted by aphids in a non-persistent manner. The use of genetic resistance appears to be the only effective and economical control strategy for SMV (Buss et al. 1989a).

Host resistance to SMV has been identified in various soybean cultivars and plant introductions. Kiihl and Hartwig (1979) found that SMV resistance in PI 96983 and Ogden was controlled by alleles at a single locus, which were designated Rsv1 and rsv¹, respectively. Subsequent studies with the cultivars York, Marshall, and Kwanggyo indicated that each contains a different allele of Rsv1 (Buss et al. 1989b, Chen et al. 1991, Roane et al. 1983). Single gene inheritance has also been reported for resistance in several other cultivars including: Raiden (Buzzell and Tu 1984), Suweon 97 (Lim 1985), Columbia (Buzzell and Tu 1989), AGS 129 (Choi et al. 1989), Buffalo and HLS (Bowers et al. 1992). SMV resistance in PI 486355 is controlled by two independent dominant genes, one of which appears to be at the Rsv1 locus (Chen et al. 1993). The resistance genes in Raiden and Columbia are at different loci and are designated as Rsv2 and Rsv3, respectively (Buzzell and Tu 1984, 1989). Lim (1985) reported that the resistance gene in Suweon 97 is not at the Rsv1 locus but he did not test for allelism with other loci. Previous studies have indicated that

the gene for SMV resistance is linked to genes conditioning resistance to peanut mottle virus (PMV) (Roane et al. 1983) and peanut stripe virus [PStV, (Choi et al. 1989)], suggesting a possible cluster of virus resistance genes.

Using RFLPs as genetic markers, virus resistance genes have already been mapped in tomato (Young et al. 1988) and maize (McMullen and Louie 1989). In soybean, Phytophthora resistance genes have been mapped recently (Diers et al. 1992), but none of the reported virus resistance genes have been mapped. Identifying the chromosomal location of Rsv1 via genetic mapping is an effective way of characterizing the Rsv1 gene. Genetic identification of Rsv1 will be instrumental in clarifying the relationship among the various resistance sources, and should facilitate the simultaneous transfer of SMV resistance with improvements of other agronomically important traits in soybean.

Short tandem repeats in DNA sequences termed microsatellites or simple sequence repeats (SSRs) have been described as an additional source of genetic markers (Weber 1990). The repeated core sequences, usually 2 or 3 nucleotides long, 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 the repeats. While SSR variation has been increasingly exploited in mammalian systems, plant microsatellites remain virtually unutilized.

The degree of polymorphism at three SSR loci was examined by Akkaya et al (1992) 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 (1993) 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 using 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 in 1988. PI 96983 contains the SMV resistance gene Rsv1 (Kiihl and Hartwig 1979), and the 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 the following winter and selfed to produce F₂ seeds. One hundred and seven F₂ plants were grown in the greenhouse in 1989. Seeds from each F₂ plant were harvested to form F_{2,3} lines.

Seven near-isogenic lines (NILs) of the cultivar Williams carrying SMV resistance genes from various sources were obtained from Dr. R.L. Bernard. Among the NILs, L78-379 and L81-4420 possess Rsv1 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 PMV resistance gene (Rpv) derived from Dorman.

DNA probes

A set of soybean genomic DNA clones used as RFLP markers (Keim et al. 1990) were kindly provided by Dr. R. C. Shoemaker at Iowa State University. Insert DNA to be used as hybridization probes were 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 PstI, ligated with a dephosphorylated PstI-linearized pUC19 plasmid, and transformed into DH5- α competent cells (GIBCO-BRL). Single-copy DNA clones were selected to serve as RFLP probes.

RFLP analysis

Soybean DNA was extracted from individual F₂ plants according to previously published procedures (Saghai-Maroo et al. 1984). Approximately eight micrograms of DNA were individually digested with one of 18 restriction enzymes. Restriction fragments were separated on 0.8% agarose gels according to standard electrophoresis procedures. DNA was then transferred to nylon membrane via Southern blotting.

Blots were hybridized with randomly primed ^{32}P -dCTP labeled insert DNA (Feinberg and Vogelstein 1983). Preliminary screening of parental DNA identified polymorphic clones which were consequently used to collect RFLP data from the F_2 progeny.

SSR analysis

Soybean sequences containing tandem di- or tri-nucleotide repeats were searched in GenBank and EMBL databases using FASTA program in the GCG Sequence Analysis Software Package (Devereux et al. 1984). SOYPRP1 [a proline-rich cell wall protein gene (Hong et al, 1987) with $(\text{TAT})_{19}$], SOYHSP176 or HSP176L [a heat shock protein 17.6 gene (Nagao et al. 1985) with $(\text{AT})_{15}$], and SOYSC514 [a lipoxygenase gene (Shibata et al. 1991) with $(\text{AT})_{14}$] were selected among them as SSR markers (see also Akkaya et al. 1992). Primers were designed using sequences flanking the tandem repeats and synthesized by Operon Technologies (Alameda, CA). The primer sequences for HSP176L are 5'TTTTG TTAAG TTAAG TTAAG TTAAG GTACT GTGG (forward primer), and 5'TATTT TAGCA GTTTT AGATG ATTCG (reverse primer).

SSR procedures were developed with the help of Dr. K. S. Wu. Briefly, a 20 μl PCR reaction contains 50 ng of genomic DNA, 0.1 μM of each primer, 1 X reaction buffer (10mM Tris-HCl, pH 8.3, 50 mM KCl); 3 mM MgCl_2 , 1.5 units of AmpliTaq DNA Polymerase (Perkin- Elmer Cetus), 160 μM each of dGTP, dTTP, dATP and 2 μM of dCTP, 1 μM of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$. Samples were covered with 15 μl of light mineral oil and subjected to 30 thermal cycles; denature (1 min, 94 $^\circ\text{C}$);

annealing (2 min, 55 °C); extension (1.5 min, 72 °C) followed by a final extension step of 72 °C for 5 min 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 8M Urea at 70 Watts constant power using a DNA sequencing unit (Model STS-45, IBI). Gels were immediately covered with plastic wrap and exposed to X-ray film for 45 minutes.

Alternatively, SSR loci with divergent products were amplified via PCR without radioactive nucleotides (cold PCR) and separated on 3% Nusieve 3:1 (FMC) agarose in 1X TAE. PCR products were visualized by ethidium bromide staining (results not shown).

SMV reaction

The Rsv1 genotype of each F₂ plant from the PI 96983 X Lee 68 cross was determined by progeny testing. At least twelve seeds from each F_{2,3} line were evaluated for response to SMV-G1 (Strain G1, Va isolate). F₃ seeds were planted in 5 inch pots filled with soil:vermiculite:peat moss mixed in a 1:1:1 ratio. Once the first trifoliolate leaves were partially expanded, unifoliolate leaves were inoculated with SMV-G1 maintained on Lee 68 as described previously (Hunst and Tolin 1982) except that inoculum contained 0.5% Celite 545 (Fisher Scientific) as an abrasive. Local reactions appeared 3-4 days after inoculation. Three weeks after inoculation, mosaic symptoms developed fully in newly formed leaves, and reactions to SMV-G1 in individual plants were recorded as resistant (symptomless) or susceptible (mosaic).

Two subsequent observations were made at one or two week intervals.

Based on the SMV reaction of the $F_{2,3}$ lines, each F_2 individual was categorized as homozygous resistant (Rsv1Rsv1), heterozygous (Rsv1rsv) 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 were also 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 (Hartwig and Hinson 1962), were used to determine the w1 genotypes.

Linkage analysis

The segregation ratios of Rsv1, 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 program developed by Suiter et al. (1983). The most probable order and map distances were determined by multiple linkage analysis using the computer program Mapmaker 2.0 (Lander et al. 1987, Whitehead Institute,

Cambridge, MA) at LOD=3.0 and a maximum Haldane distance of 50 centiMorgans (cM).

RESULTS

One hundred and seven DNA probes were initially screened using three restriction enzymes (HindIII, EcoRI and DraI) in order to detect polymorphisms between the two parents. Among them, only 20 clones (19%) were found polymorphic with at least one of the three restriction enzymes. Our preliminary linkage analysis located Rsv1 to the soybean linkage group E (Keim et al. 1990). Based on this initial analysis, six additional clones were selected from the linkage group E and tested with DNA digested by 15 additional enzymes to detect variation between the two parental lines. Three were found to be polymorphic using HaeIII, BclI or HpaII, 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. Thus, overall, segregation data for 30 genetic loci, including SMV resistance (Rsv1), anthocyanin pigmentation (w1), 25 RFLP loci (two DNA probes each detected two loci), and three SSR loci were collected from 107 F₂ individuals. F₂ segregation pattern for the SSR locus HSP176L 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 which are in the same linkage group with Rsv1 are shown in Table 1.

A linkage map for this group was constructed based on multiple linkage analyses in the MAPMAKER 2.0 program. Two RFLP markers, pK644a and pA186, and one SSR marker, HSP176L, were tightly linked to Rsv1 with a distance of 2.1, 1.5, and 0.5 cM, respectively. Anthocyanin pigmentation (w1) is also mapped to this linkage group with a distance of 48.8 cM from pK390. Associations among these molecular markers, w1, and Rsv1 are shown in Figure 2.

Our previous genetic studies have indicated that SMV resistance in Marshall is controlled by an Rsv1 allele (Chen et al. 1991). 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 HSP176L (Fig. 3 and Table 2) to confirm the close linkage detected from F₂ segregation analysis. The close linkage between Rsv1 and pA186 was evident by comparing the banding patterns of L78-379 and L81-4420 with those of the recurrent parent, Williams, and the Rsv1 donor parent, PI 96983, (Fig. 3A for EcoRI and Table 2 for all 4 enzymes). Moreover, the identical banding patterns of L84-2112 and its Rsv1-m donor parent, Marshall, for both pA186 (Fig. 3A and Table 2) and pK644a (Table 2 columns for RI, HIII and DI) provide additional evidence of the close linkages of Rsv1 with these RFLP markers.

The closely linked SSR marker, HSP176L, is not polymorphic among Williams, PI 96983 or Marshall (Fig. 3B, lanes 1, 4 & 6), thus, comparison of the corresponding

NILs is not informative. However, Williams and Buffalo are polymorphic for HSP176L (Fig. 3B, lanes 1 & 8) and the identical banding patterns of the latter with the NIL, L83-529, provides evidence for the possible linkage of the SMV resistance gene in Buffalo with HSP176L (Fig. 3B, lanes 7 & 8). This linkage is further supported by a similar observation using the RFLP marker pA186 and the enzyme EcoRV (Table 2). Allelism between the SMV resistance gene in Buffalo and Rsv1 had not been established previously (Bowers et al. 1992). The above observation indicates that the SMV resistance of Buffalo may also be controlled by an Rsv1 allele. No analogous association was found with L85-2308, L88-8431 and L29 (Fig. 3 and Table 2), which carry virus resistance genes other than Rsv1.

DISCUSSION

The simple inheritance of resistance to SMV as well as knowledge from extensive inheritance and virological studies makes SMV resistance an excellent model system for further genetic and molecular studies. The availability of molecular markers for Rsv1 is a prerequisite for the isolation of the virus resistance gene via map-based cloning. Also, molecular markers for Rsv1 should provide plant breeders with a powerful tool for i) screening for SMV resistance within advanced soybean breeding populations, ii) rapid transfer of SMV resistance to elite soybean cultivars and iii) pyramiding multiple SMV resistance genes.

Microsatellites or SSRs are ideal genetic markers in that they i) are highly

abundant, ii) appear to be evenly distributed throughout the genome (Weber 1990), iii) are highly polymorphic (Tautz 1989), iv) can be typed rapidly via PCR and v) easily disseminated 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 using 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 previously been utilized as genetic material for rapid screening of potential RFLP markers in tomato by Young et al. (1988) and recently in soybean by Muehlbauer et al. (1991). In our study, after linkages between Rsv1 and three molecular markers had been established based on F₂ segregation, molecular marker analysis of the Rsv1-carrying NILs provided supporting evidence for the existence of the observed linkage relationships. Furthermore, NIL testing using closely-linked molecular markers supported our previous genetic studies (Chen et al. 1991) on the allelism between Rsv1 and the resistance gene in Marshall. The NIL data also suggest that resistance genes in Buffalo (Bowers et al. 1992), and probably in Raiden, but not that in Hardee, are alleles of Rsv1 in PI 96983.

The RFLP markers, pA186 and pK644a, are also linked with a Phytophthora resistance gene, Rps3 (Diers et al. 1992). Earlier studies have established that linkage relationships exist between SMV resistance and resistance to other viruses

including PMV in York (Roane et al. 1983) and PStV in AGS 129 (Choi et al. 1989). Localization of PStV resistance on the same chromosome as Rsv1 is contingent on establishing allelism between Rsv1 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 ultimately clone Rsv1 gene based on its chromosomal location.

A close linkage was detected between Rsv1 and HSP176L, a low molecular weight (LMW) heat shock protein (HSP) gene. LMW HSPs, encoded by a multigene family, are among the most abundant stress proteins identified in soybean and other plant species (Czarnecka et al. 1984, Nagao et al. 1985). Upon heat shock or other stress 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 (Ellis and Van der Vies 1991). In humans, increased levels of HSPs have been found in response to infection, and autoimmune disease (Welch 1992). The observed chromosomal relationships with Rsv1 and genes controlling resistance to other diseases suggest that HSPs are possibly associated with defense responses in plants. Presently our laboratory is investigating the soybean LMW HSP multigene family, and its association with virus resistance.

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TABLE 1. Segregation of SMV resistance (Rsv), anthocyanin pigmentation (w1) and linked RFLP and SSR markers in an F₂ population from PI 96983 X Lee 68

Traits or markers	No. of F ₂ plants	Observed no.			χ^2 1:2:1	Prob.
		A ₁ A ₁ ^a	A ₁ A ₂	A ₂ A ₂		
<u>Rsv</u>	104	26	49	29	0.52	0.77
<u>w1</u>	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

^a Genotypes: A₁A₁ = PI 96983, A₁A₂ = heterozygous, A₂A₂ = Lee 68

TABLE 2. Molecular marker phenotypes of the recurrent parent Williams, various donor lines and their corresponding NILs for linked SSR and RFLPs.

Soybean lines	Virus resist. allele	SM176 (SSR)	pA186 (RFLP)			pK644a (RFLP)					
			RI ^a	HIII	DI	RV	RI	HIII	DI	RV	
Williams (R ^b)		1 ^c	1	1	1	1	1	1	1	1	1
L81-4420 (N)	<u>Rsv^d</u>	1	2	2	2	2	1	1	1	1	1
L78-379 (N)	<u>Rsv</u>	1	2	2	2	2	1	1	1	1	1
PI 96983 (D)	<u>Rsv</u>	1	2	2	2	2	1	1	1	1	1
L84-2112 (N)	<u>Rsv-m</u>	1	2	2	2	2	2	2	2	2	1
Marshall (D)	<u>Rsv-m</u>	1	2	2	2	2	2	2	2	2	1
L83-529 (N)	<u>Rsv-b</u>	2	1	1	1	2	1	1	1	1	1
Buffalo (D)	<u>Rsv-b</u>	2	1	1	1	2	1	1	1	1	1
L85-2308 (N)	<u>Rpv</u>	1	1	1	1	1	1	1	1	1	1
Dorman (D)	<u>Rpv</u>	2	3	1	1	1	1	1	1	1	1

L88-8431 (N)	<u>Rsv₂</u>	2	1	1	1	2	1	1	1	1
Raiden (D)	<u>Rsv₂</u>	2	3	3	1	1 ^c	1	1	1	1
L29 (N)	<u>Rsv₇</u>	1	1	1	1	1	1	1	1	1

^a Restriction endonucleases: RI = EcoRI, HIII = HindIII, DI = DraI, RV = EcoRV

^b "R" = the recurrent parent (Williams) for all NILs; "N" = NILs; "D" = donor parents of virus resistance genes.

^c "1", "2", and "3" within each probe/enzyme combination designate restriction fragments of different size.

^d Virus resistance genes: Rsv-m = the Rsv allele of Marshall; Rsv-b = the Rsv allele of Buffalo; Rsv₂ and Rsv₇ = non-Rsv-allelic SMV resistance genes; Rpv = PMV resistance gene.

^e 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.

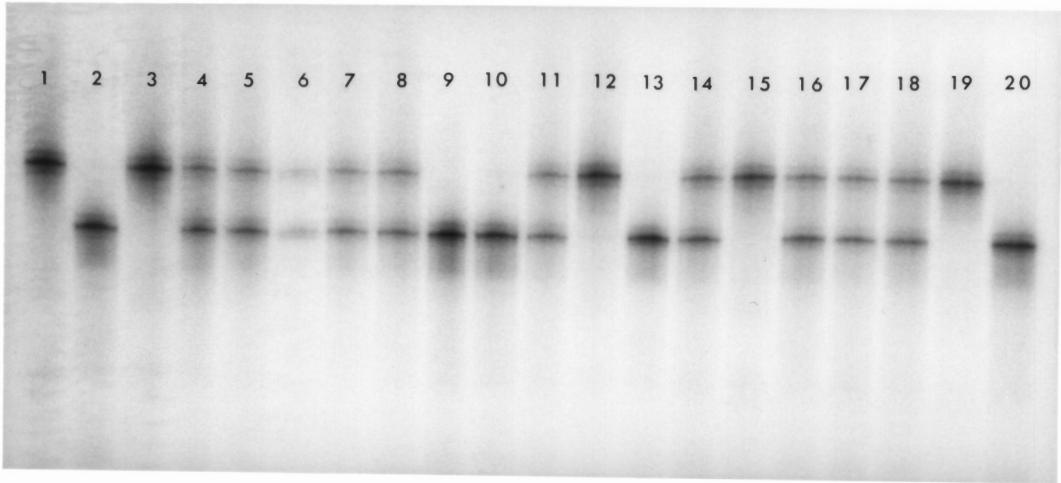


Fig. 1. F_2 segregation pattern for the microsatellite or SSR marker HSP176L. Genomic sequences containing $(AT)_n$ repeats were amplified by polymerase chain reaction (PCR) in the presence of ^{32}P -deoxynucleotides and detected by autoradiography (see Materials and Methods). Lanes 1 and 2 are parental lines, PI 96983 and Lee 68, respectively; lanes 3 through 20 are a portion of the F_2 population from the cross of the parental lines.

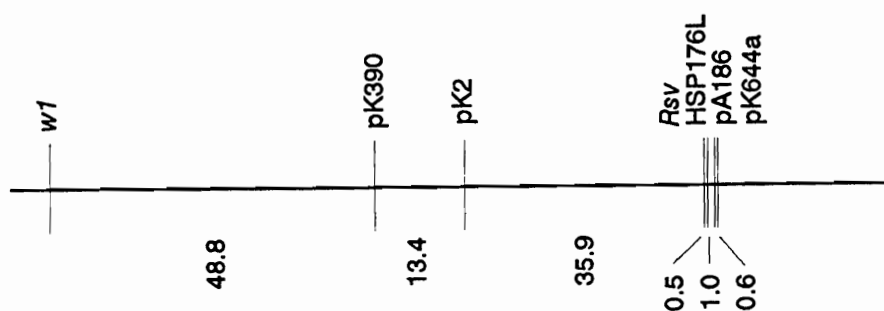
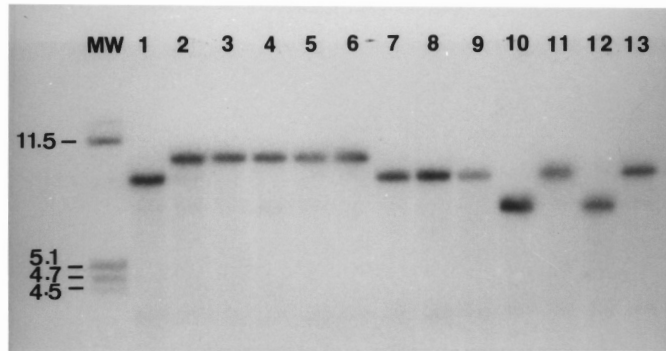
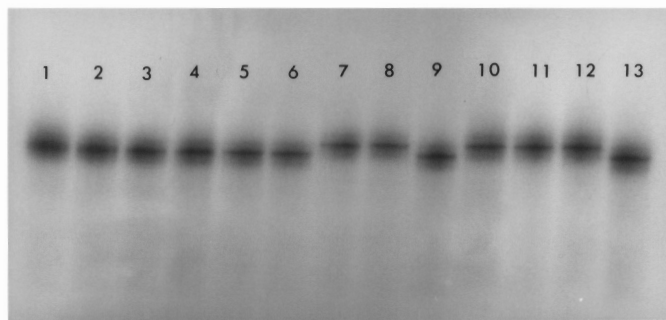


Fig. 2. A linkage map of *Rsv1* (soybean mosaic virus resistance gene), *w1* (flower color), and linked RFLP and SSR markers. The order and distances were computed by multiple linkage analysis using the MAPMAKER computer program. Distances in centiMorgans were computed from recombination frequencies using the Haldane function (19).

Fig. 3. Allelic comparison at Rsv1-linked marker loci among Williams, resistant NILs, and their corresponding donor parents. **Panel A:** Autoradiograph of the EcoRI-digested DNA samples probed with the RFLP marker pA186. DNA size standards (MW) are indicated as kilobase-pairs. **Panel B:** Autoradiograph of the PCR-amplified soybean DNA using primers for the SSR marker HSP176L. Two banding patterns are observed: lanes 1-6, 9 and 13 display the shorter product while the remaining lanes have the longer fragment. In both panels, lane 1 is the recurrent parent Williams; lanes 2 and 3 are L81-4420 and L78-379 [both of which carry Rsv1 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 [an NIL with Rpv (peanut mottle virus resistance gene) from Dorman (lane 10)]; lane 11 and 12 are L88-8431 and its Rsv2 (an SMV resistance gene independent of Rsv1) donor parent; lane 13 is L29, an NIL with SMV resistance from Hardee (not shown). See text for explanation regarding Rsv1. Neither Rsv2 nor Rpv appears to be linked to pA186 and HSP176L (see lane 1, and lanes 9 to 12 in Panels A&B).



A



B

CHAPTER III.

Divergence and Allelomorphic Relationship of A Soybean Virus Resistance Gene Based on Tightly- Linked DNA Microsatellite and RFLP Markers¹

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Abstract

The use of genetically diverse resistance sources is important in breeding for durable resistant cultivars. Detection and evaluation of resistance sources by conventional genetic experiments, however, often require laborious screening and genetic testing. In this study, the effectiveness of "marker-assisted screening" for resistance sources was examined using one microsatellite or simple sequence repeat (SSR) and two RFLP markers tightly-linked to a soybean mosaic virus (SMV) resistance gene (Rsv1). The three marker loci were used to screen sixty-seven diverse soybean (Glycine max) types including cultivars, breeding lines and plant introductions. Five alleles were found at the microsatellite locus, HSP176L. The two RFLP loci (pA186 and pK644a) near Rsv1 show a remarkably higher level of restriction polymorphism than Rsv1-independent loci, suggesting a possible association between disease resistance and rapid sequence divergence. Several specific alleles at the three marker loci were found to be highly correlated with virus resistance. These resistance markers, especially HSP176L-2 which can be detected by the polymerase chain reaction, may be useful for germplasm screening. The grouping of the 67 accessions according to their multilocus marker haplotypes agrees with the available pedigree information. Resistant cultivars within a given group, with the same Rsv1-linked marker haplotype, are likely to carry an Rsv1 allele, if all or most of the cultivars are resistant. A set of differential cultivars known to contain Rsv1 were located in several putatively Rsv1-carrying groups. Based on molecular

marker analysis and previous inheritance studies, 37 of the 45 resistant accessions derive their SMV resistance probably from Rsv1. Among the remaining eight resistant accessions, Columbia is known to carry an independent gene (Rsv3), but inheritance studies are needed for the identification of resistance genes in Holladay, Jizuka, Peking, Virginia, FFR-471, PI 507403, and PI 556949.

Key words: potyvirus, disease resistance, germ plasm, simple sequence repeat (SSR), DNA marker-assisted screening, Rsv1

Introduction

The use of genetically diverse resistance sources is a key to breeding for durable resistance to pests and diseases. Identification of different resistance genes in germplasm, however, usually requires allelism tests which involve extensive crossing and progeny testing. Therefore, a more efficient method of screening and evaluating host resistance genes would be desirable.

Closely linked RFLP (restriction fragment length polymorphism) marker loci have been identified for an increasing number of resistance genes in various crops. Alleles at these marker loci are expected to be transmitted together with the targeted resistance genes as a chromosome block, unless they are separated by recombination. Markers at the DNA level provide the best alternatives or supplements to

conventional disease screening because they 1) are more reliable due to independence from environmental influence, 2) can differentiate resistance alleles without the use of multiple strains, 3) can be used to establish allelic relationships among resistant alleles, and 4) can be used to study the co-evolution of host-pathogen interactions.

Hartl et al. (1993) found an RFLP marker for a wheat powdery mildew resistance gene (Pm3) which is capable of differentiating alleles at the Pm3 locus. Graner and Bauer (1993) reported that RFLP markers closely linked to a barley mild mosaic virus resistance gene can distinguish resistant and susceptible German barley cultivars. Nonetheless, the effectiveness of "marker-assisted screening" in assessing genetic diversity and establishing allelic relationships of disease resistance genes has not been systematically studied.

Recently, DNA microsatellites or simple sequence repeats (SSRs) have been reported as a new class of markers with a wide range of applications in plant genetic studies (Akkaya et al. 1992; Wu and Tanksley, 1993; Saghai Maroof et al. 1994). DNA sequences containing SSRs can be amplified by polymerase chain reaction, and SSR variants can be detected by gel electrophoresis of the amplified fragments (Yu et al. 1994). In the soybean genome, microsatellites with (AT)_n repeats were found to be abundant, and more polymorphic than RFLP markers (Akkaya et al. 1992; Morgante and Olivieri, 1993). This type of PCR-based markers has practical applications in plant breeding as other molecular markers, but is more rapid, and more informative. The use of closely-linked markers can include "marker-based

selection" in genetic crosses (Rafalski and Tingey, 1993), and "marker-assisted screening" prior to making crosses.

Soybean mosaic virus (SMV) is one of the most prevalent soybean viral pathogens in the world, and has been found wherever soybeans [*Glycine max* (L.) Merr] are grown. Based on the reactions in differential soybean cultivars, at least nine strains of SMV have been identified: G1-G7 (Cho and Goodman, 1979), G7A (Buzzell and Tu, 1984), and C14 (Lim, 1985). Using SMV strains G2 and G3, Kiihl and Hartwig (1979) found that host resistance in PI 96983 is controlled by a single dominant gene designated as Rsv1, and in the cultivar (cv) Ogden by rsv^t, a gene that is dominant to rsv1 but recessive to Rsv1. Roane et al., (1983) demonstrated that a single dominant gene in York confers resistance to SMV strain G1. Buss et al. (1989b) also reported that cv. Marshall and Kwanggyo each carry a single, dominant resistance gene, and the genes are allelic. In an allelism test of the genes in PI 96983, Ogden, York, Marshall, and Kwanggyo, Chen et al. (1991) concluded that resistance in each cultivar is controlled by a single dominant gene, and that genes in these cultivars are allelic. RFLP and microsatellite analysis of near-isogenic lines (NILs) indicated that cv. Buffalo may also contain a single gene at the Rsv1 locus (Yu et al. 1994).

Buzzell and Tu (1984) reported that SMV resistance in Raiden is controlled by a single gene at a locus different from Rsv1, designated as Rsv2. They (1987) also proposed that a dominant gene at a third locus (Rsv3) confers a necrotic reaction to SMV in Columbia and its derivatives. Lim (1985) found that PI 483084 and PI

486355 each had a single resistance gene, both of which are not at the Rsv1 locus. Bowers et al. (1992) reported that the single dominant genes in Buffalo and in the line HLS are located at two different loci. Using an SMV strain different from that of Lim (1985), Chen et al. (1993) concluded that resistance in PI 486355 is controlled by two independent dominant genes, one of which is at the Rsv1 locus.

Using microsatellite and RFLP markers, we recently mapped Rsv1 in an F₂ population from PI 96983 X Lee 68 (Yu et al. 1994). Three marker loci, HSP176L (SSR), pA186 and pK644a (RFLPs) were located at a distance of 0.5, 1.5 and 2.1 cM from Rsv1, respectively. The availability of these SSR and RFLP markers, and the simple Mendelian inheritance of SMV resistance provides a model system to evaluate the use of closely-linked markers in assessing the divergence of disease resistance genes from various sources. The objective of this study was to investigate the level of genetic heterogeneity near the Rsv1 locus, to detect correlation between Rsv1-linked marker alleles and SMV resistance in 67 soybean accessions, and to explore a marker-assisted classification of the SMV resistance sources.

Materials and Methods

Genetic materials

Sixty seven diverse soybean types including plant introductions, cultivars, and breeding lines were included in this study. Accessions of plant introductions were obtained from the USDA soybean germplasm collection maintained at the University

of Illinois. Cultivars and breeding lines were from our collection or the 1992 Virginia state cultivar test. Among the accessions, PI 96983, Davis, Ogden, Marshall, Kwanggyo and York are known to contain Rsv1, and Buffalo may also have an allelic SMV resistance gene (Yu et al. 1994). Approximately thirty seeds of each accession were sown in a single row one meter long in a soybean nursery in Blacksburg, Virginia.

Virus screening

Field screening of reactions of soybean accessions to SMV was according to Roane et al. (1983). To prepare a large quantity of SMV inoculum, leaves were harvested from Lee 68 inoculated 2-3 weeks earlier with SMV G1 strain (VA isolate), grounded in 2-3 ml of 0.05 M sodium citrate buffer per g of tissue. This preparation was strained through 4 layer of cheesecloth, and additional buffer was added to make a 1:10 (g:ml) dilution. Inoculations were made in the field by spraying inoculum, with 0.5% (w/v) carborundum, using an artist's airbrush at an air pressure of 4.2-5.6 kg/cm². Three-week-old soybean seedlings were inoculated, and their reactions of each plant to the virus were classified as resistant (symptomless), necrotic (stem-tip or lethal) or susceptible (systemic mosaic or wrinkling) about one month following inoculation.

Microsatellite procedure

DNA samples were prepared from soybean leaf tissues according to protocols

of Saghai Maroof et al. (1984). Leaf tissue was collected from six-week old plants grown in the field. Equal amounts of tissue from at least twelve plants per accession were pooled and frozen in dry ice. Frozen tissues were lyophilized in a Virtis Consol 25LL freeze dryer, and ground to fine powder with a sample mill, and used for DNA extraction.

The procedure published by Yu et al (1994) was used to detect SSR variants at the HSP176L (previously designated as SM176) locus. The primers 5'TTTTG TTAAG TTAAG TTAAG GTACT GTACT GTGG (forward primer) and 5'TATTT TAGCA GTTTT AGATG ATTCG (reverse primer), also described by Akkaya et al. (1992), were synthesized based on the Gmhsp17.6L sequence (Nagao et al. 1985). The genomic fragments containing the SSR region were amplified by PCR at 30 cycles of 1 min at 95°C (denaturing), 2 min at 55°C (annealing) and 1.5 min at 72°C (extension) in the presence of alpha-³²P-dCTP (ICN Biomedicals, Irvine, CA). The size variation of the PCR products were detected by 6.0% denaturing polyacrylamide gel with 8M urea followed by autoradiography.

RFLP assays

RFLP analyses also followed the protocol described by Yu et al. (1994). The soybean genomic DNA clones pA186, pK644, and pK2 were kindly provided by Dr. R. C. Shoemaker, USDA-ARS, Iowa State University. ³²P-labelled DNA probes were prepared from the insert DNA fragments by random hexamer priming and hybridized to Southern blots containing 8 ug of soybean genomic DNA digested with an

appropriate restriction enzyme.

The clones pA186 and pK2 detect a single RFLP locus, while pK644 detects two loci, pK644a and pK644b, in two different linkage groups (Diers et al. 1992a). At each of the four RFLP loci, variation in the length of restriction fragments was detected with three different restriction enzymes: HindIII (H3), EcoRI (R1) and EcoRV (R5) for pA186; H3, DraI (D1), and R1 for pK644a and pK644b; and H3, D1 and R5 for pK2.

Statistical analysis

To measure the extent of deviation from diallelism, i.e., the existence of more than two alleles, we devised a multimorphic index (MI) similar to the polymorphic index (Marshall and Allard, 1970). The formula used to calculate the MI is:

$$MI = 1 - (p_1 + p_2)^2 - \sum_{i=3}^n p_i^2$$

where p_1 and p_2 are the frequencies of the two most abundant alleles, p_i is the frequency of each additional allele and n is the total number of alleles. The value of MI is zero if only two alleles are present. The MI value becomes larger as the number of allele increases and the distribution of alleles becomes more uniform.

The association between an SSR allele or an RFLP haplotype (a combination of RFLP variants detected with different restriction enzymes in the same RFLP locus) and SMV resistance was examined by a Chi-square test. For simplicity, the one accession with necrotic reaction was considered as resistant in Chi-square calculations. The expected number of resistant (E_R) and susceptible (E_S) accessions was the

product of their respective frequencies in the total sample (67) and the number of accessions with the marker allele or haplotype. The value of Chi-square was calculated by:

$$X^2 = \frac{(O_R - E_R)^2}{E_R} + \frac{(O_S - E_S)^2}{E_S}$$

where O_R and O_S are the observed number of resistant and susceptible accessions, respectively.

The relatedness of the RFLP haplotypes at each of the two RFLP loci was calculated by cluster analysis using Statistica computer program (StatSoft, Inc., Tulsa, Oklahoma, USA). The method of clustering was single linkage (nearest neighbor), and the distance measure used was percentage disagreement.

Results

Among the 67 soybean accessions screened, 45 were found to be resistant (R), 1 was stem-tip necrotic (N), and 21 were susceptible (S) to SMV-G1. At the HSP176L locus, five variants (alleles) with different numbers of dinucleotide repeats $(AT)_n$ were detected in the 67 accessions by PCR reactions using specific primers. The variants were designated arbitrarily 1 through 5 with no regard to the number of AT repeats (Fig. 2). Variation at the four RFLP loci (pA186, pK644a, pK644b, and pK2) was detected with a combination of three different restriction enzymes in single digestions. At the pA186 locus, for instance, four variants (1, 2, 3, and 4) were

found with H3, three (1, 2, and 3) with R1, and four (1, 2, 3, and 4) with R5. At pK644a, three variants (1, 2, and 3) were detected with H3, five (1, 2, 3, 4, and 5) with D1, and four (1, 2, 3, 4, and 5) with R1. The variants detected by the same probe but with different enzymes are correlated to each other. For instance, pA186 variant R1-1 is always detected in the same lines with H3-1, R1-2 with H3-2, and R1-3 with either H3-3 or 4 (Table 1). Nonetheless, the use of multiple enzymes obviously provides more information on the level of variation at the targeted chromosomal region. A combination of variants detected at the same locus by three restriction enzymes is referred to as a haplotype, e.g., the haplotype A for pA186 represents the combination of variants H3-1, R1-1, and R5-1 (see Table 1).

The Rsv1-linked pA186 and pK644a loci exhibit a higher level of polymorphism than the pK2 and pK644b loci (Table 1). The pK2 locus is about 40 cM from Rsv1, and pK644b is on a different linkage group. Among 67 soybean accessions, six (A through F) and seven (A through G) haplotypes were found at the pA186 and pK644a, respectively. In contrast, only two haplotypes (A and B) were detected at the pK2 locus, and four (A through D) at pK644b, two of which (B and C) were observed in only one line each. The MI value is much higher for pA186 and pK644a (0.51 and 0.47, respectively) than for pK2 and pK644b (0.00 and 0.06, respectively) (Table 1). Five SSR alleles (1, 2, 3, 4, and 5) were found at HSP176L, among which HSP176L-1 and 2 are most frequent with 31 and 26 accessions each. The level of polymorphism detected by SSR analysis is generally higher than that detected by RFLP analysis (Akkaya et al. 1992). In this study, we have also observed

a high level of SSR polymorphism at HSP176L. Using probes that correspond to marker loci closely-linked to Rsv1, we have found an atypical large number of RFLP haplotypes, as opposed to the generally low level of RFLP observed in soybeans, indicating an unusually high level of DNA rearrangements in the Rsv1-containing chromosomal region.

One specific SSR allele or RFLP haplotype at each of the three marker loci is significantly associated, as shown by Chi-square tests, with SMV resistance: HSP176L-2, pA186-D, and pK644a-A (Table 2). All 26 soybean accessions that carry HSP176L-2 are resistant to SMV. Soybean accessions with pA186-D are predominantly resistant to SMV, with an R:S ratio of 24:3. At the pK644a locus, 30 of the 32 lines that carry haplotype A are resistant. These marker allele and haplotypes, HSP176L-2, pA186-D, and pK644a, are hereafter referred to as resistance markers (R markers). On the other hand, pA186-C and pK644a-F are associated with SMV susceptibility. The sample size of HSP176L-3, -4, and 5, pA186-F, and pK644a-G is too small for a Chi-square test, but they appear also to be associated with susceptibility. These marker alleles or haplotypes are referred to as susceptibility markers (S markers).

According to their multilocus Rsv1-linked marker haplotypes, the 67 soybean accessions screened were grouped into 20 classes, designated as C1-C20 (Table 3). The largest group, S12, includes 22 accessions, whereas ten smallest groups have only one accession each. The grouping of cultivars based on the Rsv1-linked marker loci agrees with the available pedigree information with respect to the source of SMV

resistance. The group C2, for instance, include 'Brim', 'Young', as well as their likely source of SMV resistance, 'Davis', (Burton et al. 1987; 1994). Kwanggyo, Suweon 97, and PI 486355, three resistant lines from Korea, are clustered in the same class, C5. SMV resistance in 'Hutcheson' is derived from 'Dorman' via York (Buss et al. 1988). These three cultivars were found to belong to the same class, C12, with a multilocus haplotype of 2 D A (Table 3). Similarly, 'Toano' is also in C12, along with PI 80837, its SMV resistance donor (Buss et al. 1987). Thus, the accessions of the same group, with the same Rsv1-linked multilocus marker haplotype, is indicative of their sharing of the same linkage block around Rsv1.

The resistance in a group of accessions are probably conferred by the same Rsv1 allele if all or most accessions in a group are SMV resistant, as these accessions have the same haplotype at the Rsv1-linked marker loci. A set of differential cultivars including PI 96983, Ogden, Marshall, Kwanggyo, and York are known from previous inheritance studies to contain Rsv1 alleles. Although no allelism test has been conducted for Buffalo, our marker analysis in NILs indicated the Buffalo gene is probably also at Rsv1 locus (Yu et al. 1994). These six Rsv1-containing cultivars were found in different putatively Rsv1-carrying groups, confirming that an Rsv1 allele is responsible for SMV resistance in each of these groups. The Rsv1-carrying groups include C1 (PI 96983), C2 (n=4 members), C3 (n=4), C5 (n=3), C11 (n=3), and C12 (n=22), a total of 37 out of 45 SMV resistant lines appear to be controlled by alleles of Rsv1. Among the remaining eight resistant accessions, Columbia is known to carry an independent gene (Rsv3), but inheritance studies are needed for

the identification of resistance genes in Holladay, Jizuka, Peking, Virginia, FFR-471, PI 507403, and PI 556949.

The association between SSR alleles and linked RFLP haplotypes at linked loci is evident from Fig. 2. Based on their distances, the six and seven RFLP haplotypes at pA186 and pK644a, can be consolidated into four and three clusters (Fig. 3). The 20 groups of 67 accessions were then located in nine combinations of haplotype clusters between the two RFLP loci. HSP176L-2 (in C10, C11, and C12) appears to be closely associated with pK644a-A, and with pA186-D. All HSP176L-3 accessions (C13, C14, and C14) were located in the two closely-related combinations of the RFLP haplotypes, so were accessions with HSP176L-4 (C16-C19). What makes all members of the C10 (Jizuka), C11, and C12 groups resistant to SMV may be related to the presence of two or three R markers in their multilocus haplotypes (Table 3, and Fig. 2). In contrast, the classes C6, C14, C15 (Pioneer 9691), C19 (CNS), and C20 contains two or three S markers. Consequently, all accessions, except Holladay, are susceptible to SMV (Table 3, and Fig. 2). Although inheritance studies are needed, it appears that SMV resistance in Jizuka is conferred by an Rsv1 allele, where as a independent gene may be responsible for resistance in Holladay.

Discussion

Molecular diversity and resistance gene clusters

A number of RFLP surveys have indicated a low level of DNA polymorphism among soybean lines. Keim et al. (1992) reported that, among 91 genomic DNA probes that revealed polymorphism in 38 cultivars and ancestral lines, 88 probes detected two alleles, and the remaining three had three alleles. This predominantly two-allele phenomenon, or diallelism, of the soybean genome is consistent with the description by Apuya et al. (1988). Roth et al. (1989) found that newly generated RFLP allele during the tissue culture process was almost always identical to the existing alternative allele at a given locus.

In contrast to the norm of diallelism, multiple alleles were found at the RFLP marker loci in the chromosomal region near Rsv1. Up to five RFLP alleles were observed at the pA186 and pK644a loci among 67 soybean types when their genomic DNA was digested with a restriction enzyme. The use of multiple enzymes enabled us to detect a larger number of RFLP haplotypes, six by the probe pA186 and seven by pK644a. The remarkably high level of polymorphism near or at the disease gene cluster may suggest an association between the molecular mechanism of disease resistance and rapid sequence divergence in plants, similar to that found in human and mouse (Murphy 1993). Several disease resistance genes including resistance to peanut mottle virus (Rpv) (Roane et al. 1983) and *Phytophthora* (Rps₃) (Diers et al.

1992b) have been located previously in the vicinity of the Rsv1-containing chromosomal region, possibly as a gene cluster.

The clustering of host resistance genes conditioning resistance to pathogenic fungi has been documented in many plant species. The best examples are rust resistance genes (Rp) on chromosome 10 of maize (Hooker 1985), mildew resistance genes (Ml-a) on chromosome 5 of barley (Wise and Ellingboe, 1985), and rust resistance genes clustered in the L group of flax (Shepard and Mayo, 1972; Islam et al. 1993). Recently, a virus tolerance gene (Bdv1) was found to be either closely linked or pleiotropic with Lr34 and Yr18 for adult plant resistance to rust in bread wheat (Singh 1993). This, together with our finding of the linkage between Rsv1 and Rps3, indicates that clustering of resistance genes is not limited to rust or fungus resistance.

Rapid divergence in DNA sequences may be a unique feature of resistance gene clusters. For instance, Hooker (1985) suggested that the location of Rp genes at the terminus of chromosome 10 of maize may be responsible for duplications at the locus. Sudupak et al. (1993) reported that unequal crossing-over may be responsible for the meiotic instability of the gene in the Rp complex.

Marker-assisted screening of SMV resistance sources

In this study, we explored the use of molecular markers as aids in plant genetics and plant breeding programs based on the association between markers and

the targeted disease resistance gene. Such uses of the resistance-correlated markers (R markers) include marker-assisted screening as alternatives or supplements to screening for the actual trait, and studies of the lineage of the target gene. R markers (HSP176L-2, pA186-D, and pK644a-A) were found at each of the three Rsv1 marker loci. Of particular interest is HSP176L-2, which was observed exclusively in 26 resistant accessions. This SSR allele was found as a rare allele (freq=0.02, or 1/43) by Akkaya et al. (1992), probably due to the lack of accessions from this type of resistance sources in their samples. These R markers, especially HSP176L-2 which can be detected by polymerase chain reaction (PCR), may be suitable in screening soybean germ plasm for SMV resistance.

Associations between SMV resistance and R markers indicate that the transmission of the targeted gene (Rsv1) can be monitored as a linkage block. Therefore, analysis of the phylogenetic relationship between the genotypes at marker loci will provide clues about the ancestral source of SMV resistance alleles associated with the marker genotypes. The grouping of the soybean accessions based on the marker genotypes was found in agreement with the available pedigree information with respect to the sources of Rsv1. Such grouping is sometimes superior to the differential response to various strains of SMV. For instance, the Buffalo group and PI 96983 can not be differentiated by SMV strains G1 to G7, but on the basis of markers the resistance genes in the two cultivars probably have different sources or lineages. Marshall and Ogden were located in the same group according to marker data. Both cultivars have identical reactions to SMV G1, G3, G4, G5, G7, and G7A

but their responses are different when inoculated with SMV G2 or G6 (Cho and Goodman, 1979). More marker data are needed to explain these differences.

Rsv1 is the likely source of SMV resistance in at least 38 of the 45 resistant accessions in this survey. The existence of a large number of alleles at or near Rsv1 warrants further study. Buzzell and Tu (1984, 1987) reported that SMV resistance in Raiden and Columbia were controlled by single genes at two different loci (Rsv2 and Rsv3), respectively. Molecular marker analysis for Rsv1 separates Columbia from Rsv1-carrying groups, supporting that Rsv3 in Columbia is independent of Rsv1. Raiden, however, carries the same R markers, and probably the same Rsv1 allele as York and 20 other resistant lines. Our previous study (Yu et al. 1994) found that L88-8431, a Williams near-isogenic line with SMV resistance from Raiden, is different from the recurrent parent at both HSP176L and pA186 (EcoRV), indicating that Raiden may contain the Rsv1 gene. Additional evidence for the presence of Rsv1 in Raiden was also provided by an inheritance study in an F₂ population from Raiden X PI 96983, which was inoculated with SMV G1 strain, and no susceptible plant was found (G. R. Ma, unpublished data). It is possible that Raiden may contain two independent resistance genes, Rsv1 and Rsv2, of which Rsv1 confers resistance at least to the G1 strain of SMV.

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TABLE 1. The number and distribution of marker alleles or haplotypes at the *Rsv1* chromosomal region (HSP176, pA186, and pK644a) and elsewhere (pK2 and pK644b) in the soybean genome among 67 soybean accessions. There is no correspondence between different columns (loci) in this table.

HSP176		pA186		pK644a		pK2		pK644b	
SSR ^a allele	No. Obs	H ₃ R ₁ R ₃ ^b type ^c	No. Obs	H ₃ D ₁ R ₁ type	No. Obs	H ₃ D ₁ R ₅ type	No. Obs	H ₃ D ₁ R ₁ type	No. Obs.
1	31	1 1 1	12	1 1 1	32	1 1 1	39	1 1 1	18
2	26	2 2 1	3	2 2 2	5	2 2 2	28	3 1 1	1
3	4	2 2 2	18	2 2 3	4			3 1 3	1
4	4	3 3 2	27	2 2 4	1			2 2 2	42
5	2	4 3 3	3	2 3 2	5				
		4 3 4	4	2 4 3	16				
				3 5 2	4				
N ^d	5		6		7		2		4
MI ^e	0.268		0.509		0.468		0.000		0.063

^a SSR alleles that differ in the number of AT repeats.

^b RFLP haplotypes or combination of RFLP variants as detected using the following restriction enzymes: H₃ (HindIII), R₁ (EcoRI), R₅ (EcoRV), and D₁ (DraI).

^c Letter designations of RFLP haplotypes which will appear in Table 2 and Fig. 2 and 3.

^d Number of SSR alleles or RFLP haplotypes observed in 67 soybean accessions.

^e Multimeric index, see Materials and Methods.

TABLE 2. Number of soybean lines that exhibit resistance or necrotic (R) or susceptible (S) reaction to SMV G1 strain as tabulated according to alleles or haplotypes at the three Rsv1-linked SSR (HSP176L) and RFLP (pA186 and pK644a) loci.

SSR ^a	HSP176L			pA186			pK644a							
	R ^b	S	χ^2 ^c	Class ^d	RFLP	R	S	χ^2	Class	RFLP	R	S	χ^2	Class
1	18 ⁿ	13	1.7		A	10	2	1.1		A	30	2	9.1 ^{**}	R
2	26	0	11.8 ^{**}	R	B	3	0	1.3		B	4	1	0.2	
3	1	3	3.9 [*]	S	C	7	11	7.6 ^{**}	S	C	2	2	0.7	
4	1	3	3.9 [*]	S	D	24 ⁿ	3	5.1 [*]	R	D	0	1	2.3	
5	0	2	4.6 [*]	S	E	2	1	0.0		E	3	2	0.2	
					F	0	4	9.3 ^{**}	S	F	7 ⁿ	9	4.7 [*]	S
					G	0	4	9.3 ^{**}	S	G	0	4	9.3 ^{**}	S

^a "SSR" indicates SSR alleles; "RFLP" indicates RFLP haplotypes or combinations of RFLP variants with three different restriction enzymes, see Table 1.

^b Number of G. max lines that exhibit resistance or necrotic (R) or susceptible (S) reaction to SMV G1 strain. An superscript 'n' following a number indicates that one (1) necrotic accession (PI 507389) is included as a resistant line for the purpose of χ^2 calculation.

^c In Chi-square test for the independence of SMV resistance from marker alleles or haplotypes, an * or ** sign following a χ^2 value indicates significant (p<0.05) or highly significant (p<0.01), respectively.

^d Marker alleles or haplotypes associated with SMV resistance belong to either resistance (R) or susceptibility (S) classes.

TABLE 3. The grouping of 67 soybean accessions according to their multilocus haplotypes at Rsv1-linked HSP176L, pA186, and pK644a, and tentative allelism of the SMV resistance genes.

Group	Multilocus		Accessions ^c	Resistance genes ^d
	Geno. ^a	Class ^b		
C1	1 A A	- - R	<u>PI 96983</u>	<u>Rsv1</u>
C2	1 A B	- - -	<u>Davis</u> , <u>Brim</u> , <u>Young</u> , <u>HT3550</u> , <u>G83-198</u>	<u>Rsv1</u>
C3	1 A F	- - S	<u>Marshall</u> , <u>Ogden</u> , <u>V73-178</u> , <u>N87-325</u> , <u>PI 507376</u>	<u>Rsv1</u>
C4	1 C A	- S R	<u>Peking</u> , <u>Williams</u> , <u>Md78L-0198</u> , <u>FFR-471</u> , <u>PI 556949</u>	<u>R?</u>
C5	1 C E	- S -	<u>Kwanggvo</u> , <u>Suweon 97</u> , <u>PI 486355</u>	<u>Rsv1</u>
C6	1 C F	- S S	<u>Bay</u> , <u>Essex</u> , <u>Holladay</u> , <u>DP105</u> , <u>FFR-396</u> , <u>KS5292</u> , <u>Pioneer 9442</u> , <u>HT4290</u>	-
C7	1 D F	- R S	<u>PI 507389^e</u> , <u>507453</u>	<u>R?</u>
C8	1 E C	- - -	<u>Columbia</u>	<u>Rsv3</u>
C9	1 E G	- - S	<u>Hartwig</u>	-
C10	2 A A	R - R	<u>Jizuka</u>	<u>R?</u>
C11	2 B A	R - R	<u>Buffalo</u> , <u>PI 507391</u> , <u>507477</u>	<u>Rsv1</u>
C12	2 D A	R R R	<u>York</u> , <u>Dorman</u> , <u>Hutcheson</u> , <u>Raiden</u> , <u>Toano</u> , <u>Youbian 30</u> , <u>Doujiao 44</u> , <u>TN6-90</u> , <u>V71-370</u> , <u>DP415</u> , <u>DP425</u> , <u>FFR-544</u> , <u>PI 80837</u> , <u>399012</u> , <u>468408A</u> , <u>495020</u> , <u>507474</u> , <u>507690</u> , <u>509096</u> , <u>509098</u> , <u>509106</u> , <u>556950</u>	<u>Rsv1</u>
C13	3 E C	S - -	<u>Virginia</u>	<u>R?</u>

C14	3 F E	S S -	Underwood 607, TN85-157	-
C15	3 F G	S S S	Pioneer 9691	-
C16	4 D C	S R -	PI 508295	-
C17	4 D D	S R -	PI 508298	-
C18	4 D F	S R S	<u>PI 507403</u>	<u>R?</u>
C19	4 F C	S S -	CNS	-
C20	5 C G	S S S	Lee 68, Cumberland	-

^a The multilocus haplotypes at the order of HSP176L, pA186, and pK644a as shown in Table 1.

^b The resistance-correlated marker class (R or S) of SSR allele or RFLP haplotypes in the order of HSP176L, pA186, and pK644a. See Table 2 for their designations. A "-" indicates no significant association with SMV resistance was detected for the SSR allele or RFLP haplotype.

^c All 45 underlined accessions are SMV-resistant. An superscript "n" following the accession PI 507389 indicates its necrotic reaction to SMV-G1. The remaining 21 accessions are susceptible.

^d Allelism among SMV resistance genes. Rsv3 is independent of Rsv1 (Buzzell and Tu 1989), R? indicates that the SMV resistance genes in these groups need to be determined by inheritance studies, and "-" indicates in a group indicates that no SMV resistance was detected.

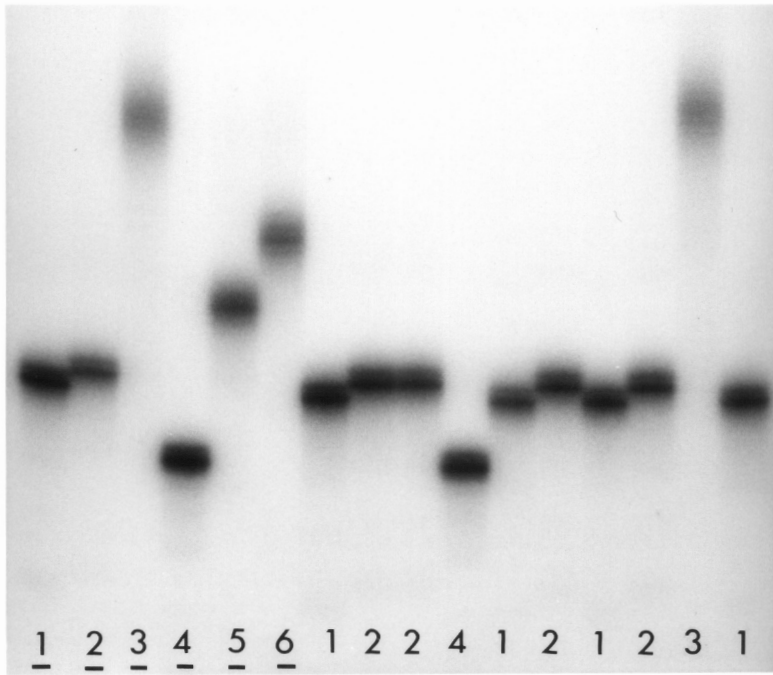


Figure 1. SSR alleles at the HSP176L locus. Lanes 1 through 5 (underlined) show the five SSR alleles observed in the 67 soybean accessions. lane 6 is an allele found in one *G. soja* accession (PI 407162) which is not included in this study; lanes 7 through 16 are random samples of the soybean accessions screened.

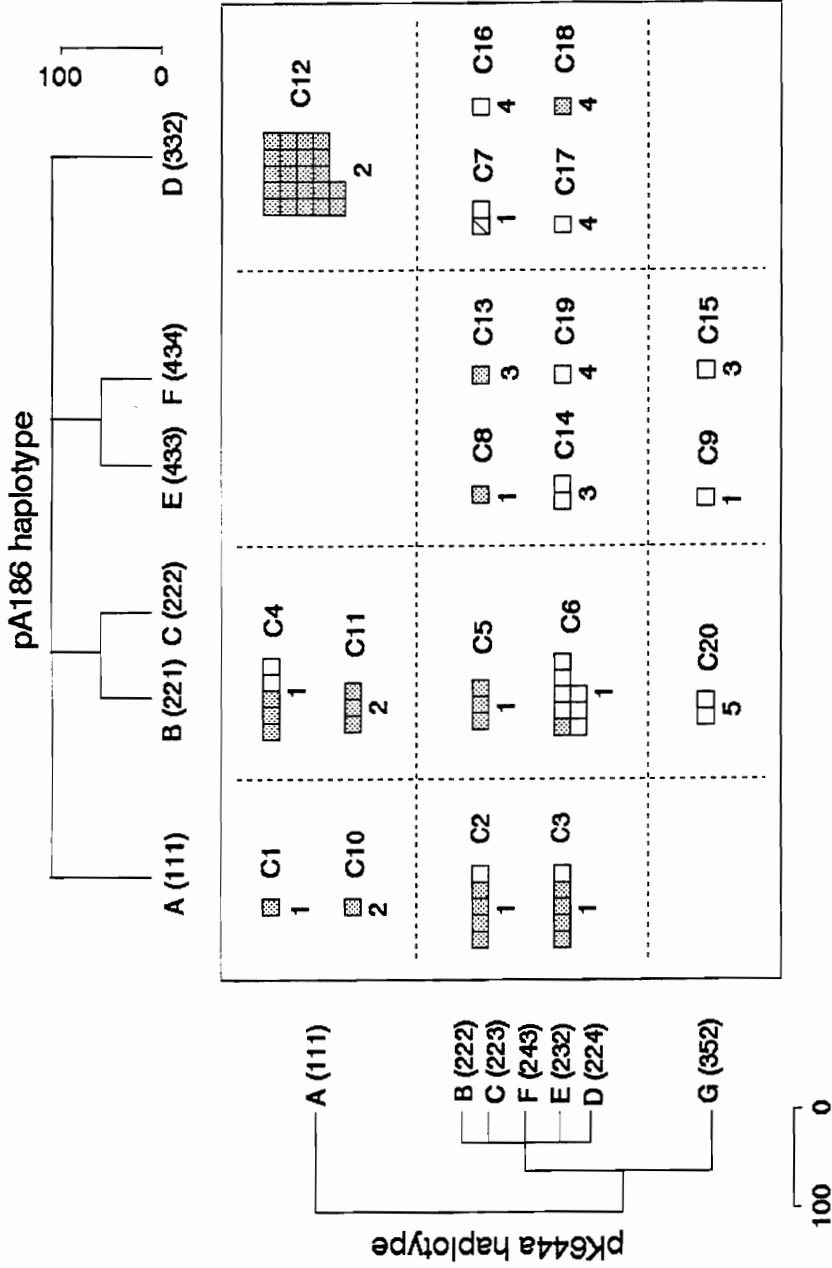


Figure 2. Association of marker alleles or haplotypes at closely-linked HSP176L, pA186, and pK644a. Each \blacksquare , \square , and \square designates one (1) resistant, necrotic, or susceptible accession, respectively. The number below each group, 1 through 5, indicates the SSR allele at HSP176.

CHAPTER IV.

A Soybean Mosaic Potyvirus Resistance Gene Is Closely Linked to a Multigene Family¹

¹Manuscript to be submitted to Molecular Plant-Microbe Interaction

ABSTRACT

The Rsv1 gene confers host resistance to soybean mosaic potyvirus (SMV) in PI 96983 and other soybean lines. Close linkage of Rsv1 to another potyvirus (Rpv1) and phytophthora (Rps3) resistance genes indicates that it may belong to a resistance gene cluster. We have previously mapped a heat shock protein (HSP) gene, HSP176L, near Rsv1 in group F of the USDA/ISU soybean linkage map. The objectives of this study were to determine the chromosomal locations of the Class I small HSP multigene family, which includes HSP176L, with respect to Rsv1. A technique termed amplified sequence length polymorphism (ASLP) was developed to convert known DNA sequences of small HSP genes and an auxin down-regulated gene (ADR11) to PCR-based genetic markers. Among seven pairs of primers used, three (HSP175E, 185C, and ADR11) detected ASLPs between the parents, PI 96983 and Lee 68, and the corresponding loci were mapped using 174 F₂ individuals. HSP175E is closely-linked (0.7 cM) to HSP176L, both of which are Class I small HSP genes. HSP185C is independent of the Class I genes, suggesting that it may belong to a different family. ADR11 was selected because it was mapped (using probe P11) to group 1 of the Du Pont soybean map in close linkage to HSP176B, another Class I gene that detected no ASLP in our mapping population. ASLP analysis using ADR11 in a set of near-isogenic lines of Rsv1 indicates that this locus is linked to Rsv1, and the ASLP marker co-segregates with HSP175E in the F₂ population. Thus, a family of Class I small HSP genes including HSP176L, 175E, and 173B, and possibly a family of ADR genes, is located near the Rsv1 gene cluster.

INTRODUCTION

The potyviruses, a group of single-stranded RNA viruses multiplying through a polyprotein strategy, is the largest and economically most important group of plant viruses (Matthews 1991). Host resistance is the most effective way of preventing crop loss from potyviruses. Potyvirus resistance genes have been identified for soybean mosaic (eg. Rsv1, Kiihl and Hartwig, 1979), bean common mosaic (I, Ali, 1950), maize dwarf mosaic (Mdm1, McMullen and Louie, 1989; Rmd1, Roane et al. 1989), and potato Y (Ry, Cockerham 1970) viruses. Nonetheless, none of the potyvirus resistance genes has been cloned, and the mechanism of host resistance remains unknown.

Soybean mosaic potyvirus (SMV) causes one of the most important viral diseases in soybean worldwide (Buss et al. 1989). Based on the symptoms that SMV isolates cause on a set of differential soybean cultivars, Cho and Goodman (1979) classified them into seven strain groups (G1-G7). The soybean line PI 96983 is resistant to SMV owing to a single dominant gene, Rsv1 (Kiihl and Hartwig, 1979), but the G7 strain causes lethal systemic necrosis in PI 96983 (Buss et al 1989a). Alleles at the Rsv1 locus are also responsible for SMV resistance in York, Ogden, Kwanggyo and Marshall (Chen et al 1991). Another gene, which is independent of Rsv1, confers a necrotic reaction to SMV strains G1-G4 in the cultivar Columbia (Buzzell and Tu, 1989). The line PI 486355 contains two independent genes for resistance to G1 (Chen et al. 1993).

At least 16 other potyviruses infect soybean naturally or by inoculation. Among them, peanut mottle, bean yellow mosaic, and peanut stripe potyviruses are also economically important in soybean (Ford et al. 1989). A dominant gene (Rpv1) that controls peanut mottle resistance in York soybean is closely linked to the SMV resistance gene Rsv1 (Roane et al. 1983). Resistance to peanut stripe in the soybean line AGS 129 is controlled by a single dominant gene that is also closely linked to SMV resistance (Choi et al. 1989). Resistance or hypersensitive response to a number of potyviruses in pea (Provvidenti 1988) and common bean (Kyle and Provvidenti, 1993) is controlled by clusters of closely-linked genes. Based on the conserved genomic structure among related species (Helentjaris 1993), it is likely that Rsv1 also belongs to a potyvirus resistance gene cluster.

The recent success in map-based cloning of disease resistance genes (Martin et al. 1993) offers a feasible approach to isolate Rsv1. In order to accomplish the positional cloning of Rsv1, however, a more detailed understanding of the genetic structure of the Rsv1-containing chromosomal region is required. We have recently located the Rsv1 gene in the soybean linkage map with closely-linked RFLP and microsatellite markers (Yu et al. 1994a). The map position of Rsv1 is in the vicinity of a *Phytophthora* resistance gene (Rps3) located by Diers et al. (1992). An investigation using Rsv1-linked molecular markers found an extraordinarily high level of diversity (Yu et al. 1994b), supporting the hypothesis that clustered resistance genes may be members of multigene families, each has a specificity generated by genetic variation (Pryor, 1987).

Among genetic loci that are closely-linked to Rsv1 is Gmhsp17.6L (HSP176L, previously described as SM176), a soybean small heat shock protein (HSP) gene which contains a microsatellite or simple sequence (AT) repeats (Yu et al. 1994a). The small HSPs, at sizes of 15-18 kD, are the most abundant HSPs in soybean and other plant species. There are several multigene families of small HSP genes, each made up of a few to 13 closely-related genes (Nagao et al. 1985). Clustering of multigene families has been reported for rbcS and cab genes in pea (Polans et al. 1985), and chalcone synthetase genes in petunia (Koes et al. 1987). A previous mapping study with three small HSP genes in soybean, however, has located each of these genes in a different linkage group (Rafalski and Tingey 1992). Whether each of the small HSP genes exists as a cluster remains undetermined. A cluster of small HSP multigene family that is closely linked to Rsv1, if verified, would be valuable in saturating this chromosomal region with molecular markers. The objectives of this study were to determine the chromosomal locations of small HSP multigene families with respect to Rsv1, and to provide additional information on the organization of the resistance gene cluster.

MATERIALS AND METHODS

Plant materials

The mapping population was constructed by crossing PI 96983 and Lee 68, and selfing the resultant three F₁ plants in the greenhouse. One hundred and seventy

four F_{2.3} lines were produced by harvesting seeds from each F₂ plant. PI 96983 is resistant, and Lee 68 is susceptible to SMV G1 strain. PI 96983 has white flowers and green hypocotyl (w1), and Lee 68 has purple flowers and purple hypocotyl (W1).

Virus testing

Reactions to the SMV were determined by manual inoculation on F_{2.3} plants. The G1 strain (VA isolate) was maintained on Lee 68. The SMV inoculation was as previously described (Yu et al. 1994a). For testing the reactions of each F_{2.3} line to SMV, 8 seeds were sown in each of 2 clay pots containing a soilless potting mix, and grown in the greenhouse. Fully-expanded unifoliate leaves were inoculated with inoculum of SMV G1 strain prepared from infected Lee 68 soybean leaves 2 to 3 weeks after inoculation in 0.01M phosphate buffer. The symptom development was observed in two to eight weeks after inoculation.

DNA extraction and RFLP procedures

DNA was extracted from freeze-dried leaf tissues of F₂ plants according to Saghai Maroof et al. (1984). DNA probes for RFLP analysis were obtained from R. C. Shoemaker, USDA-ARS, Iowa State University, Ames, IA. The RFLP procedure has been described previously (Yu et al. 1994a).

SSR and ASLP techniques

The protocol of microsatellite or SSR analysis for the HSP176L locus has been

described previously (Yu et al. 1994a). The technique of ASLP is essentially the same as SSR analysis. Published DNA sequences are retrieved from Genbank / EMBL databases (ver. 7.3) using University of Wisconsin Genetics Computer Group (GCG) package (Deveux et al, 1987). The targeted sequence for amplification in ASLP analysis are non-coding sequences, for they are more tolerant to insertions and deletions than coding sequences. The size of amplified products are generally in the range of 200-300 bp, such that enough sequence area is surveyed, yet changes as small as a few base pairs in length can still be detected by polyacrylamide gel electrophoresis. The product size of ADR11 is smaller (126 bp) because only a short non-coding sequence is available (Datta et al, 1993). Because the insertion or deletion at the targeted DNA sequence can range from a few base-pairs to as large as 100 bp (unpublished data), the percentage of polyacrylamide gel and running time differ for each ASLP locus. The sequences of oligonucleotide primers, sizes of expected PCR products, and references of published sequence for the three ASLP loci that we have mapped are presented in Table 2.

Linkage analysis and map construction

The Linkage-1 computer program (Suiter et al. 1983) was used to test the goodness of fit to expected segregation ratios (1:2:1, or 3:1), and to detect two-point linkages for each genetic locus. The Mapmaker/exp (ver 3.0) (Lander et al. 1987) was used for multiple linkage analysis, and for the construction of the linkage map. The default linkage criteria for map construction was a LOD score of 4.0, and a

maximum Kosambi distance of 40.0 cM.

RESULTS

Since the initial mapping of Rsv1 in linkage group F (Yu et al. 1994a), various types of molecular markers have been used in order to saturate the area around Rsv1. Seven additional RFLP clones that were previously mapped in this linkage group (Diers et al. 1992) were used to probe the DNA of the parental lines, PI 96983 and Lee 68, digested with each of 18 restriction enzymes. Only four (pA245, 401, 708, and 806) of these new clones detected polymorphism with at least one enzyme. The segregation of RFLP markers, as exemplified by pA186 and pK644a, in the F₂ population fit the expected ratio of 1:2:1 (Table 1).

An small HSP gene, Gmhsp17.6L or HSP176L (hereafter the prefix Gmhsp- is simplified by HSP-), contains simple sequence (AT)_n repeats, and was mapped in PI 96983 X Lee 68 using a PCR-based, and highly polymorphic, microsatellite or SSR marker (Yu et al. 1994a). The other five published sequences of small HSP genes including HSP175E, 185C, 179D, 173B, and 175M (Schoeffl et al. 1984, Czarnecka et al. 1985, Nagao et al. 1985, and Raschke et al. 1988) do not have SSRs long enough for microsatellite analysis. In order to generate PCR-based markers, a pair of primers were designed for each of the small HSP genes, in such a way that a sequence smaller than 300 bp can be amplified via polymerase chain reaction (PCR). Although no SSR is present in the targetted sequences, a size variation was frequently

detected due to the sensitivity of a polyacrylamide gel. This type of variation is, hereafter termed amplified sequence length polymorphism (ASLP) was found at the HSP175E locus between PI 96983 and Lee 68, and segregates co-dominantly in the resultant F₂ population (Fig. 1). The segregation of this ASLP marker in the F₂'s fits the expected 1:2:1 ratio (Table 1). HSP175E was then mapped as an ASLP locus to linkage group F, 0.7 map unit from the HSP176L locus which is closely-linked to Rsv1 (Fig. 2). The close linkage between the two small HSP genes provides evidence for the existence of a multigene family near Rsv1.

ASLP analysis of HSP185C indicated that the parents are polymorphic for this small HSP gene, and subsequently it was mapped to linkage group M, 28.9 map units from pK24. HSP179D, a class II (formerly class IV, Raschke et al. 1988) gene, detected two amplified sequences with a size difference of at least 50 bp in a random sample of 12 soybean lines, but is not polymorphic between PI 96983 and Lee 68. Segregation analysis of these two small HSP genes in a cross of PI 486355 X Essex indicates that they are independent of each other (data not shown).

Two pairs of primers were synthesized for two discrete portions of HSP173B for ASLP analysis. None of these two primer pairs detected polymorphism between PI 96983 and Lee 68. Neither did a pair of primers for the HSP175M locus. Therefore, we were not able to determine the map location of HSP173B and HSP175M. Subsequently, a marker that is closely linked to a locus detected by the HSP173B probe (Rafalski and Tingey, 1992) was selected to determine whether the HSP173B locus is linked to Rsv1. This marker, termed P11, belongs to a soybean

auxin down-regulated multigene family, and has been sequenced (Datta et al. 1993). ASLP analysis of one member of this multigene family, ADR11, showed that three Rsv1 near-isogenic lines, L81-4420, L78-379, and L84-2112, are different from the recurrent parent but identical to their respective Rsv1 donor (Fig. 2), indicating that ADR11 is closely-linked to Rsv1. Polymorphism between PI 96983 and Lee 68 was also found as one band is absent in PI 96983 but present in Lee 68. This band segregates as a dominant marker (3 presence : 1 absence) in the F₂ population (Table 1). No F₂ recombinant was found between ADR11 and HSP175E, indicating that ADR11, as well as HSP173B, is closely linked to HSP175E, HSP176L, and Rsv1.

The most probable map position of Rsv1 on linkage group F, as shown in Fig. 3 with respect to pK644a, pA186, HSP176L, HSP175E, and ADR11, was determined by multiple linkage analysis using Mapmaker /exp program (Lander et al. 1987). The map order of Rsv1 with respect to the four closely-linked marker loci is provisional, since an alternative map order with an LOD (log-likelihood) only 1.30 lower was found (not shown). The nine recombinants from the 174 F₂ plants contain too many double crossing-over in this chromosomal region according to the current map order (Table 3). Whether this is indicative of error in genotyping or unusual chromosomal behavior (rearrangement or unequal crossing-over) requires further studies to clarify. The two point linkage of Rsv1 to ADR11, HSP175E, HSP176L, pA186 and pK644a is 2.1, 2.4, 1.5, 2.1, and 1.7 cM, respectively.

DISCUSSION

Chromosomal locations of small HSP multigene families

The existence of multigene families in the vicinity of Rsv1 will provide a substantial number of closely-linked markers. The entire family of class I small HSP genes, at least 13 (Nagao et al. 1985), will be valuable in saturating this chromosomal region, and in physical mapping and positional cloning of the Rsv1 gene. In this study, we developed a new procedure, ASLP, to convert known DNA sequences of small HSP genes to polymorphic genetic markers. We found that two class I genes, HSP175E and 176L, are closely linked to each other (0.7 cM), and to the Rsv gene. Another class I gene, HSP173B, is also closely-linked to Rsv1, based on ASLP analyses of the HSP173B-linked ADR11 among a set of Rsv1 near-isogenic lines and in an F₂ population. The linkages between three members of the class I multigene family of small HSPs, HSP175E, 176L, and 173B, indicates that this multigene family exists as a gene cluster.

Multiple linkage analysis indicates that the exact position of Rsv1 with respect to small HSP genes could not be determined in this population of 174 F_{2,3}, due to the lack of a unique linear map order among these closely-linked loci. Two-point linkages of Rsv1 to HSP176L and HSP175E are 1.5 and 2.4 cM, respectively. ADR11, which is also linked to Rsv1 with a two-point linkage of 2.1 cM, belongs to another multigene family whose expression is down regulated by auxin (Datta et al. 1993). Thus, it is likely that more than one multigene family may be located in the

Rsv1 resistance gene cluster, and could be used as genetic markers to saturate this chromosomal region.

HSP185C was previously reported as a class I gene (Raschke et al. 1988), but its independent chromosomal location suggests that it should belong to a different class (family). The coding sequence of HSP185C differs from the class I gene (HSP175E, 176L, 175M, and 173B) because of an insertion of 21 bp (or 7 amino acid residuals, positions 40-46) (Raschke et al. 1988). We propose that HSP185C belongs to a new multigene family (Class III). In an RFLP analysis (Rafalski and Tingey 1992), probe HSP173B detected two independent loci, one of which corresponds to class I and the other probably to a class III gene. HSP179D is a class II (formerly VI) gene (Raschke et al. 1988), and its independence from both class I (HSP176L) and III (HSP185C) genes (segregation data not shown) is consistent with the map location reported by Rafalski and Tingey (1992). So, there are at least three multigene families of small HSP genes (for 15-18 kD), class I, II and III, each located in a different chromosome as a gene cluster.

Clustering of potyvirus resistance genes

The linkages of SMV resistance to peanut mottle resistance (Rpv1) (Roane et al. 1983; and Yu et al. unpublished data) and to peanut stripe resistance (Choi et al. 1989), suggests that a cluster of distinct genes are responsible for potyvirus resistance. In addition, the Rsv1 gene in soybean is mapped to a position close to a Phytophthora resistance gene Rps3 (Yu et al. 1994; Diers et al. 1992), indicating

that the genes in the Rsv1 cluster may not be restricted to potyvirus resistance.

Clustering of potyvirus resistance genes has been reported in other crops. In common bean, a potyvirus resistance gene (I) or a gene complex reportedly confers necrotic response to SMV, watermelon mosaic virus 2 (WMV2), and three other potyviruses (Kyle and Provvidenti, 1993). Another gene for SMV resistance (Smy) is independent of I (Provvidenti et al. 1982), as is the Wmv gene for WMV2 resistance (Kyle and Provvidenti, 1987). The allelic relationship between Wmv and Smy has not been determined. Resistance to each of the potyviruses that cause disease in pea is controlled by one of the resistance genes clustered in chromosome 2 or 5 (Provvidenti 1991). In maize McMullen and Lourie (1991) mapped a gene for resistance to wheat streak mosaic virus, Wsm1, to the same chromosomal region as a maize dwarf mosaic virus resistance gene, Mdm1 (McMullen and Lourie, 1989).

A possible correlation between HSPs and virus resistance

Close linkages between resistance gene clusters and multigene families have been found previously. For instance, Wise and Ellingboe (1985) reported that the barley Ml-a resistance gene cluster is flanked by Hor1 and Hor2, members of a seed storage protein gene family. Paran et al. (1992) mapped nine loci of a multigene family detected by a random cDNA probe, four of them to three resistance gene clusters. These genetic data support the hypothesis that resistance genes may be members of multigene families with common origin and function yet diverged specificity (Pryor, 1987). Nonetheless, no definitive correlation has been established

for a resistance gene cluster and a multigene family.

In this study, two gene clusters, one of class I small HSP genes and the other of potyvirus resistance genes, are mapped to the same soybean chromosomal region in very close linkage. The small HSPs are among the abundant HSPs induced by heat shock or other stress conditions. Cytoplasmic small HSPs have been reported to form large aggregates termed as heat shock granules (Nover et al. 1983). No physiological function has been established for small HSPs (Vierling 1991). On the other hand, potyvirus resistance conferred by the I gene in common bean is temperature-dependent (Kyle and Provvidenti 1993), and SMV resistance in soybean is reportedly temperature sensitive (Mansky et al. 1991). The tomato genes, Tm-2 and Tm-2², confer symptomless resistance to tobacco mosaic virus (TMV) at normal temperature, but cause severe systemic necrosis at elevated temperature (Cirulli and Alexander, 1969). High temperature affects the expression of N and N' genes (van Loon 1975) and coat-protein mediated resistance (Nejidat and Beachy, 1989) to TMV in tobacco.

HSPs (small HSPs and HSP60, 70, and 90) are essential to protein folding and assembly, and replication of small viruses in bacteria depends on two HSPs named groEL and groES (Georgopoulos et al. 1973). A small HSP (HSP26) homologue in potato is induced by fungal infection (Taylor et al. 1990) and an HSP90 gene in barley is activated during powdery mildew infection (Walther-Larsen et al. 1993). Koonin et al. (1991) reported the existence of amino acid similarity among movement proteins of diverse groups of plant RNA and DNA viruses, suggesting that a common

mechanism of cell-to-cell movement for these viruses. Interestingly, a large subset of these movement proteins was found to share a conserved motif with HSP90, indicating that the chaperon-like function of HSPs may be essential to mediate virus transport. Whether the positional relationship between the Rsv1 gene cluster and the class I small HSP multigene family is indicative of a functional association deserves further investigation.

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TABLE 1. Segregation of three types of molecular markers (simple sequence repeats or microsatellite, amplified sequence length polymorphism, and restriction fragment length polymorphism), a morphological marker (W1), and resistance to SMV (strains G1) in the F₂ population from PI 96983 X Lee 68.

Marker type ^a	Loci	No.	segregation			χ^2 (1:2:1)	p
			aa ^b	ab	bb		
SSR	HSP176L	163	39	83	41	0.10	0.95
ASLP	HSP175E	154	36	75	43	0.74	0.69
ASLP	ADR11	118	28	90		0.10 ^c	0.75
RFLP	pA186	166	41	85	40	0.11	0.95
RFLP	pA245	97	34	48	15	9.30	0.01
RFLP	pA401	148	45	103		2.31 ^c	0.13
RFLP	pA708	149	37	75	37	0.01	0.99
RFLP	pA806	152	52	66	34	6.90	0.03
RFLP	pK2	172	47	84	41	0.51	0.77
RFLP	pK390	168	56	75	37	6.22	0.04
RFLP	pK644a	170	43	87	40	0.20	0.90
NEM	<u>W1</u>	148	37	78	33	0.65	0.72
SMV	<u>Rsv1</u>	144	38	76	30	1.33	0.51

^aSSR = simple sequence repeats or microsatellites; ASLP = amplified sequence length polymorphism; RFLP = restriction fragment length polymorphism; NEM = naked eye morphology; SMV = resistance to soybean mosaic virus strains G1.

^bAt each of the loci shown, aa = PI 96983; ab = heterozygous; bb = Lee 68.

^cADR11 and pA401 segregate as dominant markers, thus their χ^2 tests are to the 3:1 ratio.

TABLE 2. Polymerase chain reaction (PCR) primers that detected amplified sequence length polymorphism (ASLP) between PI 96983 and Lee 68.

Locus	Primers ^a	size ^b (bp)	sequence references
HSP175E	(F) 5'TATTTTTTCCACTTCAATTCCTCC (R) 5'CTTCTGGACATCATTCAATAAATAC	279	Czarnecka et al. 1985
HSP185C	(F) 5'GTCCCTTGTATATTTGTATTGTTCAA (R) 5'TTTCTACAGAAAACCAGGGGC	294	Raschke et al. 1988
ADR11	(F) 5'AAATCGACATGAGAGGATGTC (R) 5'AAACCATACATGCAACGATCTC	126	Datta et al. 1993

^a Primers for each locus, including forward (F) and reverse (R) primers, were designed by computer program Primer 0.5 (Whitehead Institute, Cambridge, MA).

^b The size of amplification products expected from the published sequences.

TABLE 3. F₂ plants from PI 96983 X Lee 68 that contain crossing-overs between the six closely-linked loci (ADR11, HSP175E, HSP176L, pA186, pK644a, and Rsv1). pK2 and pA708 flank distantly the left and right side of this six-locus cluster, respectively.

		Genotype ^a						
	(K2)	ADR11	H175E	H176L	A186	K644a	<u>Rsv1</u>	(A708)
PL129	(aa)	aa	aa	aa	aa	ab	ab	(ab)
PL86	(aa)	b-	bb	ab	ab	ab	ab	(ab)
PL52	(ab)	b-	-	-	aa	ab	aa	(aa)
PL14	(ab)	b-	ab	ab	ab	ab	aa	(ab)
PL104	(ab)	b-	ab	ab	bb	bb	ab	(ab)
PL106	(ab)	b-	bb	bb	bb	bb	ab	(ab)
PL80	(bb)	b-	bb	ab	ab	aa	aa	(-)
PL20	(bb)	b-	bb	bb	ab	ab	bb	(ab)

^a At each of the loci shown, aa = PI 96983; ab = heterozygous; bb = Lee 68.

Notes: 1. The distances (cM) among the above seven loci are shown in Figure 2.

2. No crossing-over was detected between the six above closely-linked loci (HSP175E, 176L, pA186, pK644a, and Rsv1) in the remaining 166 F₂ plants of PI 96983 X Lee 68.

PI 96983
Lee 68

F₂

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

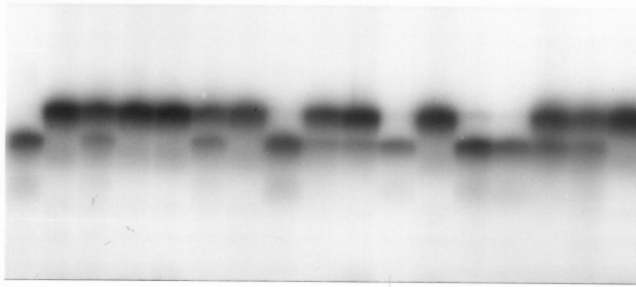


FIGURE 1. The co-dominant segregation of an amplified sequence length polymorphism (ASLP) locus, HSP175E, in the F₂ population from PI 96983 X Lee 68. The targeted sequences of HSP175E was amplified by PCR in the presence of alpha-³²P-dCTP, seperated in a 7% polyarylamide gel, and detected by autoradiography.

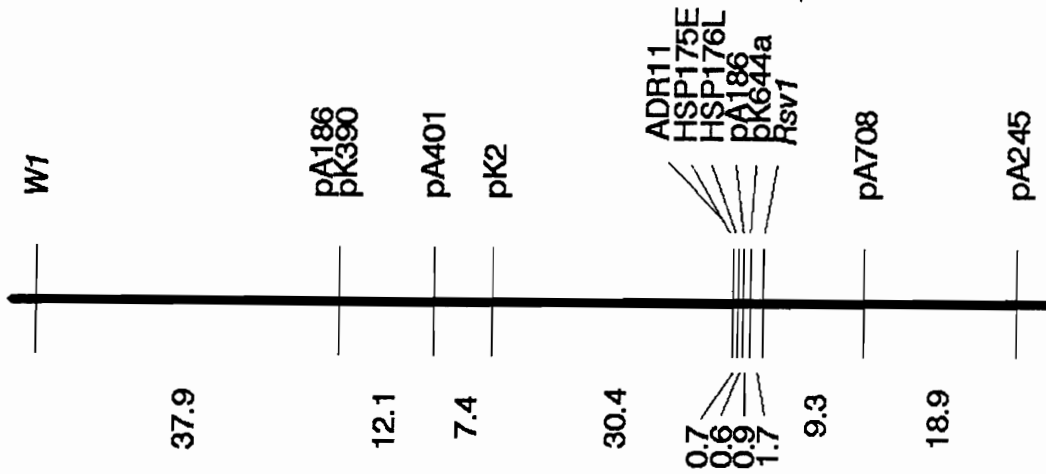


FIGURE 2. The linkage map of Rsv1 showing morphological, RFLP, microsatellite or SSR, and ASLP markers. The order and kosambi distances shown were obtained from linkage data of 174 F₂ of PI 96983 X Lee 68 by Mapmaker / exp. An alternative map order exists with an LOD (log likelihood) score slightly (1.30) lower than the map order above. The two point linkage of Rsv1 to ADR11, HSP175E, HSP176L, pA186, and pK644a is 2.1, 2.4, 1.5, 2.1, and 1.7 cM, respectively.

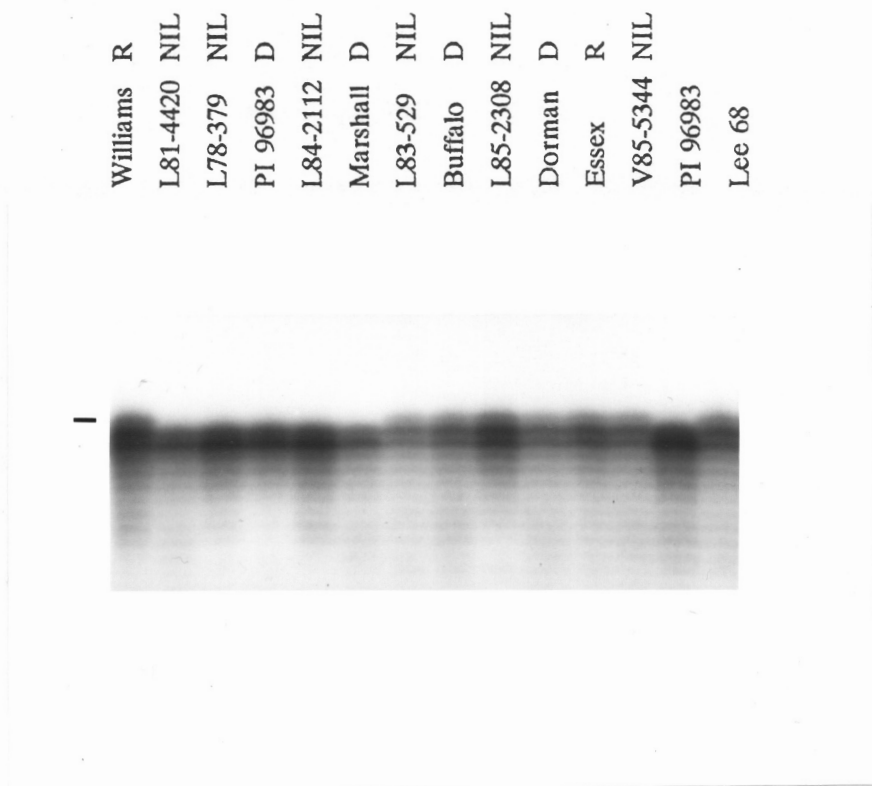


FIGURE 3. Analysis of an ASLP locus, ADR11, in a set of near-isogenic lines (NILs) indicates that this locus is linked to Rsv1. Three NILs, L81-4420, L78-379, and L84-2112, exhibits the same HSP173B genotype as their respective donor parents, PI 96983 and Marshall, and different from the recurrent parent Williams. By definition, these NILs are nearly identical to Williams except Rsv1 and a small flanking chromosomal segment. Thus, ADR11 is closely-linked to Rsv1. Also shown is the polymorphism between PI 96983 and Lee 68, which is used as a dominant marker in the F_2 population.

CHAPTER V.

Summary and Future Prospects

Host resistance is the only practical means currently available to control soybean mosaic virus (SMV), a potyvirus pathogen of soybean detected worldwide. Rsv1, a single dominant gene conferring symptomless resistance to SMV, is present in PI 96983 and the majority of resistant soybean cultivars studied. The soybean-SMV system can be regarded as a model for gaining insights into host-virus interactions. Genetic analyses of the Rsv1 gene were conducted to facilitate the development of disease resistant cultivars, and to enhance our understanding of the molecular mechanism of host resistance.

1. Genetic mapping of Rsv1 using molecular markers

The chromosomal location of Rsv1 was determined by genetic mapping in an F₂ population from a cross between PI 96983 and a susceptible cultivar. The genetic markers used include restriction fragment length polymorphisms (RFLPs) and microsatellites or simple sequence repeats (SSRs), and a morphological marker (W1). Twenty five RFLP and three SSR loci polymorphic between the parental lines were analyzed in 104 F₂ individuals. Genotypes of Rsv1 were determined by inoculating F₂₋₃ progeny with the G1 strain of SMV. Analyses of the data revealed that the SSR marker, HSP176L, and two RFLP markers, pA186 and pK644a, are closely linked to Rsv1, with a distance of 0.5, 1.5, and 2.1 cM, respectively.

The close linkages of the three markers to Rsv1 were confirmed by analysis of three Williams near-isogenic lines (NILs) each carrying an Rsv1 allele derived from

PI 96983 or Marshall. Marker analysis also indicated that the SMV resistance gene in Buffalo is probably at the Rsv1 locus.

To our knowledge, this was the first successful use of microsatellite markers in mapping important plant genes. Microsatellites are ideal genetic markers because they are highly polymorphic and can be rapidly typed by polymerase chain reaction (PCR). Another type of PCR-based markers, random amplified polymorphic DNA (RAPD) (Martin et al. 1991), has been used in a variety of genetic studies. After screening over 200 random oligonucleotide primers for RAPD markers that can distinguish a set of Williams NILs, however, we found that RAPD products are frequently not reproducible (Yu et al. unpublished data). RAPD markers can be misused or misinterpreted in mapping resistance genes (e.g. Dickinson et al. 1993), whereas microsatellites are specific and robust.

2. Marker-assisted screening of SMV resistance sources

The three molecular markers tightly-linked to Rsv1 were used to screen 67 diverse soybean types including cultivars, breeding lines, and plant introductions. The three marker loci show a remarkably high level of restriction polymorphism, indicating a possible association between disease resistance and rapid sequence divergence. Several specific alleles at the three marker loci were found to be highly correlated with virus resistance. These resistance markers (R markers), especially an HSP176L allele (HSP176L-2) which can be detected by the polymerase chain reaction

(PCR), may be useful for germplasm screening.

The grouping of the 67 soybean accessions according to their multilocus haplotypes agrees with the available pedigree information. The lines of a group are likely to carry an Rsv1 allele if their haplotype contains an R marker at one or more of the three loci, and all or most of the accessions in the group are resistant. A set of differential cultivars known to contain Rsv1 were located in several putatively Rsv1-carrying groups. Based on molecular marker analysis and previous inheritance studies, 37 of the 45 accessions that are resistant to SMV probably derive their resistance from Rsv1.

Among the remaining eight resistant accessions, Columbia is known to carry an independent resistance gene, Rsv3, but further inheritance studies is required to determine the allelic relationships for Holladay, Jizuka, Peking, Virginia, FFR-471, PI 507403, and PI 556949. It appears from marker data, subject to inheritance test, that Jizuka contain carry Rsv1 gene, and genes independent of Rsv1 condition SMV resistance in Virginia and Holladay.

3. Rsv1 and a possible resistance gene cluster

Rsv1 has been found to be linked (3.5 cM) to Rpv1, a peanut mottle virus (PMV) resistance gene in York (Roane et al. 1983), and SMV resistance in AGS 129 is linked to peanut stripe virus (PStV) resistance (Choi et al. 1989). The map position of Rsv1 is in the vicinity of Rps3, a Phytophthora resistance gene (Yu et al.

1994; Diers et al. 1992). These observations, and the large number of "alleles" of Rsv1 we have found, suggest a possible resistance gene cluster in the Rsv1 chromosomal region, as the resistance gene complexes found in other crops (e.g., Wise and Ellingboe, 1985; Kesseli et al. 1993). In order to determine whether resistance to PMV and the necrotic reaction to SMV-G7 in PI 96983 are pleiotropic effects of Rsv1, a portion of the F₂₃ lines of PI 96983 X Lee 68 were inoculated with PMV and SMV-G7. PMV resistance segregated as a single dominant gene, and two point linkage analysis found it is 4.4 ± 2.5 cM from Rsv1. Among 49 F₂₃ lines inoculated with both strains of SMV, two lines appear to contain a cross-over between G1 and the gene conferring necrotic reaction to G7, indicating a necrosis gene may exist in PI 96983, 3.3 cM away from Rsv1 (Yu et al. unpublished data). The number of F₂₃ lines used to study the necrosis gene, however, is too small to make a confident conclusion. A future study using a larger population is necessary to clarify the relationship between the necrosis gene and Rsv1.

4. Closely-linked multigene families, and consideration for positional cloning of Rsv1

HSP176L, which is closely-linked to Rsv1, belongs to the class I small heat shock protein (HSP) multigene family. A technique termed amplified sequence length polymorphism (ASLP) was developed to convert known DNA sequences of small HSP genes to PCR-based genetic markers. Among six pairs of HSP primers

used, two (HSP175E and 185C) detected ASLPs between the parents, PI 96983 and Lee 68, and the corresponding loci were mapped using 174 F₂ individuals. HSP175E was found to be closely-linked (0.7 cM) to HSP176L in the linkage group F, both of which are class I small HSP genes. HSP185C was mapped to linkage group M, suggesting that it may belong to a family other than class I.

The chromosomal location of an auxin down-regulated gene (ADR11), which belongs to an ADR multigene family, was studied because of its close linkage to HSP173B. HSP173B is a class I gene that was not mappable in this population due to the lack of ASLP. ASLP analysis using ADR11 in a set of Rsv1 NILs indicates that this locus is linked to Rsv1, and the ASLP marker co-segregates with HSP175E in the F₂ population. Thus, the ADR multigene family (ADR11), and the class I small HSP genes (HSP176L, 175E, and 173B), are located near Rsv1.

An immediate future goal should be the isolation and sequencing of Rsv1, which will enhance our capability of developing durable virus resistance. The finding of two closely-linked multigene families, ADR and small HSP, in the Rsv1 chromosomal region will facilitate the high resolution mapping by providing molecular markers. The single gene control of SMV resistance, the relatively small genome size of soybean, the well established virus-plant interactions, and the amenability of soybean for transformation make it feasible to clone Rsv1 via positional cloning. More importantly, both multigene families consist of expressed genes, which are related to stress conditions but for which no function has been established. Based on

their position information from this study, these genes can be used as "candidate genes" (Ballabio 1993) to examine their possible role in disease resistance, which may lead to the cloning of Rsv1 and other resistance genes.

5. References

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APPENDIX I**REFEREED JOURNAL PUBLICATIONS**

Yu, Y. G., Saghai Maroof, M. A., Buss, G. R., Maughan, P. J., and Tolin, S. A. 1994. RFLP and microsatellite mapping of a gene for virus resistance in soybean. *Phytopathology* 84:60-64.

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Yu, Y. G., Saghai Maroof, M. A., and Buss, G. R., and Tolin, S. A. 1994. A cluster of soybean potyvirus resistance genes is closely-linked to a multigene family. *Mol. Plant Microbe Interact.* (to be submitted)

Ragab, R. A., Saghai Maroof, M. A., Dronavalli, S., and Yu, Y. G. 1994. Construction of sorghum RFLP linkage map with maize and sorghum probes. *Genome* (accepted)

Saghai Maroof, M. A., Van Scoyoc, S. W., Yu, Y. G., and Stromberg, E. L., 1993. Gray leaf spot disease of maize: rating methodology and inbred line evaluation. *Plant Disease* 77: 583-587.

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Saghai Maroof, M. A., Yu, Y. G., Xiang, Z., Stromberg, E. L., and Rufener K. 1994. Identification of quantitative trait loci controlling resistance to gray leaf spot in maize.

Saghai Maroof, M. A., Yu, Y. G., and Warren, H. 1994. Quantitative trait loci controlling resistance to H. leaf spot in maize.

PRESENTATIONS AND ABSTRACTS

Yu, Y. G., Saghai Maroof, M. A., Buss, G. R., Maughan, P. J., and S. A. Tolin. 1994. A soybean virus resistance gene (Rsv) is tightly linked to a multigene family. Plant Genome II, San Diego, CA

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Yu, Y. G., Saghai Maroof, M. A., Xiang, Z., and Stromberg, E. L. 1991. RFLP mapping of quantitative trait loci controlling resistance to gray leaf spot of maize. 3rd annual meeting of The Chinese Agricultural Society of Students and Scholars.

VITA

Yong Gang Yu was born October 15, 1965 in Xinchang County, Zhejiang Province, People's Republic of China. Mr. Yu completed his elementary through high school education when he graduated from Xiaojiang High School in July 1980. In October, 1980, he was admitted to Zhejiang Agricultural University (ZAU), Hangzhou, Zhejiang. He received a B.S. degree with a major in Agronomy in 1984, and subsequently a M.S. degree in Agricultural Biophysics in 1987, both from ZAU. He then worked in the Institute of Nuclear Agricultural Science at ZAU until February 1989, when he left for Rutgers University, New Brunswick, NJ, as a visiting scientist in plant molecular biology. He came to Virginia Polytechnic Institute and State University, Blacksburg, VA, in October 1989 to participate in a molecular genetics research project. He was admitted to the graduate program of Crop and Soil Environmental Sciences in Fall 1990, and to the interdepartmental program of Genetics in Fall 1991. Mr. Yu was elected to the honor society of Phi Kappa Phi in 1992, and Gamma Sigma Delta in 1994. He is a member of the Genetic Society of America, American Society of Agronomy, Crop Science Society of America, and American Association for the Advancement of Science. He is a recipient of Charles I. Rich Graduate Fellowship from the Department of Crop and Soil Environmental Science in 1993, and Gerald O. Mott Meritorious Graduate Student Award from the Crop Science Society of America in 1994.



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