MOLECULAR MAPPING OF A SOYBEAN MOSAIC VIRUS (SMV) RESISTANCE GENE IN SOYBEAN (*Glycine max*)

By

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MOLECULAR MAPPING OF A SOYBEAN MOSAIC VIRUS (SMV) RESISTANCE GENE IN SOYBEAN (*Glycine max*)

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Abstract

Soybean mosaic virus (SMV) is the major virus disease reported all over the world in soybean. This disease causes reduction in the yield and quality of the soybean crop. Three independent genes *Rsv1*, *Rsv3*, and *Rsv4*, were found to provide host resistance in soybean. *Rsv1* confers resistance to all but most virulent strains of SMV. *Rsv1* has been mapped to soybean molecular linkage group (MLG) F by using molecular markers. The purpose of this study is to investigate the location of the *Rsv3* gene on the soybean genomic map using molecular markers. The *Rsv3* gene of soybean confers resistance to the most virulent strains (G5-G7) of SMV. In order to map the gene, an F_2 population was constructed from a cross between L29, an *Rsv3* isoline of 'Williams', and 'Lee 68', a susceptible cultivar. *Rsv3* genotypes of 183 F_2 plants were determined by inoculating $F_{2:3}$ progeny with the G7 strain of SMV. A preliminary survey of two parental lines, near isogenic lines (NILs), and bulk segregants with 136 restriction fragment length polymorphism (RFLP) markers yielded 36 markers showing variation between the two parents. These polymorphic RFLP markers were unable to provide any indication of linkage to *Rsv3*. As an alternative strategy, amplified fragment length polymorphic (AFLP) marker analysis of the two parental lines, NILs and bulk segregants was performed using 64 primer combinations. Initial breakthrough came in the form of AFLP primer combination of Eco+AAC/Mse+CTG, which exhibited polymorphism between NILs, bulk segregants, and two parental lines. This AFLP marker was isolated and cloned to convert it into a RFLP clone to further investigate the linkage to *Rsv3* by F_2 segregation analysis. A mapping population constructed by crossing *Glycine max* x *Glycine soja* was employed in determining the location of the AFLP-derived RFLP clone on the soybean linkage map. This population has densely mapped molecular marker data that enabled determining the location of AFLP-derived RFLP clone ACR1 on soybean MLG B2 between the markers pA516 and pA519. This finding made it

easy to establish the linkage of markers pA519, pA516, and pA593 in L29 x Lee 68 population by F_2 segregation analysis. The closest linked marker, pA519, was 0.9 cM away from *Rsv3*. Results of this study are useful in marker-based selection (MAS), pyramiding viral resistance genes, and in cloning the *Rsv3* gene.

DEDICATION

I would like to dedicate this thesis to
my beloved parents,
my wife with whose support and sacrifice I reached at this point of journey
and everyone who supported me along the way.

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INTRODUCTION

Diseases caused by viruses are important from an economic standpoint. The use of resistant cultivars is the cheapest and most effective way of combating virus diseases (Walkey, 1991). The cost of growing a resistant cultivar is likely to be no greater than growing a susceptible one, and savings are made in the form of not having to take expensive preventive or control measures to keep the vector under control (Walkey, 1991) and in reduced yield losses.

Soybean mosaic virus (SMV) is the most commonly occurring virus disease on soybean plants. Control through developing host resistance is the only effective solution for this disease. In the majority of reported cases, a single dominant gene at the locus *Rsv1*, confers SMV resistance (Kiihl and Hartwig, 1979). Yu et al. (1994) reported mapping the *Rsv1* gene to the linkage group 'F' (USDA-ARS Soybean map) by using DNA-based markers. None of the reported alleles of *Rsv1* gives resistance to all the strains of SMV. Each allele gives resistance to some of the strains, while being necrotic or susceptible to other strains (Ma et al., 1995). Since *Rsv1* has been the most widely used SMV resistance gene in soybean breeding programs (Ma, 1996), a condition of genetic uniformity exists.

To achieve durable resistance, it is better to have different sources of resistance accumulated into a single line or cultivar, which is referred to as gene pyramiding. The first step in the process of gene pyramiding is to locate and characterize multiple sources of resistance. As a result of the search for additional sources of SMV resistance in soybean, *Rsv3* was discovered in 'L29' (Ma, 1996), a BC_5-derived isoline of 'Williams' containing a resistance gene from 'Hardee' (Bernard et al. 1991). This gene is reported to be a single dominant gene at a locus independent of *Rsv1* and *Rsv2* (Ma, 1996). In contrast to most *Rsv1* alleles, *Rsv3* confers resistance to the higher strains of SMV (G5-G7), while being susceptible to lower strains (G1-G4).

Ma et al., (1995) reported yet another resistance gene, *Rsv4*, derived from PI 486355. It confers resistance to all seven strain groups of SMV. The segregation analysis of $F_{2:3}$ population involving *Rsv4* did not show necrotic reaction (Ma et al., 1995).

Molecular markers are useful in marker-assisted selection in breeding for resistance as well as in pyramiding multiple sources of resistance. By tagging various sources of resistance with molecular markers, the ultimate objective of gene-pyramiding could be achieved relatively easily (Melchinger, 1989). The molecular markers can be utilized to select genotypes with multiple resistance genes from segregating populations or rapid transfer of resistance genes to otherwise agronomically superior cultivars by backcross breeding. A backcrossing program can also be accelerated by using marker-based selection for the genotype of the recurrent parent (Tanksley and Rick, 1980). Molecular mapping of a resistance gene is an initial step towards map-based cloning and isolation of the gene itself.

Once the gene is isolated, it could be rapidly transferred to susceptible cultivars by transgenic means. This process eventually helps in understanding the host-pathogen interaction. The objective of this study was to map the *Rsv3* gene in the population of L29 x Lee 68, using DNA-based molecular markers.

LITERATURE REVIEW

Soybean mosaic virus (SMV) is a potyvirus that causes one of the most prevalent soybean [*Glycine max* (L.) Merr.] viral diseases in the world (Sinclair, 1982; Thottapilly and Rossel, 1987). The disease symptoms caused by SMV on soybean range from mild mosaic to lethal necrosis (Ross, 1969; Tu and Buzzell, 1987; and Chen et al, 1991; Kwon and Oh, 1980). The losses due to SMV are in the form of reduction in yield and deterioration of seed quality. Yield losses have been reported from all over the world, wherever soybean is grown (Sinclair, 1982; Thottappilly and Rossel, 1987). The yield losses due to SMV disease can be as high as 50% and deterioration of seed quality results in low price in the market (Demski et al., 1989). Disease epidemics and severe yield losses were reported from Asian countries; China, Korea, Indonesia, Japan, and the former Soviet Union (Irwin and Goodman, 1981).

The virus is seed borne and also transmitted by over 30 aphid species in a non-persistent manner (Demski et al., 1989; Abney et al., 1976). The pervasiveness of SMV is mainly attributed to its seed borne nature. The SMV disease is distributed to any part of the world, wherever the seed

from infested fields is transported (Sinclair, 1982). Infected soybean seed is the primary source of initial inoculum (Hill and Benner, 1980a) and is further spread by aphids.

Properties and molecular aspects of SMV

SMV belongs to the potyvirus group of viruses (Bos, 1972). According to the classification system proposed by Lindbo and Dougherty (1994), SMV is a member of the family *Potyviridae*, genus *Potyvirus*. The *Potyvirus* genus is the largest and economically the most important group of plant viruses, infecting a wide range of species (Mathews, 1991).
Physical properties: SMV is a flexuous particle about 750 *nm* in length (Bos, 1972). The thermal inactivation point is reported to be 55-60 °C for 10 min. The dilution end-point is around 10^{-3}. The infectivity in leaf sap commonly is 2-3 days at room temperature (Bos, 1972). Longevity of the virus *in vitro* is 14 to 15 days in plant sap stored at 4 °C (Galvez, 1963). The virus is most stable at pH 6.0 in expressed sap and loses infectivity at pH levels below 4 and above 9 (Galvez, 1963).

Molecular and genomic properties

SMV, as a species of the Potyvirus genus, consists of a single stranded RNA genome encapsidated in a coat protein with a single structural subunit. The molecular weight of the coat protein is 28,300 daltons (Hill and Benner, 1980a), whereas its ssRNA genome which comprises 5.3% of the virus particle, has a molecular weight of 3.02×10^6 (Hill and Benner, 1980b). Jayaram et al. (1992) reported the complete nucleotide sequences of the two SMV strains, G2 and G7, which differ in their 5 prime region. The SMV genome measuring 9588 nucleotides long encodes for nine mature proteins and a genome linked VPg protein.

SMV induces three distinct reactions in soybean: resistance (symptomless), necrosis, and susceptible (mosaic). SMV has been classified into seven strain groups based on the reactions of 98 SMV isolates in a set of differential soybean cultivars (Cho and Goodman, 1979).

SMV is principally a seed-borne virus (Bos, 1972). Under adverse conditions, seed transmission could be as high as 30% or more (Koshimizu and Iizuka, 1963). Transmission through seed is influenced by factors such as time of infection, soybean genotype, and virus strain (Iizuka, 1973; Irwin and Goodman, 1981; Bowers and Goodman, 1979). SMV is also transmitted by over 30 aphid species in a non-persistent manner (Koshimizu and Iizuka, 1963; Abney et al., 1976; Irwin and Goodman, 1981; Maury, 1985; Demski et al., 1989).

SMV is readily transmitted by sap inoculation (Bos, 1972). Various methods of mechanical inoculations are available from studies conducted earlier; however, hand inoculation by rubbing the surface of leaves dusted with an abrasive by a pestle dipped in inoculum gave 100% infection and is an effective method (Cho and Goodman, 1979; Roane et al., 1983).

Maintenance of pure cultures of different virus strains can be achieved by desiccating infected tissue over calcium chloride and storage at 4 °C or preparing a liquid nitrogen powder and storage at -20 °C (Roane et al., 1983). Another method is simply freezing infected leaf tissue at -70 °C (Yong Yu, personal communication). SMV strains can be maintained in a pure form in soybean callus culture induced from infected leaf explants. Transfers of callus need to be made about every two months (Chen et al., 2003).

Host resistance to SMV

Resistance to SMV has been identified in various sources of soybean germplasm. In the majority of the sources studied, the resistance was conferred by a single dominant gene, *Rsv1* (Kiihl and Hartwig, 1979; Roane et al., 1983; Buzzell and Tu, 1984; Shigemori, 1988; Buss et al., 1989a; Chen et al., 1991).

The gene at the *Rsv1* locus and its allelic forms have been widely reported from different inheritance studies (Kiihl and Hartwig, 1979; Buzzell and Tu, 1984; Shigemori, 1988; Buss et al., 1989b; Chen et al., 1991), but no one allele of *Rsv1* gives resistance to all the strains of SMV. Each allele gives resistance to some of the strains of SMV, while being necrotic or susceptible to other strains (Cho and Goodman, 1979; Shigemori, 1988; Gai et al., 1989).

Kiihl and Hartwig (1979) found single genes conferring resistance to the strain SMV-1 in PI 96983 and Ogden. The dominant resistance gene found in PI 96983 was designated as *Rsv* (Kiihl and Hartwig, 1979). The symbol *rsv-t* was proposed for the resistance gene found in cv. Ogden, which was allelic to *Rsv* and showed resistance to SMV-1 strain, but susceptible to SMV-1-B. However, it was later shown to be dominant in cv. York allele and was redesignated *Rsv1-t* by Chen et al. (1991). In the classification system of Cho and Goodman (1979) the strains that were identified as SMV-1 and SMV-1-B were found to be similar to SMV-G2 and SMV-G3, respectively.

In addition to *Rsv1* and *Rsv1-t*, seven other resistance alleles have been identified at the *Rsv1* locus. *Rsv1-y, Rsv1-m*, and *Rsv1-k* have been identified in cvs. York, Marshall, and Kwanggyo (PI 406710), respectively (Chen et al., 1991), while *Rsv1-s* and *Rsv1-sk* are respectively present in PI 486355 (SS74185) and PI 483084 (Suweon 97) both developed at the Crop Experiment Station, Suweon, South Korea (Ma et al., 1995; Chen et al., 2002). The allele present in cv. Raiden (PI 360844) is *Rsv1-r* (Chen et al., 2001). An allele at the *Rsv1* locus in PI 507389 (cv. Tousan50 from Japan) has been tentatively designated *Rsv1-n.* This allele exhibits a severe necrotic reaction with SMV-G1 and is susceptible to the other strain groups (Ma et al., 2003). Cultivars Hill, Essex, and Lee 68, as well as numerous other cultivars, contain the susceptible allele *rsv1*.

Buzzell and Tu (1984) reported the presence of a dominant gene for resistance in the breeding line 'OX 670' which presumably derived its resistance from the 'Raiden' cultivar, and gave resistance to all strains of SMV. Their allelism tests showed the gene was not at the *Rsv1* locus and it was designated as *Rsv2*. However, 'Raiden' the reported source of *Rsv2*, recently has been shown to contain a resistance gene at the *Rsv1* locus rather than at an independent locus (Ma, 1996, Chen et al., 2001). Further analysis of the inheritance of resistance in OX670 by Gunduz et al. (2001) revealed that it actually contained two resistance genes, *Rsv1* and *Rsv3*. The *Rsv3* was derived from cv. Harosoy, but Buzzell and Tu (1984) were likely unaware of its existence.

Tu and Buzzell (1987) observed a dominant gene expressing stem-tip necrosis to strains SMV-G1 and G4 in a breeding line 'OX 686', which was derived from Columbia. This gene was reported to be at a locus independent of *Rsv1* and *Rsv2* and it was assigned the symbol *Rsv3* (Buzzell and Tu, 1989). Ma et al., (2002) found that Columbia is resistant to all known SMV strains G1–G7, except G4. Genetic studies indicate inheritance of resistance to SMV strains in Columbia is due to interactions of two resistance genes. These two genes were denoted as *R3* and *R4* in this article. *R3* appears to be the same gene previously reported as *Rsv3* in OX686, which was derived from Columbia. The *R3* gene confers resistance to G7, but necrosis to G1. The other gene, *R4,* conditions resistance to G1 and G7 at the early seedling stage and then a delayed mild mosaic reaction (late susceptible) 3 weeks later. Plants carrying both the *R3* and *R4* genes were completely resistant to both G1 and G7, indicating that the two genes interact in a complementary fashion. Plants heterozygous for *R3* or *R4* exhibited systemic necrosis or late susceptibility, suggesting that the resistance is allele dosage dependent. The *R4* gene appeared epistatic to *R3* since it masked expression of necrosis associated with the response of *R3*. The complementary interaction of two resistance genes, as exhibited in Columbia, can be useful in development of soybean cultivars with multiple and durable resistance to SMV. An allele of this *Rsv3* gene was reported in L29 (Ma, 1996, Buss et al., 1999) a BC_5 isoline of Williams, containing a resistance gene from Hardee (Bernard et al., 1991). It seems that the *Rsv3* alleles in Columbia and Hardee are not identical since they respond differently to SMV strain groups G1 and G4.

Ma et al. (1995) reported that PI 486355 contained two resistance loci, one which is allelic to *Rsv*1 and another one (*Rsv*4) is not allelic to either the *Rsv*1 or *Rsv*3 locus. Gunduz et al., (2004) subsequently found that the single dominant resistance gene in PI 88788 was allelic to the non-*Rsv1* gene in PI 486355 and both were nonallelic with *Rsv3* and *Rsv1*. The gene symbol *Rsv4* was tentatively assigned to the new locus. The *Rsv*4 locus is a completely dominant gene, in contrast to *Rsv1* alleles. The *Rsv*4 locus from PI 486355 shows resistance without necrosis in both the heterozygous and homozygous states. The resistant allele present in cv. Peking also was demonstrated to be at the *Rsv4* locus (Gunduz, 2000).

Molecular genetics of host-pathogen interactions

Plants defend themselves against pathogens such as viruses, bacteria, fungi, nematodes, insects and even other parasitic plants (Bent et al., 1994). Flor (1947) proposed a "gene-for-gene" model of plant and pathogen interaction based on studies of the fungal rust pathogen of flax, *Melampsora lini.* Pryor and Ellis (1993) explain "Plant defenses are often activated by specific interaction between the product of a disease resistance (R) gene in the plant and the product of a corresponding avirulence (*Avr*) gene in the pathogen". If either the plant or pathogen partner lacks a functional allele of the corresponding gene pair, then resistance is not triggered and the plant becomes diseased (Whitham et al., 1994).

With the advent of burgeoning molecular technology, understanding the interaction between plants and their pathogens has been made easier. Several achievements have been made in understanding and applying molecular genetics of plant disease resistance. On the application side, DNA-based molecular markers are used for locating disease resistance genes. The most commonly used molecular markers at present are: restriction fragment length polymorphism (RFLP) (Botstein et al. 1980; Yu et al., 1994), random amplified polymorphic DNA (RAPD) markers (Martin et al., 1991, Williams et al., 1990), simple sequence repeat (SSR) markers (Yu et al., 1994; Akkaya et al., 1992) and amplified fragment length polymorphic (AFLP) markers (Vos et al., 1995). There is a plethora of examples in the literature. There are a number of economically important disease resistance genes in soybean located by molecular markers to date.

Diers et al., (1992) reported linkage of RFLP markers to five of the six *Rps* loci (*Rps* 1-6) conferring resistance to the Phytophthora root and stem rot disease. Polzin et al. (1994) reported three economically important loci linked together on molecular linkage group (MLG) J (USDA-ARS Soybean map). They are Phytophthora root and stem rot resistance locus (*Rps2*), powdery mildew resistance gene (*Rmd*), and non-nodulation gene (*Rj2*). The *Rh*g4 locus conferring resistance to the soybean cyst nematode (SCN), which is tightly linked to the *I* locus, has been mapped to MLG A and two additional quantitative trait loci (QTLs) associated with SCN resistance were located within 3 cM of RFLP markers on molecular linkage groups G and M

(Webb et al., 1995). Vierling et al. (1996) reported loci associated with SCN resistance: a major locus A006 (MLG B) and three minor loci A567 (MLG S), A487 (MLG A), and A112 (MLG G).

Several soybean disease resistance genes were reported to be located on molecular linkage group (MLG) F forming a disease resistance gene cluster. This cluster of genes include *Rpv1* which confers resistance to peanut mottle virus (Roane et al. 1983), *Rps3* which confers resistance to Phytophthora root rot (Diers et al. 1992), and *Rpg1* which confers resistance to bacterial blight (Ashfield et al. 1996). Tamulonis et al. (1997) reported mapping of a quantitative trait loci for resistance to javanese root-knot nematode to the MLG F. Resistance genes to corn ear-worm and Sclerotonia stem rot are also present in this chromosomal region (Rector et al. 1999; Arahana et al. 2001). *Rsv1*, a single dominant gene which confers resistance to G1-G6 strains of SMV disease has been mapped to the location of gene cluster at MLG F using RFLP and SSR markers (Yu et al., 1994). In order to understand the divergence and allelomorphic relationship of SMV resistant loci, Yu et al. (1996) investigated 67 diverse soybean cultivars, breeding lines, and plant introductions using one tightly linked DNA microsatellite and two RFLP markers. Based on marker variants it was found that *Rsv1* is the most likely source of SMV resistance in 38 resistant lines. According to Yu et al. (1996), the resistant locus in Raiden, was suggested as an allele of *Rsv1* in disagreement with Buzzell and Tu (1984), who reported it as an independent allele at *Rsv2*. However, this finding supports the allelism tests of Ma (1996) which show Raiden to have an *Rsv1* allele.

Further characterization of plant disease resistance genes is found to encode NBS (N-terminal nucleotide-binding site) and LRR (C-terminal leucine-rich repeat) domains (Bent et al. 1994). These are the proteins involved in the process of defense signal transduction cascade. Conserved NBS-encoding sequences have been amplified from numerous plant species using degenerate PCR primers. Many of these resistance-gene candidate sequences have been shown to either map near or cosegregate with resistance-gene loci (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996).

Yu et al. (1996) isolated two classes of NBS sequence, classes b and j, that map to the resistance gene cluster on MLG F. The full length NBS5 (class b) gene is highly homologous to the Toll–

Interleukin-1 cytoplasmic receptor (TIR) subclass of NBS–LRR disease-resistance genes and maps to a single locus on MLG F (Hayes et al. 2000a). Conversely, NBS61 (class j) resembles a second subclass of NBS–LRR disease resistance genes lacking a TIR domain (non-TIR) in the transcribed product. This NBS sequence is one member of a high-copy NBS–LRR gene family mapping to MLG F (Yu et al. 1996). R14, another member of this gene family, was isolated using a modified AFLP approach (Hayes and Saghai Maroof 2000). Twelve non-TIR–NBS class j members from the *Rsv1* resistant line, PI 96983, have been characterized and map to a single locus (or several tightly linked loci) flanking the *Rsv1* gene (Jeong et al. 2001). Some of these sequences may encode portions of functional resistance-gene products.

Gore et al. (2002) further studied resistance gene cluster at MLG F by developing a high resolution mapping employing over 20 RFLP, RAPD and microsatellite markers to map 38 loci covering 6.8-cM region. The main purpose of this study was to understand the disease reaction of potyviruses *Rsv1* and *Rpv1* in soybean. This study demonstrates that *Rsv1* and *Rpv1* are tightly linked at a distance of 1.1 cM. This study also provides support to the hypothesis that several tightly linked genes recognize and respond to SMV infection and that these genes work in concert.

Determination of the molecular map location of the non-*Rsv1* resistant allele reported in Columbia, *Rsv3* (Buzzell and Tu, 1989), is the objective of the current study. Hayes et al. (2000b) reported mapping of SMV resistance loci *Rsv4* to soybean MLG D1b. The *Rsv4* locus confers resistance to all the known strain groups of SMV (Ma et al, 1995). It has been reported that the *Rsv4* locus is flanked by the microsatellite markers, Satt542 at 4.7 cM and Satt558 at 7.8 cM (Hayes et al, 2000b).

Plant breeders can directly employ molecular markers closely linked to the disease resistance genes in their breeding programs. Marker-based selection in disease resistance breeding saves a stupendous amount of time in transferring the genes to susceptible cultivars. Further steps can be taken in the direction of isolating the disease resistance genes and transferring them to susceptible plants with superior agronomic traits.

Importance and objective of the experiment

Rsv1 gene has been mapped by using molecular markers. Efforts are under way to construct a high-resolution map in the vicinity of *Rsv1* to assist in map based cloning (Yu et al, 1994). Since the *Rsv1* gene confers resistance to only some strains of SMV, there is a need for identification of new sources of resistance in order to achieve complete and durable resistance to the SMV disease. In contrast with *Rsv1* and its alleles, *Rsv3* has been reported to be resistant to higher strains of SMV (G5-G7), while being susceptible to lower strains (G1-G4) (Ma, 1995). An allele of *Rsv3* was reported in L29 (Ma, 1996) a BC_5-derived isoline of Williams, containing a resistance gene from 'Hardee' (Dr. R. L. Bernard - Personal communication). The objectives of this study were to locate the *Rsv3* gene to a MLG in the population of L29 X Lee 68, using DNA-based molecular markers.

MATERIALS AND METHODS

Plant materials

Seeds of L29, a backcross-derived isoline of Williams carrying the *Rsv3* resistance gene from Hardee, were obtained from Dr. R. L. Bernard at the University of Illinois (Urbana Champaign, IL). A cross was made between L29 and the susceptible cultivar Lee 68 to investigate the inheritance of resistance (Ma, 1996). The F_1 plants were raised in the greenhouse. The F_2 plants were grown to yield $F_{2:3}$ seeds at the Virginia Crop Improvement Association's Foundation Seed Farm at Mt. Holly, Virginia (Ma, 1996). $F_{2:3}$ seeds harvested from F_2 plants were provided by Dr. G. Ma for this study. In total, 183 $F_{2:3}$ lines were evaluated from this mapping population.

SMV disease reaction

The virus-reaction genotype of each F_2 plant from the L29 X Lee 68 cross was determined by inoculating $F_{2:3}$ plants with SMV strain G7 (supplied by Dr. S. A. Tolin at Virginia Tech) in the greenhouse. The choice of conducting the experiment in the greenhouse rather than in the field was to prevent the risk of spreading the virulent SMV-G7 strain into the environment. Twenty seeds from each $F_{2:3}$ line were planted in six-inch plastic pots containing a 1:1 mixture of top soil and commercial potting soil. A set of six SMV strain differentials, 'PI 96983', 'York', 'Ogden', 'Hardee', 'Lee 68', and 'L29' were included as checks in the experiment. The inoculum was maintained on the susceptible cultivar York, which is resistant to G1 and susceptible to G7, thus eliminating the possibility of contamination with G1. Inoculations were performed approximately 10 days after planting when the unifoliolate leaves were fully expanded (Hunst and Tolin et al., 1982). Local necrotic symptoms appeared on inoculated leaves. One to two weeks after inoculation, mosaic symptoms were fully developed on newly formed trifoliate leaves. Plants were scored for disease reaction at two weeks and again at four weeks after inoculation. Reactions were recorded as either resistant (symptomless) or susceptible (mosaic).

Molecular Mapping

Soybean leaf tissue was used for DNA extraction. Soybean leaf tissue collection and extraction of DNA from freeze-dried tissue was done according to the procedures described previously by Saghai Maroof et al., (1984). Parental lines L29 and Lee 68, as well as Williams and Hardee, the recurrent and donor parents for L29, respectively, were screened with molecular markers to detect polymorphism. Resistant and susceptible pools for bulked segregant analysis (Michelmore et al., 1991) consisted of DNA from 15 homozygous resistant and 15 homozygous susceptible F_2 plants. About 10 μg DNA was taken from each source plant for the purpose of bulking. DNA extracted from mapping populations of F_2 and F_3 generations were used in this study. Two sets of DNA samples were prepared for segregation analysis. Leaf tissue samples from 90 F_2 plants were used to constitute one set. Later the second set with an increased population size of 183 DNA samples was extracted from leaf material harvested from F_3 plants. Each sample in the second set was prepared by bulking at least 15 segregating F_3 plants, which represents their F_2 genotype. For RFLP analysis, DNA digestion and hybridization were carried out essentially as described previously (Yu et al., 1994). The restriction enzymes *BamHI*, *DraI*, *EcoRI*, *EcoRV*, and *HindIII* were initially used. For the clones that did not detect polymorphism with those enzymes, *BclI*, *TaqI*, and *XbaI* were employed to obtain polymorphism. Eight micrograms of DNA from each sample were digested with a restriction enzyme for 24 hours. Digested DNA fragments were separated on 0.8% agarose gels according to standard electrophoresis procedures. Separated DNA fragments were then transferred to nylon membrane by Southern blotting. These filters containing DNA samples were used in genotyping by RFLP markers. In order to identify genomic clones located near *Rsv3*, clones from three different sources were examined: i) Publicly available RFLP soybean genomic DNA probes from the Iowa State University/USDA soybean genomic library; ii) Soybean *NBS* probes (Yu et al., 1996); iii) A few cowpea and mung bean genomic clones (obtained from Dr. N. Young, Univ. of Minnesota).

Genomic DNA inserts were prepared either by excision of the insert by restriction digestion of vectors or by amplification via PCR. The inserts were radioactively labeled with ^{32}P-dCTP by random priming procedure (Feinberg and Vogelstein, 1983). The labeled probes were

introduced to Southern blot membranes containing digested and separated DNA fragments. After hybridization, membranes were rinsed in 2x SSC, 0.5 % SDS at room temperature, and washed in 0.5x SSC, 0.1% SDS for 20 min at 65 °C before autoradiography. Filters were wrapped in plastic sheets (Cling wrap) and exposed to X-ray film at -70 °C for 3-7 days.

In pursuit of mapping *Rsv3,* AFLP marker (Vos et al., 1995) analysis of near-isogenic lines Williams and Hardee, and bulk segregants was also conducted with the collaboration of Dr. P. J. Maughan, and Dr. Hayes. AFLP is a PCR-based molecular marker that allows screening of large numbers of loci in a short period of time (Vos et al., 1995). AFLP analysis was carried out following the protocols as described previously (Vos et al., 1995; Maughan et al., 1996). As Hayes (1998) described, genomic DNA is double digested with a four- and six-cutter restriction enzyme. Specific nucleotide adaptors are then ligated to the restriction ends by DNA ligase. A subset of the generated fragments is amplified using adaptor specific primers that contain three selective nucleotides at the 3' end of the primer. The six-cutter-specific primer is end-labeled using γ^{32}P-ATP and polynucleotide kinase. DNA was digested with restriction enzymes EcoRI and MseI followed by ligation with specific adaptors for +1 and +3 amplification. AFLP products were visualized by means of a ^{32}P-end labeled Eco primer and electrophoresis through a 7 M urea, 6% (w/v) polyacrylamide gel for 3 hours at 45 Watts. AFLP fragments of interest were cloned into a plasmid according to the methods of Upender et al. (1995) and Hayes and Saghai Maroof (2000). In brief, a polymorphic AFLP band eluted from a gel slice was reamplified with specific +3 primers, by cold PCR. Product of the reamplification was resolved on an agarose gel and visualized by ethidium bromide staining to estimate the fragment size. This fragment was then cloned into the pCNTR shuttle vector (5prime-3prime, Boulder, CO) or cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturers' protocols. The plasmid insert was then used as a probe for RFLP analysis.

Once the single AFLP marker showing polymorphism among bulk segregants and NILs has been detected, this AFLP fragment was excised from the dried polyacrylamide gel and DNA was eluted by Dr. P. J. Maughan according to the procedure described by Madhvi et al (1995). This eluted AFLP fragment was re-amplified using specific + 3 primers, by cold PCR. Products of the re-amplification were resolved on an agarose gel and visualized by ethidium bromide to

estimate the fragment size. This fragment was cloned into PUC Plasmid vector system. Upon sequencing, the fragment size was determined as 79 bp (Dr. P. J. Maughan, personal communication). This was used as RFLP clone to further investigate the segreagation analysis of the F_2 population.

Statistical Analysis

The segregation ratios for SMV disease reaction and molecular marker data from screening the F_2 population were tested for goodness of fit to a 1:2:1 genotypic ratio by Linkage-1, a Pascal computer program developed by Suiter et al. (1989). The most probable order and map distances were determined by multiple linkage analysis using the computer program MapMaker 3.0b (Lander et al., 1987) at log likelihood 3.0, with a maximum Haldane distance of 50 cM.

RESULTS

RFLP probes from three different sources were employed to detect polymorphism between parental lines, NILs, and bulked segregants (Table 1). A preliminary survey with 136 RFLP markers gave 36 polymorphic markers with at least one restriction enzyme used. However, this initial screening did not detect any differentiation between sets of either NILs (Williams vs. L29) or bulked segregants (resistant vs. susceptible). Out of 36 polymorphic markers, 17 were used to collect segregation data from 183 F_2 genotypes.

At this stage AFLP marker analysis of parental lines, bulk segregants and NILs was conducted using 64 primer combinations. This analysis yielded an AFLP marker exhibiting consistent polymorphism between parental lines, bulk segregants, as well as NILs, with a primer combination of Eco+AAC/Mse+CTG. An AFLP fragment of approximately 80 base pairs (bp) was converted to an RFLP probe named ACR1. The initial linkage analysis of this ACR1 probe using 90 F_2 lines indicated tight linkage to the *Rsv3* gene. Even though this probe is linked to the gene as indicated by disease response data, its genomic position was unknown, because of its origin from random AFLP primer analysis. The genomic location of this linked AFLP fragment-based probe was determined by mapping it in an F_2 population of V71-370 X PI 407.162 containing 167 mapped RFLPs (a cross between *Glycine max* and *G soja*) (Maughan et al., 1995). This AFLP-based RFLP probe ACR1 was mapped to MLG B2 (USDA-ARS map), between the markers pA516 and pA519a. This step was necessitated, because available marker data were inadequate in the original population of L29 x Lee 68 to determine the linkage group of AFLP marker linked to the gene.

In addition to the original 136, ten RFLP markers known to be from MLG B2 in the vicinity of predicted *Rsv3* region were screened for polymorphism between parental lines, bulk segregants, as well as NILs. Five of them were polymorphic between parental lines, bulk segregants and isolines Williams and L29. The probes, namely pA516, pA519, pA593, pB124 and pB153 appeared to be linked to *Rsv3*, from this analysis. Data were collected from 183 $F_{2:3}$ DNA samples for the three markers pA519, pA516, and pA593 known to be located adjacent to the

AFLP-based probe ACR1 from an earlier analysis in a different population (V71-370 x PI 407.162). After examining the relative positions of these markers to the AFLP marker linked to the gene, the other two probes pB124 and pB153 were excluded from segregation analysis because they are located at farther distances (> 20 cM) from the gene compared to others based on previously published genomic maps of this region. All markers linked to the *Rsv3* gene segregated codominantly, and gave good fits to the 1:2:1 ratio (Table 2). Only those markers linked to the *Rsv3* gene are presented in Tables 2 and 3. The linkage map (Figure 1) constructed based on the results of multiple linkage analysis using Mapmaker 3.0b showed that three RFLP markers pA519, pA516, pA593 and AFLP based marker ACR1 were tightly linked to the *Rsv3* gene, at distances of 0.9 cM, 1.8 cM, 3.7 cM, and 3.7 cM respectively. These markers exhibited distinct polymorphic bands as shown in Figure 2.

In this study, a total of 146 RFLP markers from three different sources were screened (Table 1). Out of the 146 probes tested, 41 were polymorphic between the parental lines, with at least one of the enzymes used. Out of the 41 polymorphic markers found, segregation data were collected only from 21 markers. Once the linkage group of *Rsv3* was discovered by mapping the linked AFLP marker, the remaining 20 polymorphic markers, which are known to be from different linkage groups (from published maps), were excluded from this study.

DISCUSSION

This study reports the mapping of *Rsv3* gene conferring resistance to *Soybean mosaic virus* to MLG B2. The other two reported independent SMV resistance genes *Rsv1* and *Rsv4* have been mapped to MLG F (Yu et al., 1994) and MLG D1b (Hayes et al., 2000b), respectively. In order to achieve durable resistance it is desirable to have different sources of resistance accumulated into a single line or cultivar. By tagging various sources of resistance with molecular markers the ultimate objective of gene-pyramiding could be achieved relatively easily (Melchinger, 1989). Since *Rsv1* has been the most widely used SMV resistance gene in soybean breeding programs (Ma, 1996) a condition of genetic uniformity exists. In addition, *Rsv1* confers resistance only to some strains of SMV while being susceptible or necrotic to others (Chen et al., 1994; Ma, 1996). The *Rsv3* gene is particularly interesting in the efforts for pyramiding SMV resistance genes, because it confers resistance to the more virulent strain groups, G5 to G7. Accumulation of numerous resistance genes can be complicated, because it will be difficult to distinguish the presence of multiple genes, unless they can be identified by different reactions to different disease strains. Use of molecular markers enables combining different sources of SMV resistance into a single elite line or cultivar with the objective of achieving durable resistance.

Search for *Rsv3* was hastened by the use of NILs and bulked segregants in combination with the use of AFLP markers. NILs show DNA polymorphism only in those regions of the chromosome for which they were selected (Young et al. 1988; Michelmore et al. 1991; Martin et al., 1991; Yu et al. 1994), whereas bulk segregants will show random segregation at all loci except the gene of interest and closely linked markers. NILs Williams and L29 were produced as a result of backcross breeding, whereas bulk segregants are constituted based on the phenotypic reaction of the plants to virus inoculation. Sets of NILs and bulked segregants were subjected to screening for polymorphism using RFLP probes. A preliminary survey for linked markers using 136 RFLPs could not produce fruitful results. As an alternative AFLP marker analysis was attempted, these are a new generation of high throughput markers (Vos et al., 1995). Meksem et al. (1995) observed 100-200 AFLP loci in a single experiment, in contrast with RFLP markers, which can detect only one or two loci. The initial linkage to the *Rsv3* gene was observed by AFLP analysis. An AFLP primer combination of Eco+AAC/Mse+CTG exhibited polymorphism

between parental lines, bulks, as well as NILs, suggesting the putative linkage to the *Rsv3* gene. Majority of the AFLP markers are dominant markers like RAPDs (Meksem et al., 1995). Therefore, to enhance the utility of linked AFLP marker it was converted into codominant RFLP probe. Once the location of AFLP-based marker was determined, further search for the RFLP markers associated with the gene was made easy. The linkage group information obtained by mapping AFLP marker helped in finding the other RFLP markers linked to the gene. The linkage to *Rsv3* with RFLP markers, pA519, pA516, pA593 and with an AFLP based marker was indicated by polymorphism between NILs, Williams and L29, as well as between susceptible and resistant bulks. The linkage was further corroborated by F_2 segregation analysis with the same probes. The genomic distances among the MLG B2 markers are approximately similar to the previously published results. Two markers, pA516, and pA593, were mapped on top of each other. This is in agreement with the soybean genomic map published by Shoemaker and Olson (1993). The closest marker, pA519, being located at 0.9 cM distance away from the gene, exhibits very tight linkage to *Rsv3* (Figure 1).

The clustering of disease resistance genes has been reported in many plants (for a review, see Michelmore and Meyers, 1998). In soybean, a cluster of several closely linked resistance genes occurs in the vicinity of the SMV resistance locus, *Rsv1*. Genes for resistance to peanut mottle virus (*Rpv*) (Roane et al. 1983), *Phytophthora* (*Rps3*) (Diers et al. 1992) and Javanese root-knot nematode (Tamulonis et al. 1997) have been mapped to linkage group F (USDA-ARS map). The chromosomal region in the proximity of *Rsv3* appears to contain a cluster of disease resistance genes. The presence of *Rps4* and *Rps5*, conferring resistance to some races of *Phytophthora sojae* root and stem rot disease (Diers et al. 1992), as well as significant quantitative associations with resistance to two races of soybean cyst nematode, have been reported (Qiu et al., 1999) indicating the possibility of another resistance gene cluster.

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TABLE 1: The sources and numbers of RFLP molecular markers tested, detected polymorphism and used in segregation analysis.

Marker source	**Number of markers tested**	**Number of markers polymorphic between L29 and Lee 68**	**Number of polymorphic markers mapped in F_2**
Soybean genomic library (ISU)	97	28	16
N-terminal nucleotide-binding site (*NBS*) markers	23	8	5
Cowpea and mung bean genomic library	26	5	0
Total	146	41	21

TABLE 2: Segregation analysis of disease response to Soybean mosaic virus (SMV) strain G7 conditioned by the resistance gene *Rsv3*, and RFLP markers linked to it, in an $F_{2:3}$ population from L29 X Lee 68.

Loci	No. of $F_{2:3}$ plants	No. of F_2 lines observed for			χ^2 1:2:1	Probability
		AA*	AB	BB		
Rsv3	185	54	93	38	2.77	0.25
ACR1	80	26	39	15	4.19	0.12
pA519	172	49	88	35	2.04	0.36
pA516	174	48	90	36	4.39	0.11
pA593	174	48	90	36	4.39	0.11

- Genotype: AA = L29, resistant to SMV G7, AB =segregating, BB = Lee 68, susceptible to SMV G7.

Table 3: Summary of marker genotypes of $F_{2:3}$ plants grouped according to plant disease reaction classes for response to Soybean mosaic virus strain G7.

Markers	Number of F_2 lines									χ^2	P
	Rsv3 Rsv3			*Rsv3 rsv3*			*rsv3 rsv3*				
	AA	AB	BB	AA	AB	BB	AA	AB	BB		
ACR1	24	2	0	0	39	0	0	2	13	136.52	<0.001
pA 519	46	2	1	0	88	0	0	1	34	320.53	<0.001
pA 516	43	5	0	5	84	1	0	4	32	265.17	<0.001
pA 593	43	5	0	5	84	1	0	4	32	265.17	<0.001

- Genotype: AA = L29, resistant to SMV G7, AB =segregating, BB = Lee 68, susceptible to SMV G7.
- Plant disease reactions denoted as *Rsv3 Rsv3*: resistant, *Rsv3 rsv3*: segregating F_2 lines, *rsv3 rsv3*: susceptible.

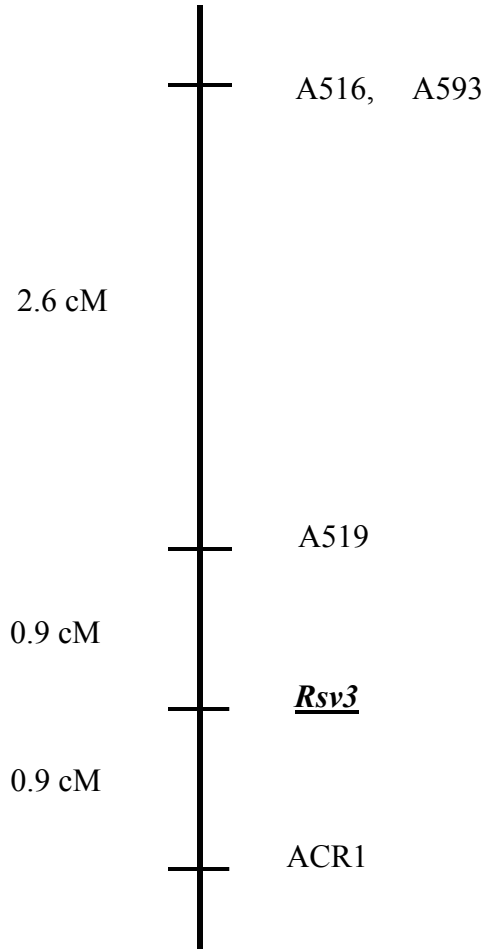

Figure 1. Genetic map of the soybean molecular linkage group B2 surrounding the Soybean mosaic virus resistance gene, *Rsv*3. Markers were mapped in segregating population constructed by crossing L29 (*Rsv*3) X Lee 68 (*rsv*3).

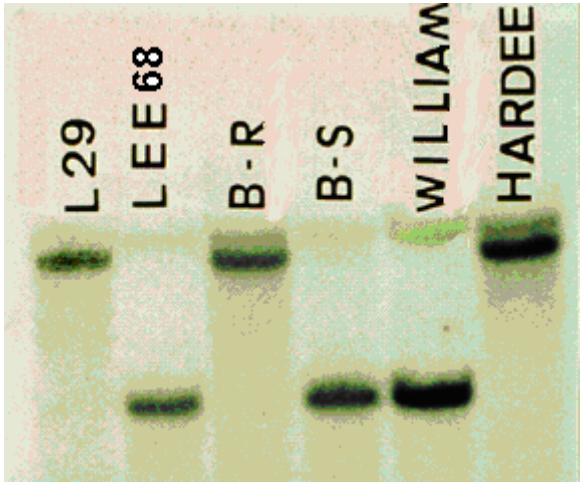

Figure 2. A marker linked to *Rsv3*, exhibiting clear polymorphism among parents (L29 X Lee 68), resistant and susceptible bulk segregants (B-S; B-R = bulk segregant susceptible and resistant, respectively), and near-isolines Williams and Hardee (carrying the *Rsv3* resistance gene).

Vita

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