Saturation mapping of a disease resistance gene in soybean [Glycine max (L.) Merr.]

by

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ABSTRACT

Saturation mapping of a disease resistance gene (Rps1-c) in soybean [Glycine max (L.) Merr.] was conducted using near-isogenic lines (NILs) and three high-through-put marker techniques: RAPD, AFLP and SSR. Eight hundred twenty random primers were screened against the recurrent parent 'Williams' (containing the susceptible recessive allele rps1-c) and the near-isogenic cultivar 'Williams 79' (containing the resistant dominant allele Rps1-c) using RAPD assays. Twelve PCR-amplified products were identified that were present in one but not the other parent. The frequency of polymorphism was 0.31% based on an average of 4.7 PCR-amplified fragments per primer. $F_{2:3}$ segregation analysis confirmed that ten of twelve polymorphic markers (83%) were linked to the Rps1-c gene. One marker, OPP11, was positioned 0.9 centiMorgans (cM) from Rps1-c, and another marker, OPV18, was 1.4 cM from the gene. Twenty-five AFLP primer combinations were screened against the same pair of parents used in the RAPD analysis. Two amplified products were identified that were present in one but not the other genotype. The frequency of polymorphism was 0.2% based on an average of 40 fragments per primer combination. One marker, AFT24E21 was linked to Rps1-c at a distance of 8.9 cM. One SSR marker was located 2 cM from Rps1-c. The size of the introgressed region in the NIL was approximately 39.9 cM. The usefulness of saturation mapping using high-volume marker techniques for map-

CHAPTER 1. INTRODUCTION

Literature Review

The cultivated soybean [Glycine max (L.) Merr.] is one of the major oilseed crops in the world. The crop, which is thought to have originated in China, is currently grown commercially in 35 countries. Approximately 55% of the world soybean production is in the United States (Foreign Agricultural Service, 1985, cited in Fehr, 1987).

There are many different diseases that attack the soybean, but there are relatively few that consistently cause injury of economic importance and that are given major consideration by breeders. One of the most commonly considered is Phytophthora root rot. This disease recently originated in the United States and was first observed in northeastern Indiana in 1948 and in northwestern Ohio in 1951 (Schmitthenner, 1985). The first comprehensive report of this disease by Kaufmann and Gerdemann (1958) identified the pathogen as Phytophthora sojae. A year later in a second definitive report, Hildebrand (1959) changed the pathogen name to P. megasperma var. sojae. In 1980, the pathogen was renamed P. megasperma f. sp. glycinea (Kuan and Erwin). In 1991, Hansen and Maxwell renamed it back to P. sojae because of significant differences in host specificity, enzyme patterns and RFLPs between P. sojae and P. megasperma. Phytophthora root rot of soybean is a rather unique disease which may result in plant losses and yield reductions of 100% in very susceptible

soybean cultivars. It is the only severe Phytophthora disease of a major grain crop (Schmitthenner, 1985). It was estimated that approximately 5 million hectares were infested in the north central region and 3 million hectares in other areas of the United States (Schmitthenner, 1989) and that average losses of $188 million per year occurred during the period 1989-1991 (Doupnik, 1993). About 25% of early season damping off of soybean is caused by this disease in Iowa during a typical wet spring (Rizvi et al., 1993).

Genetic control of Phytophthora root rot is feasible through the use of several major resistance genes. There are 32 physiological races of the pathogen (Schmitthenner et al., 1994), but relatively few are of major importance at the present time. It has been common practice to incorporate major genes for resistance into susceptible cultivars by backcrossing and forming near-isogenic lines (NILs) (Fehr, 1987).

NILs of soybean can be used to integrate the separate molecular marker and conventional marker linkage maps (Muehlbauer et al., 1988). Gene mapping with NILs is based on the premise that when a conventional marker is introgressed from a donor parent into a recurrent parent through backcrossing, the resultant NIL retains a small portion of the donor genome surrounding the introgressed gene. Markers unique to the donor parent can be used to identify the residual regions of the donor genome in the NIL. Most of these retained markers will be linked to the introgressed conventional marker. For a hypothetical species possessing n= 20 chromosomes, with each having

a length of 50 cM, Muehlbauer et al. (1988) calculated that approximately four of one hundred molecular markers with recurrent parent/donor parent allelic contrasts would be retained in BC_5S_1-derived NIL. Of these four markers, two or three could be expected to be located in the introgressed region containing the gene of interest, with the others dispersed randomly among the other chromosomes. Therefore, a soybean NIL formed through five backcrosses would contain, on average, 4% of the genome of the donor parent.

The soybean genome contains an estimated 1.29×10^9 base pairs (bp) (Gurley et al., 1979) to 1.81×10^9 bp (Goldberg, 1978) for 1N DNA content. Though certainly not the largest or most complex of the plant genomes, the genome size of the soybean is many times larger and more complex than that of Arabidopsis. Mapping of the soybean genome has been recent compared with maize (Zea mays L.). Because of the complexity of the soybean genome, the lack of genetic variation in soybean germplasm, the paucity of cytogenetic markers, and the difficulties in producing large numbers of hybrid soybean seed (Shoemaker et al., 1992), only 63 morphological, pigmentation, and isozyme markers have been mapped to the soybean classical genetic map (Palmer and Hedges, 1993).

RFLP technology has provided many molecular markers for the soybean genome. Several maps have been constructed. Keim et al. (1990) used an interspecific cross between G. max and G. soja to construct a genomic map for soybean based upon 150 polymorphic markers that identified 26 linkage groups and a genomic length of 1200

cM. This map now contains approximatly 600 markers, 23 linkage groups, and spans approximately 3000 cM (Shoemaker and Olson, 1996). A soybean RFLP map was also made by researchers at Du Pont de Nemours. The map contained about 550 markers covering 2700 cM in 23 linkage groups (Rafalski and Tingey, 1990). Lark et al. (1992) constructed a genetic linkage map of an intraspecific G. max cross. The map consisted of 132 RFLP, isozyme, morphological, and biochemical markers, 31 linkage groups, and spanned 1550 cM.

Seven loci are known with alleles that provide race specific resistance of soybean to Phytophthora in the soybean genome. The first gene found conferring Phytophthora sojae resistance was Rps1 (Bernard et al., 1975), a resistance gene, Rps2, was next found (Kilen et al., 1974), followed by Rps3 (Mueller et al., 1978), Rps4 (Athow et al., 1980), Rps5 (Buzzell et al., 1981), Rps6 (Athow and Laviolette, 1982) and Rps7 (Anderson and Buzzell, 1992). At some loci, more than one resistance alleles have been reported (Athow, 1987). There are six alleles known at the Rps1 locus (Athow, 1987; Buzzell and Anderson, 1992; Kilen and Keeling, personal communication). Resistant sources of these six alleles are from different varieties that provide race specific resistance of soybean to Phytophthora (Table 1).

Three Rps loci have been mapped onto the classical linkage map: Rps1 on linkage group 10 (Kilen and Barrentine, 1983), Rps2 on linkage group 19 (Devine et al., 1991) and Rps7 linked 12.5 ± 2.7 cM from Rps1 on linkage group 10 (Anderson and Buzzell, 1992). Five Rps loci have been placed on the USDA-ARS, ISU molecular linkage map. Rps1 was

Table 1. Genes for resistance to physiologic races of P. sojae (Schmitthenner et al., 1994)

Source	Gene	Resistance to physiologic races
Williams	rps1	None (susceptible to all physiologic races)
Mukden	Rps1-a	1, 2, 10, 11, 13, 15, 16, 17, 18, 24, 26, 27, 31, and 32.
Sanga	Rps1-b	1, 3, 4, 5, 6, 7, 8, 9, 13, 14, 15, 18, 21, and 22.
Arksoy	Rps1-c	1, 2, 3, 6, 7, 8, 9, 10, 11, 13, 15, 17, 21, 23, 24, 26, 28, 29, 30, and 32.
PI103091	Rps1-d	1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 13, 14, 15, 16, 18, 20, 21, 22, 23, 24, 25, 27, 28, 29, 30, and 32.
PI172902	Rps1-e	1, 2, 3, 4, 5, 7, 8, 9, 14, 15, 16, and 19.
Kingwa	Rps1-k	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 17, 18, 21, 22, 24, and 26.

mapped to linkage group N, Rps2 to linkage group L, Rps3 to linkage group E, and Rps4 was linked to PT-5 and PA-586 (Diers et al., 1992). A later report mapped Rps7 to linkage group N (D. Lohnes, 1996, personal communication).

RFLP markers are a powerful tool in genetic mapping. However, it is not the best method for applications involving thousands of individuals, or large numbers of markers. In general, RFLP technology requires radioisotope and is time-consuming and labor intensive (Walton, 1993).

Over the last few years, polymerase chain reaction technology (PCR) has led to the development of several novel genetic assays based on selective DNA amplification (Krawetz, 1989; Innis et al., 1990). PCR reactions are simple to perform and preferable in experiments where the genotype of a large number of individuals needs to be determined at a few genetic loci. Unfortunately, because of a prerequisite for DNA sequence information, these assays are limited in their application.

In 1990, a new genetic assay was developed independently by two different laboratories (Welsh and McClelland; Williams et al.). This procedure was called the RAPD (Random Amplified Polymorphic DNA) assay. RAPD assays combine PCR technology with non-species-specific DNA primers to amplify random DNA fragments. RAPD markers are four to six times more efficient, on a per assay basis than RFLP assays, and are ten-fold more efficient in time and labor (Tingey and del Tufo, 1993). The RAPD assays are an efficient tool to identify molecular markers tightly linked to genes of interest and have been used

successfully in many plant species including pine (Devey et al., 1995), sugar cane (Sobral and Honeycutt, 1993), wheat (D'Ovidio., et al., 1990), birch (Roy et al., 1992), sorghum (Pammi et al., 1994), turfgrass (Sweeney and Danneberger, 1995), and tomato (Kawchuk et al., 1994). The RAPD assay can also be used to quickly construct well-saturated genetic maps in a variety of organisms. Metzenberg and Grotelueschen (1990) placed eighty-eight RAPD markers in the context of an existing Neurospora map using a standard mapping population. Reiter et al. (1992) has constructed a well-saturated map of Arabidopsis thaliana using 250 RAPD markers in only four person-months. Chaparro et al. (1992) created a 191 RAPD marker map of loblolly pine in only six person-months, clearly demonstrating the utility of RAPDs for quickly saturating genetic maps.

Several groups have used the RAPD assay as an efficient tool to identify molecular markers that lie within regions of a genome introgressed during the development of near-isogenic lines (Klein-Lankhorst et al., 1991; Martin et al., 1991; Paran et al., 1991). By definition, any region of the genome that is polymorphic between two near-isogenic plants is potentially linked to the introgressed trait. Thus, Klein-Lankhorst et al. (1991) were able to identify RAPD markers specific to chromosome 6 of tomato by screening a Lycopersicon esculentum substitution line, and Martin et al. (1991) were able to confirm linkage of RAPD markers to the Pto locus in tomato after screening two near-isogenic lines. Paran et al. (1991) used two different sets of near-isogenic lettuce lines to identify RAPD markers

linked to the Dm1, Dm3, and Dm11 locus, which also complemented the RFLP map of lettuce in regions containing few RFLP markers.

SSRs (Simple Sequence Repeats), also known as microsatellite DNA markers, have been described as an additional source of genetic markers (Weber, 1990) which are ubiquitously interspersed throughout eukaryotic genomes (Tautz and Renz. 1984). A SSR is composed of tandemly repeated two to five nucleotide DNA core sequences such as $(CA)_n$ or $(ATT)_n$. The DNA sequences flanking microsatellites are generally conserved within individuals of the same species, allowing the selection of polymerase chain reaction primers that will amplify the intervening SSR in all genotypes. Variation in the number of tandem repeats, n, results in different PCR product lengths (Weber and May, 1989; Litt and Luty, 1989; Tautz, 1989). The high level of polymorphism, combined with broad genomic interspersion, makes them an abundant source of genetic markers. Extensive genetic maps have been constructed using SSRs for human (Weissenbach et al., 1992) and various other mammalian species (Copeland et al., 1993).

In plants, SSR markers are being developed in a number of species including tropical forest trees (Condit and Hubbell, 1991), maize (Senior and Heun, 1993), Brassica (Lagercrantz et al., 1993), wheat (Devos et al., 1995), grape (Thomas and Scott, 1993), barley (Saghai-Maroof et al., 1994), rice (Wu and Tanksley, 1993; Zhao and Kochert, 1993), sunflower (Brunel, 1994), avocado (Lavi et al., 1994), and Arabidopsis (Bell and Ecker, 1994). In soybean, Akkaya et al. (1992) examined the degree of polymorphism at three SSR loci. Morgante and

Olivieri (1993) also demonstrated that SSRs were present in soybean and exhibited high levels of length polymorphism. More recently, Cregan et al., (1994) and Jiang et al. (1995) described SSR loci with as many as 26 alleles. The Soybean Rsv gene (resistance to soybean mosaic virus) was recently mapped by Yu et al. (1994) with SSR and RFLP markers. SSR markers in plants can be up to tenfold more variable than other marker systems. SSRs, therefore, may represent a very useful genetic marker system for genetic mapping of inbreeding species that generally contain little intraspecific polymorphism (Roder et al., 1993).

More recently, Keygene, a European biotechnology firm, has developed a new molecular marker technology, AFLP (Amplified Fragment Length Polymorphism). AFLPs detect DNA polymorphism through length differences in restriction fragments. The technique uses universal primers for PCR reactions. Adapters are used to tag the restriction fragments and to serve as the template for PCR primers. Selective amplification is achieved by using PCR primers containing one or more selective nucleotides. By altering the number of selective nucleotides included in the PCR primer, one can obtain meaningful banding patterns. This technique offers researchers valuable and possibly novel technology compared to RFLPs and RAPDs. Smith et al. (1993) analyzed genetic associations among inbred lines used AFLPs and found that a clustering of inbred lines in maize were related by pedigree. Lin and Kuo (1995) described AFLP analysis of Arabidopsis and E.coli strains.

Little is known about the application of AFLPs as molecular markers in soybean. In this study, we used RAPD, AFLP, SSR and RFLP techniques to screen the Phytophthora susceptible recurrent parent 'Williams' and the resistant near-isogenic line 'Williams 79'. Williams 79 differs from Williams for an introgressed region of the genome that contains the Rps1-c gene. The objectives of this study were to identify molecular markers tightly linked to the Rps1-c locus and suitable for YAC or BAC landing, and to estimate the minimum size of the introgressed region containing Rps1-c.

Importance of This Study

Because of the importance of Phytophthora root and stem rot as a soybean disease, fine-structure mapping of Rps loci would be useful for both plant breeding and molecular studies.

Traditionally, plant breeders screen for resistance genes by inoculating plants with the pathogen. In many cases, the environment affects the expression of the genotype, so that the phenotype provides an imperfect measure of a plant's genetic potential. Also, simultaneous or sequential screening of plants with several different pathogens can be difficult or impractical. In other instances, breeders are unable to screen for new pathogen, because of quarantine restriction on their shipment and use. In contrast, detecting disease resistance genes by their linkage to molecular markers makes it practical to screen for many different disease resistance genes

simultaneously without the need to inoculate the population (Tanksley et al., 1989).

Molecular markers also may be used in marker-assisted selection programs, and also to give plant breeders new opportunities for utilization of exotic germplasms and identification of adapted cultivars. Shoemaker et al. (1992) discussed the application of molecular marker genetic maps for a retrospective analysis of the soybean genome among breeding lines in an attempt to correlate genomic regions to breeder manipulations of agronomic traits. Lorenzen et al. (1995) conducted a detailed molecular genetic analysis of soybean pedigrees.

Methods are well-established for isolating and cloning a gene if the product of that gene is known (Maniatis et al., 1982). Unfortunately, the products of many disease resistance gene are unknown. One approach to cloning such genes without knowledge of the gene product is transposon tagging. Lawrence et al. (1995) reported the successful transposon tagging and cloning of the L6 rust resistance gene in flax using the maize transposon Activator. This approach has proven useful in cloning genes in species in which transposons are well-characterized (Federoff et al., 1984). If transposons are not well-characterized, the genes controlling such traits will likely require alternative gene isolation and cloning strategies.

Map-based gene cloning offers an independent method for gene isolation. Often referred to as reverse genetics, this approach is based on physical linkage of a gene to a cloned molecular marker(s). Reverse

genetics has been used to clone genes involved in hereditary diseases of human (Orkin, 1986). Construction of saturated genetic maps has allowed the precise location of a few disease resistance genes in plants, including the Pto bacterial resistance locus in tomato (Martin et al., 1993), the Xa21 bacterial blight resistance locus in rice (Ronald et al., 1992), the Rp1 rust resistance locus in maize (Hulbert and Bennetzen, 1991), and the Dm3 downy mildew resistance locus in lettuce (Paran et al., 1991). Mapping at high resolution is an essential step in map-based cloning of resistance genes. The availability of saturated maps also provides a ready starting point to search for markers tightly linked to a target gene, then to "walk" to a target gene by overlapping clones (chromosome walking). Another procedure is to directly identify molecular markers that are physically close to the target gene so that little or no chromosome walking is required. These tightly linked markers are used to screen a genomic library to isolate directly a single clone containing the gene of interest (chromosome landing). The latter method is likely to become the main strategy by which map based cloning is applied to isolate disease resistance genes (Tanksley et al., 1995).

I have been involved in a long term project to clone the Phytophthora root rot resistance gene, Rps1-c from soybean. The first step of this project was to identify molecular markers tightly linked to the Rps1-c gene and to construct a saturated genetic map immediately flanking the resistance gene. My roles in this project were : 1) to conduct all RAPD and SSR assays, 2) to develop AFLP procedures and

screen 25 primer combinations, 3) to inoculate approximately 40 $F_{2:3}$ seedlings in the greenhouse, and 4) to collect and analyze data using Chi-square, Linkage1 and Mapmaker computer programs.

Most of the inoculation data and RFLP data were provided by Cindy Clark, a technician in Dr. Shoemaker's Lab.

CHAPTER 2. MATERIALS AND METHODS

Plant Materials

The recurrent parent 'Williams' and the resistant near-isogenic line L75-3735 (Williams 79) used in this study, were obtained from Dr. R. L. Nelson, the curator of USDA-ARS soybean germplasm collection, Urbana, Illinois. Williams 79 derived its resistance allele (Rps1-c) from Lee 68 (Caviness and Walters, 1968). It has undergone five backcrosses to Williams and a final selfing generation (Bernard and Cremeens, 1988).

The isoline Williams 79 and the cultivar Williams were crossed to form F_2 populations for testing linkage among the RAPD, AFLP, SSR and RFLP markers and Rps1-c locus. The crosses were made in a greenhouse during the fall of 1992. The resulting F_1 plants were grown during the spring of 1993 in a greenhouse. A total of 335 F_2 plants representing 3 crosses were grown in a field near Ames, IA during the summer of 1994. Each F_2 plant was harvested individually to form F_2-derived lines in the F_3 generation ($F_{2:3}$).

Scoring Plant Reaction to Phytophthora Sojae

Scoring plant reaction to Phytophthora sojae was conducted in a greenhouse by testing a minimum of 11 F_3 seedlings from each selfed F_2 plant, affording a probability of 0.95 of detecting at least one

susceptible plant in a population segregating 3 resistant/1 susceptible (Sedcole, 1977). A modified hypocotyl-puncture method for inoculation (Morgan and Hartwig, 1965) was used for disease testing. Seeds were planted in clay pots with standard greenhouse potting soil. Seedlings were inoculated at the unifoliate leaf stage, 7-10 days after planting, with a culture of Phytophthora sojae, race1. Oatmeal Agar (OMA) was used for inoculum production. The hypocotyls were slit 1 cm below the cotyledons and a 1 mm^2 piece of mycelium was inserted into the slit. After 3-4 days post-inoculation, reaction to Phytophthora was scored. Each F_2 individual was categorized as homozygous susceptible, homozygous resistant or heterozygous resistant. Evaluations were reported as numbers of dead and diseased seedlings divided by the total numbers of inoculated seedlings. The check cultivars Williams and Williams 79 were included in each analysis. The optimum temperature in greenhouse for disease development was 24-27°C.

DNA Isolation

DNA was prepared from leaves using a modified CTAB (hexadecylatrimethylammonium bromide) procedure (Saghai-Maroof et al., 1984). The leaf material for Williams and Williams 79 was obtained from seedlings grown in a greenhouse. The leaf material used to determine the genotypes of the F_2 individuals was obtained from twenty or more F_3 seedlings derived from an individual selfed F_2 plant. The F_3 seedlings were grown in a sandbench in a greenhouse. After

three weeks, the leaf material was harvested, frozen in liquid nitrogen, lyophilized, and ground. One gram of powdered leaves was placed in a 50 ml polypropylene tube and 25 ml of 1x CTAB extraction buffer (50 mM Tris-HCl, PH 8.0; 50 mM EDTA, PH 8.0; 0.7 M NaCl; 1% CTAB; 0.5% 2-mercaptoethanol) was added. The leaf powder and buffer were mixed well, placed in a water bath and incubated for 60 minutes at 60°C. The samples were removed from the water bath and allowed to cool for 10 minutes at room temperature. After adding 20 ml of chloroform : isoamyl ethanol (24 : 1) and mixing thoroughly by inversion, the samples were centrifuged at 3750 rpm in a Beckman rotor. The upper aqueous phase was decanted into a 50 ml tube containing a 2/3 volume of 2-isopropanol. The samples were inverted several times and placed at -20°C for 20 minutes to facilitate DNA precipitation. The DNA precipitate was removed with a glass hook and placed in a 15 ml tube containing 10 ml 80% ethanol/15 mM ammonium acetate for at least 30 minutes, and then transferred to a 1.5 ml tube. Excess ethanol was removed from the DNA pellet by centrifuging 2 minutes and decanting onto a piece of paper. The DNA pellet was allowed to dry for several hours and was then resuspended in 750 ml of TE buffer (10 mM Tris-Cl; 1 mM EDTA, PH 8.0) containing 10 µl of RNAse (10 mg/ml). DNA suspensions were quantified using a Hoefer mini-flourometer.

RAPD Assays

A total of eight hundred and twenty primers was screened against the cultivars Williams and Willliams 79. Two hundred of these primers were synthesized by the University of British Columbia. Six hundred primers were synthesized by Operon Technologies, Inc. Each of these eight hundred primers consisted of ten nucleotides. Twenty primers were synthesized by GIBCO BRL, and consisted of twenty-one nucleotides.

PCR amplification reactions were performed in a volume of 25 µl containing 10 mM Tris-Cl, PH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelation, 100 µM of each of dATP, dCTP, dGTP, and dTTP, 10 picomoles of primer, 25 ng of genomic DNA, and 1 unit of Taq polymerase (Promega Corp., Madison, WI). Amplification was performed in a Perkin-Elmer/Cetus DNA thermal cycler programmed for 45 cycles of 1 minute at 94°C, 1 minute at 34°C, and 2 minutes at 72°C. Amplification products were analyzed by electrophoresis in 1.4% agarose gels and detected by staining with ethidium bromide.

AFLP Assays

500 ng of genomic DNA was double digested with Taq1 restriction enzyme at 65°C for 3 hours and then with EcoR1 enzyme at 37°C for 3 hours. The DNA fragments were ligated with 25 pMole Taq1 and EcoR1 adapters. For selective templates with adapters, paramagnetic

streptavidine beads (Dynal) were used to separate Biotinylated fragments (EcoR1-EcoR1 fragments and EcoR1-Taq1 fragments) from non-biotinylated fragments (Taq1-Taq1 fragments). For selective amplification, Taq1 primers (designated AFT21, AFT22, AFT23, AFT24, and AFT25) with 1-3 selective nucleotides were ^{32}P-labeled using the T4 polynucleotide Kinase. One µl of ^{32}P labeled Taq1 primer (5 ng) and 0.5 µl of unlabeled Taq1 primer (2.5 ng) were mixed with 0.6 µl of EcoR1 primer (designated AFE21, AFE22, AFE23, AFE24, and AFE25), 1 µl of template DNA (100 ng), 2 µl of 10x PCR buffer, 4 µl of 1.25 mM dNTPs, 1.2 µl of 25 mM MgCl, and 1 unit of Taq polymerase, using sterile H_2O bringing the final volume to 20 µl. The mixture was amplified for 1 cycle of 94°C for 30s, 65°C for 30s, and 72°C for 60s, then by lowering the annealing temperature 0.7°C each cycle for 13 cycles, and then 23 cycles of 94°C for 30s, 56°C for 30s, 72°C for 60s. After the PCR reaction was completed, 10 µl aliquots of the reaction mixture was taken out, put in a fresh tube and mixed with 8 µl of sequencing loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenolblue). The resulting mixtures were heated 3 minutes at 95°C and then 3 µl of the samples were loaded on a 5% polyacrylamide sequencing gel with 8 M Urea. The gel was run at constant power of 110 watts to prevent inconsistant heat development during electrophoresis. Under these conditions the field strength of the gels corresponded to 40 to 50 volt/cm. After electrophoresis, the gel was dried and exposed to X-ray film.

SSR Assays

We selected one SSR marker, SATT-9 on linkage group N of the soybean genome map (Akkaya et al., 1995), to test the distance between Rps1-c and this marker using our population.

Reaction mixtures contained 60 ng of genomic DNA, 3 mM MgCl, 0.2 µM of 3' and 5' end primers (sequence from Beltsville Agricultural Reaction Center, synthesized by GIBCO BRL, 1x reaction buffer (10 mM Tris-HCl, PH 8.3, 50 mM KCl, 2 mM $MgCl_2$, and 0.001% gelation), 200 µM of each of dATP, dCTP, dGTP, and dTTP, and 1.5 units of Taq polymerase in a total volume of 30 µl. PCR reaction consisted of 30s at 94°C, 30s at 47°C, and 30s at 68°C for 32 cycles on a Perkin-Elmer/Cetus thermal cycler. PCR products were separated on 2.5% Meta Phor agarose gel (FMC byproducts, Riceland, ME) in 1x TAE buffer (Tries-acetate, EDTA) instead of a sequencing gel, since the size difference between DNA amplification products of the two alleles at this SSR locus was about 50 bp in length greater than the 8-10 bp minimal size difference distinguished by Meta Phor agarose gel. The PCR products were visualized by ethidium bromide staining.

RFLP Analysis

RFLP analysis used the same restriction enzymes and probes as reported by Diers et al. (1992). Samples of 8 µg of DNA were individually digested with DraI and Taq I restriction enzymes.

Restriction fragments were separated on 0.8% agarose gels. DNA was transferred to nylon membrane via Southern blotting (Southern, 1975). Blots were hybridized with randomly primed ^{32}p-labeled dCTP insert DNA. Preliminary screens of parental DNA identified polymorphic clones that subsequently were used to get RFLP data from F_2 derived lines in F_3.

Linkage Analysis

The segregation ratios of Rps1-c and each molecular marker in the F_2 population were tested for goodness of fit to a 3:1 (dominant markers) or a 1:2:1 (codominant markers) genotypic ratio using Chi-square analysis. Linkage analyses between the loci were conducted using the maximum likelihood program 'Linkage-1' (Suiter at al., 1983). When data were collected from three or more loci in a linkage group, the most probable order and map distances were determined by multipoint linkage analysis with the computer program 'Mapmaker 3.0' (Lincoln et al., 1993; Lander et al., 1987) at LOD = 3.0 and a maximum distance of 50 cM.

CHAPTER 3. RESULTS

Disease Evaluation Results

Reaction of the soybean $F_{2:3}$ seedlings to Phytophthora sojae, race 1 was scored after 3-4 days post-inoculation in the greenhouse. The homozygous susceptible seedlings were dead or withered with necrosis spreading from the inoculation slit, whereas the resistant seedlings were healthy and had slight necrosis only at the point of inoculation. Heterozygous seedlings segregated to 3 resistant / 1 susceptible (Figure 1). The results of disease evaluation indicated that the segregation ratio did not deviate from the expected 1:2:1 ratio based on a Chi-square test (Table 2).

RAPD Results

Williams and Williams 79 were screened with 820 RAPD primers. Approximately 3850 discrete products, ranging from 200 to 3000 base pairs, were amplified by the 820 primers tested (average of 4.7 products per primer). The majority of the products were identical in both Williams and Williams 79. However, twelve primers produced fragments that appeared in one parent but not the other (Fig. 2; shows products of nine of twelve primers). The frequency of polymorphism was 0.31%. Primers OPH4, OPK2, OPK10, OPL14, OPP10, OPP11, OPR4, OPV18, OPAA7, OPAE20, and UBC150 each generated one polymorphic product between Williams and Williams 79. The molecular weights

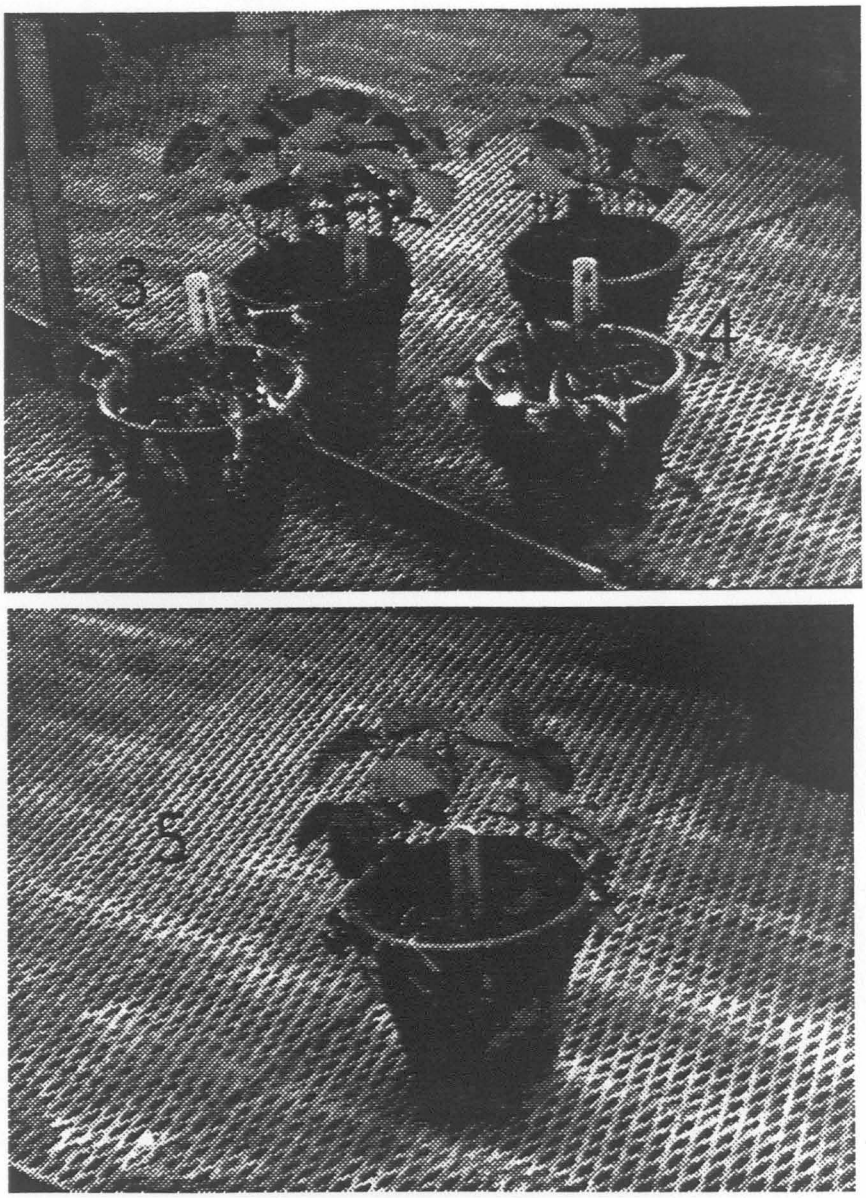


Fig. 1. Scoring plant reaction to Phytophthora sojae race 1 in a greenhouse. Pots 1 and 2 represent homozygous resistant $F_{2:3}$ individuals, Pots 3 and 4 represent homozygous susceptible $F_{2:3}$ individuals, pot 5 represents a heterozygous resistant $F_{2:3}$ family after 3-4 days post-inoculation.

Table 2. Segregation ratio of the Rps1-c gene and molecular markers

Marker types	Loci	Expected ratio	Observed ratio	n†	χ^2	P‡
R. Phen.§	Rps1-c	1:2:1	67:129:89	285	5.95	0.051
RAPD	OPK-02	3:1	183:57	240	0.20	0.655
RAPD	OPK-10	3:1	179:61	240	0.02	0.888
RAPD	OPL-14	3:1	47:11	58	1.13	0.288
RAPD	OPP-10	3:1	182:56	238	0.27	0.603
RAPD	OPP-11	3:1	237:92	329	1.63	0.202
RAPD	OPR-04	3:1	180:61	241	0.00	1.000
RAPD	OPV-18	3:1	239:93	332	1.61	0.205
RAPD	OPAA-07	3:1	73:24	97	0.00	1.000
RAPD	OPAC-11	3:1	190:50	240	2.22	0.136
RAPD	UBC-150	3:1	188:54	242	0.93	0.335
RFLP	PA-280	1:2:1	20:41:26	87	1.12	0.571
RFLP	PA-71	1:2:1	53:121:66	240	1.43	0.489
SSR	SATT-9	1:2:1	79:153:93	325	2.32	0.314
AFLP	T24E21	3:1	42:22	64	3.00	0.083

†Number of $F_{2:3}$ individuals tested in this study.

‡Probability of a greater value of χ^2.

§Resistance phenotype.

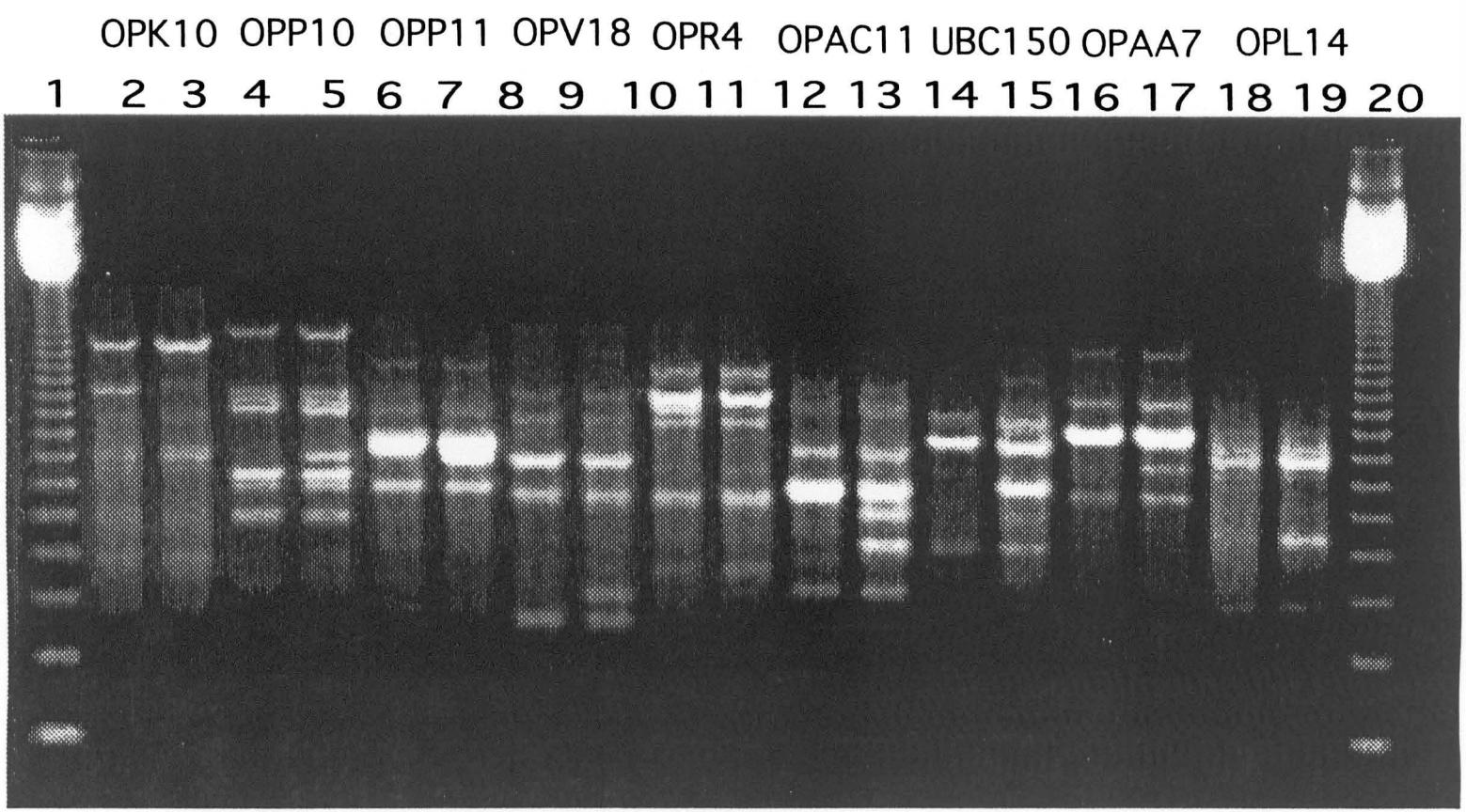


Fig. 2. Amplification products using RAPD primers to identify markers linked to Rps1-c. Lanes 1 and 20 are 123-bp molecular ladders; lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18 are the susceptible recurrent parent Williams; lanes 3, 5, 7, 9, 11, 13, 15, 17, and 19 are the resistant near-isogenic line Williams 79. Primers are, from left to right: OPK10, OPP10, OPP11, OPV18, OPR4, OPAC11, UBC150, OPAA7, and OPL14.

of the polymorphic fragments were 980bp, 1720bp,1230bp, 520bp, 870bp, 860bp, 1100bp, 380bp, 820bp, 610bp, and 720bp, respectively (Table 3). Primer OPAC11 produced two polymorphic products at 510bp and 610bp.

To test for linkage between the twelve RAPD markers and the Rps1-c gene, we analyzed $F_{2:3}$ families from the cross of Williams and Williams 79. The number of $F_{2:3}$ families tested varied from 58 to 329 depending on the proximity of the marker to the gene. Figures 3 and 4 show examples of the $F_{2:3}$ segregation pattern for marker OPP11 and OPV18, respectively. The results of linkage tests indicated that ten of twelve markers linked to Rps1-c gene and fit a 3:1 segregation ratio (Table 2). Linkage data for the most closely linked markers, OPP11 and OPV18, are presented in Table 4. These two markers showed highly significant linkage to Rps1-c based upon 276 $F_{2:3}$ individuals (χ^2 = 266.90, $P < 0.001$; χ^2 = 262.26, $P < 0.001$, respectively). Only individuals for which segregation data existed for all markers were used in the analysis of linkage using Linkage-1. For this reason the number, n, used to caculate linkage relationships between markers and the resistance locus varied for each marker.

Table 3. Primer sequences and sizes for polymorphic RAPD fragments identified in this study

Primers	Nucleotide sequence 5'-3'	Total No. of fragments amplified	No. of polymorphic fragments	Size of polymorphic fragment DNA (bps)
OPH-4	GGAAGTCGCC	4	1	980
OPK-2	GTCTCCGCAA	6	1	1720
OPK-10	GTGCAACGTG	5	1	1230
OPL-14	GTGACAGGCT	3	1	520
OPP-10	TCCCGCCTAC	7	1	870
OPP-11	AACGCGTCGG	3	1	860
OPR-4	CCCGTAGCAC	6	1	1100
OPV-18	TGGTGGCGTT	6	1	380
OPAA-7	CTACGCTCAC	5	1	820
OPAC-11	CCTGGGTCAG	5	2	510, 610
OPAE-20	TTGACCCCAG	7	1	610
UBC-150	GAAGGCTCTG	3	1	720

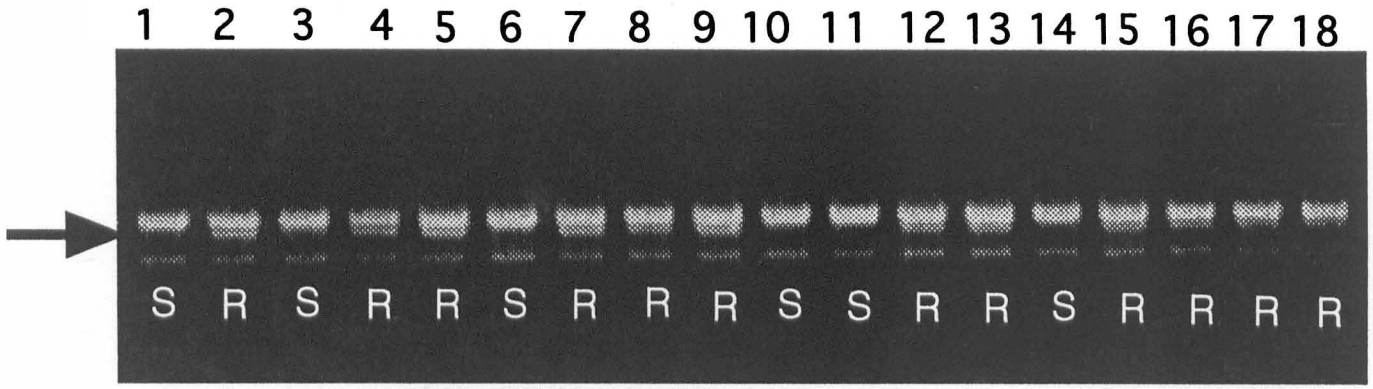


Fig. 3. $F_{2:3}$ segregation pattern for RAPD marker OPP11. Lanes 1 and 2 are Williams and Williams 79, respectively; lanes 3-18 are examples of the $F_{2:3}$ population from a cross between the two parents.

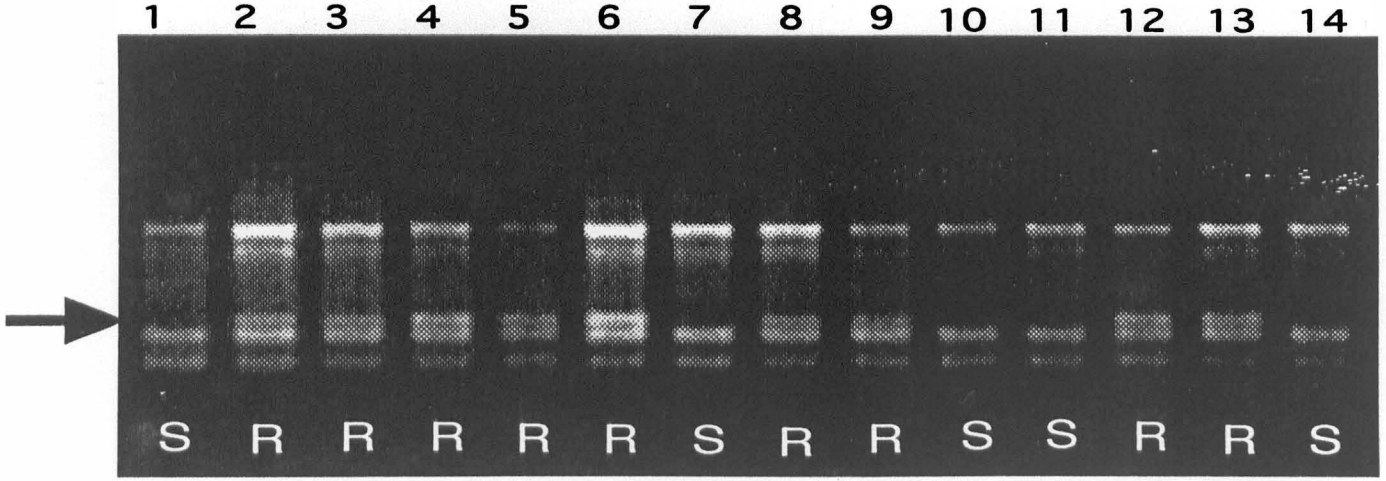


Fig. 4. $F_{2:3}$ segregation pattern for RAPD marker OPV18. Lanes 1 and 2 are Williams and Williams 79, respectively; Lanes 3-14 are examples of the $F_{2:3}$ population from a cross between the two parents.

Table 4. Linkage data for polymorphic markers and the Rps1-c resistance gene

Genotype	Genotypic classes†									n‡	X^2	P§	R¶	SE#	CM††	SE‡‡
	ef	ghi	jk	l	m	n										
Rps1-c/OPP-11	63	125	0	1	1	86				276	266.90	0.000	0.099	0.006	0.99	0.60
Rps1-c/OPV-18	63	125	1	1	1	85				276	262.26.	0.000	0.013	0.007	1.34	0.70
Rps1-c/AFT24E21	14	19	1	1	1	17				53	40.70	0.000	0.070	0.036	7.00	3.60
	e	f	g	hi	j	k	l	m	n							
Rps1-c/SATT_9	61	5	2	121	1	0	1	0	85	276	494.90	0.000	0.020	0.006	2.03	0.61
Rps1-c/PA-71	35	4	6	86	1	1	1	5	59	198	288.99	0.000	0.052	0.011	5.22	1.15
Rps1-c/PA-280	18	3	2	26	1	2	2	2	17	72	82.36	0.000	0.108	0.027	10.98	2.74

†Class designations per Allard (1956). a=A-B, b=A-bb, c=aaB-, d=aabb, a'=A-b-, b'=A-BB, c'= aab-, d'=aaBB, e=AABB, f=AaBB, g=AABb, hi=AaBb, j=AAbb, k=Aabb, l=aaBB, m=aaBb, n=aabb. A = allele from the resistance parent, a=allele from the susceptible parent from the first locus listed, and B=allele from the resistance parent and b=allele from the susceptible parent from the second locus listed.

‡ Number of $F_{2:3}$ individuals tested in this study.

§ Probability of a greater value of X^2.

¶ Estimate of frequency of recombination.

Standard error of recombination estimate.

†† Estimate of genetic distance (centiMorgan) between two loci.

‡‡ Standard error of distance estimate.

AFLP Results

Twenty-five primer combinations were used to screen Williams and Williams 79, and approximately 1000 discrete products were amplified. Two primer combinations (Table 5) produced fragments that appeared in one parent but not the other. The frequency of polymorphism was 0.2%. To test for linkage between these two AFLP markers and the Rps1-c gene, we tested 64 $F_{2:3}$ individuals. The results indicated that only AFT24E21 was linked to Rps1-c and did not deviate from the expected 3:1 ratio based on Chi-square test (Table 1). For this marker, we tested only a small population, since the distance was too far from Rps1-c to be useful for future cloning experenments.

Table 5. AFLP primers for the polymorphic DNA fragments in this study

Primers	Enzyme	Nucleotide sequence 5'-3' (primers)
AFT 24	Taq I	TGAGTCCTGACCGAAGT
AFE 21	EcoR I	CTGCGTTACCAATTCCAA
AFE 24	EcoR 1	CTGCGTTACCAATTCTC

SSR Results

Only one SSR primer (Table 6) was used to screen Williams and Williams 79. Figure 5 shows examples of the $F_{2:3}$ segregation pattern. Chi-square test indicated that the segregation ratio fit the expected 1:2:1 ratio (Table 2) and that the marker was linked to Rps1-c (χ^2 = 494.90, P< 0.001).

Table 6. SSR primer for the polymorphic DNA fragments in this study

Primer	Core motif	5' end primer	3' end primer
SATT9	$(AAT)_{14}$	CCAACTTGAAATTACTAGAGAAA	CTTACTAGCGTATTAACCCTT

RFLP Results

Two RFLP probes, PA71 and PA280, were used to screen Williams and Williams 79 and segregation data was collected from 240 and 87 $F_{2:3}$ individuals, respectively. Both fit the expected 1:2:1 ratio (χ^2 = 1.43, P = 0.489; χ^2 = 1.12, P = 0.571), and as expected, both were linked to Rps1-c (χ^2 = 288.99, P< 0.001; χ^2 = 82.36, P< 0.001).

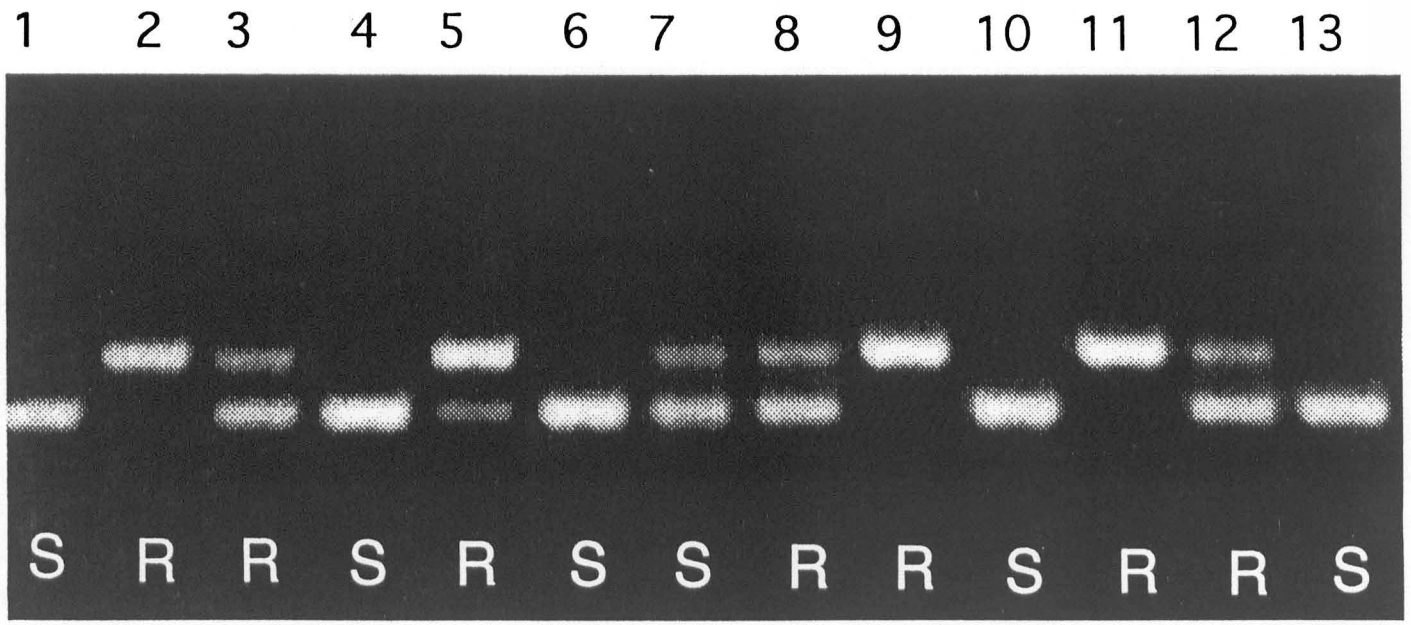
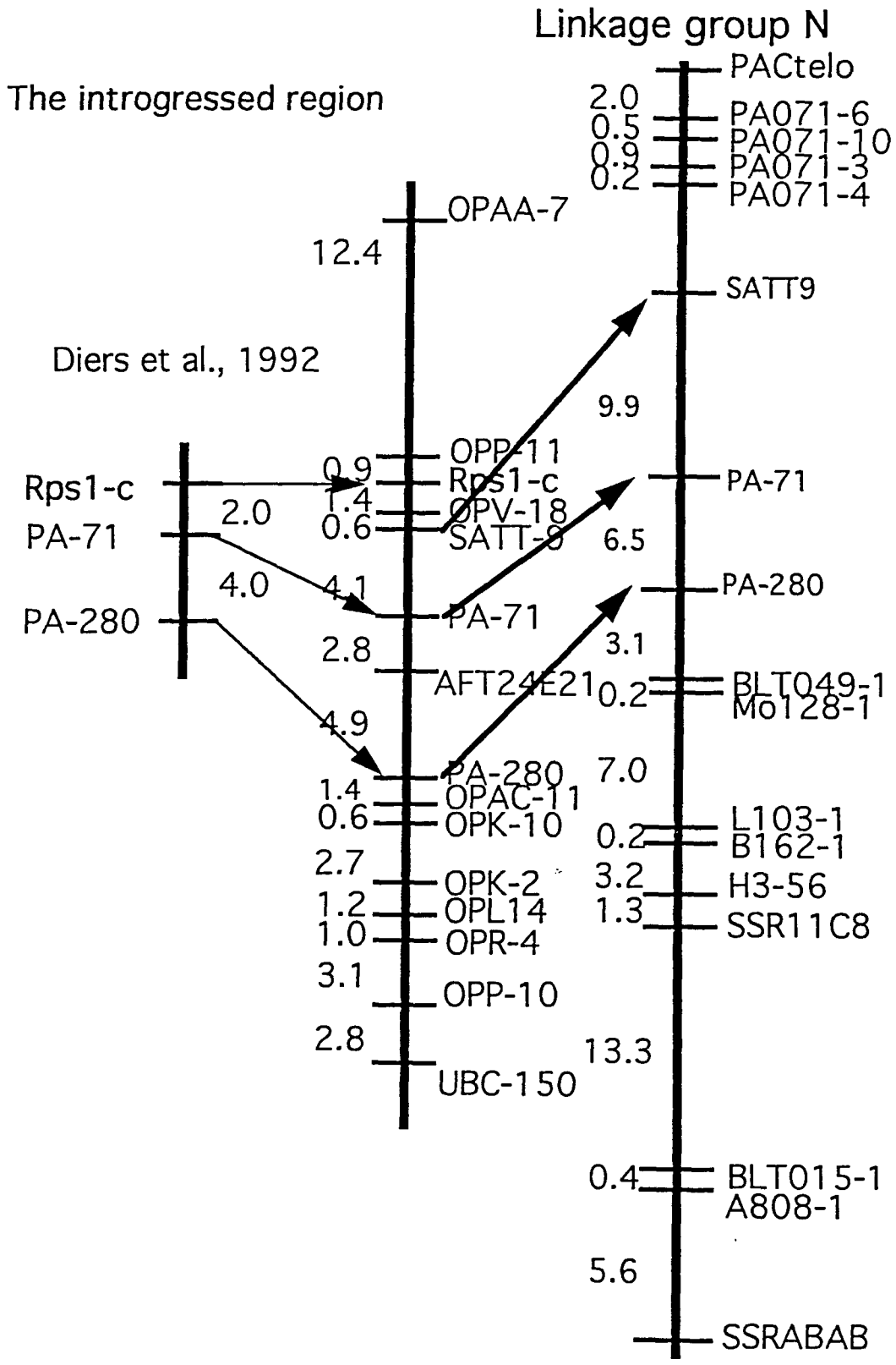


Fig. 5. $F_{2:3}$ segregation pattern for the Simple Sequence Repeat (SSR) marker SATT9. The genomic sequence containing AAT repeats was amplified by polymerase chain reaction, run on a agarose gel, and stained with ethidium bromide. Lanes 1 and 2 are Williams and Williams 79, respectively: lanes 3-13 are examples of $F_{2:3}$ families from a cross between the two parents.

Linkage Map

A linkage map was constructed based on multipoint linkage analysis in the Mapmaker 3.0 computer program. Two RAPD markers, OPP11 and OPV18, and one SSR marker SATT9, were closely linked to Rps1-c, with distances of approximately 0.9, 1.4, and 2.0 cM, respectively. Associations among these markers and the Rps1-c gene are shown in Figure 6.

Figure 6. Map of the introgressed region encompassing the Rps1-c region located on linkage group N of the soybean genome. This map was constructed from the cross of Williams and Williams 79 using RAPD, AFLP, SSR, and RFLP markers, and compared with a genetic map of the same region constructed by Diers et al., 1992 using only RFLP markers. An OP prefix indicates a locus detected by a RAPD primer obtained from Operon Technology; a UBC prefix indicates a locus detected by a RAPD primer obtained from the University of British Columbia; a PA prefix indicated a locus detected by a RFLP probe from the USDA-ARS, ISU; an AF prefix indicates a locus detected by an AFLP primer synthesized by GIBCO BRL; and a SATT prefix indicated a locus detected by a SSR primer from the Beltsville Agricultural Research Center. The order and distances were computed by multipoint linkage analysis with the Mapmaker 3.0 computer program. Genetic distances shown in cM are to the left of the vertical line. Arrows indicate markers in common between maps.


Linkage group N
The introgressed region
Diers et al., 1992
Rps1-c
PA-71
PA-280
2.0
4.0
4.9
4.1
OPAA-7
12.4
0.9
1.4
0.6
OPP-11
Rps1-c
OPV-18
SATT-9
PA-71
2.8
AFT24E21
PA-280
1.4
0.6
OPAC-11
OPK-10
2.7
1.2
1.0
OPK-2
OPL14
OPR-4
3.1
OPP-10
2.8
UBC-150
PACtelo
2.0
0.5
0.9
0.2
PA071-6
PA071-10
PA071-3
PA071-4
SATT9
9.9
PA-71
6.5
PA-280
3.1
0.2
BLT049-1
Mo128-1
7.0
0.2
L103-1
B162-1
3.2
1.3
H3-56
SSR11C8
13.3
0.4
BLT015-1
A808-1
5.6
SSRABAB

CHAPTER 4. DISCUSSION

Several approaches have been suggested to saturate genomic regions of interest with molecular markers. These include preselection using NILs (Williams et al., 1990) and bulked segregant analysis (Michelmore et al., 1991). To construct a saturated map in preparation for chromosome walking (Rommens et al., 1989) or chromosome landing (Tanksley et al., 1995), it is necessary to identify markers tightly linked to the target gene. RFLP markers can be used to find such linked markers, but these require the screening of many clones. In this study, we used three alternative marker techniques RAPD, AFLP, and SSR in conjunction with a susceptible recurrent parent, Williams, and a resistant NIL, Williams 79. We identified ten RAPD markers, one AFLP marker, and one SSR marker linked to the target gene, and developed a linkage map of the introgressed region surrounding the Rps1-c gene. Two RAPD markers, OPP11 and OPV18, were closely linked to the Rps1-c gene, with recombination distances of 0.9 cM and 1.4 cM, respectively. One SSR marker, SATT9, was 2.0 cM from the resistance gene. In order to reduce the standard errors, we used larger populations for testing segregation of the markers most closely linked to Rps1-c, and to more accurately place these markers within the linkage group.

The amount of linked and unlinked donor DNA in NILs that has been removed by backcrossing can vary considerably (Young and Tanksley 1989). According to formulas summarized by Muehlbauer et al. (1988), a soybean NIL formed through five backcrosses would have, on average,

4 % of its genome originating from the donor parent. However, in this study, only 12 RAPD polymorphisms were observed within the 3850 amplified fragments, and only 2 AFLP polymorphisms were observed within the 1025 amplified fragments, indicating a lower percentage of the donor DNA or a higher than expected similarity of the introgressed region of DNA with the region from the recurrent parent. One RAPD marker, OPAE20, segregated in the $F_{2:3}$ population, but did not link to the Rps1-c gene. On the basis of this observation, it is clear that the NIL Williams 79 still carries donor DNA segments not associated with the Rps1-c gene. One marker, OPH4, repeatedly presented a polymorphism between the two parents, but did not segregate in the $F_{2:3}$ population. The reason for this is not clear. It is clear that segregation analysis is necessary to confirm linkage to the target gene when relying upon NIL to identify putatively linked markers. The probability of obtaining a marker within a specified distance of a target gene in NILs depends not only on the number of primers screened but also on genome size and the degree of DNA sequence divergence between the NIL and the recurrent parent in the region surrounding the targeted gene (Martin et al., 1991).

We also tested two RFLP markers, PA71 and PA280 and found that the order of these two markers was the same as Diers et al. (1992), but that the genetic distances were different. The reason for this may be due to the different population size, the numbers of markers tested, or genotype-specific difference in rates of recombination.

We also estimated the minimum size of the introgressed region. Our data indicated this region spans at least 39.9 cM of linkage group N. Of particular interest are the markers OPP11 and OPV18 which flank the Rps1-c by 0.9 and 1.4 cM respectively. We have developed a map of the introgressed region and generated recombinants within the Rps1-c complex.

The availability of molecular markers tightly linked to the Rps1-c gene not only is a prerequisite for the isolation of the Phytophthora resistance gene via map-based cloning, but also provides plant breeders with a powerful tool for efficient screening for Rps1-c resistance without relying on inoculations with the pathogen. This dramatically increases both the numbers that can be screened and the reliability of the results, and facilitates the rapid transfer of the Rps1-c resistance to elite soybean cultivars.

RFLP, RAPD, AFLP, and SSR are all good molecular marker systems. The RFLP assay is usually practiced with specific probes which detect polymorphisms due to point mutation at the restriction site or due to chromosome aberrations (e.g., translocations, inversions, and deletions). DNA blotting and hybridization require several micrograms of DNA (5-8 µg), and radioactive probes are usually used, with each probe detecting one or more genetic loci that share sequence homology. More than one allele can be detected at a locus using RFLP markers. This allows RFLPs to be codominant markers and allows them to distinguish heterozygous loci.

In contrast, the RAPD assay does not require species-specific primers. There is also no limitation to the numbers of primers available. Small amounts of DNA (25-50 ng) are needed for PCR (100 times less than for RFLP's). RAPD assays are also simple, fast, and no radioisotope is needed. Therefore, marker-assisted selection is practical for the plant breeder using this technology. However, RAPDs are usually found to be dominant and rarely co-dominant in soybean (Williams et al., 1990). In our study, we found that all RAPD markers were dominant. It was therefore impossible to distinguish between dominant homozygote and dominant heterozygote genotypes.

Because of their dominant nature, RAPD markers are less informative than co-dominant isozyme or RFLP markers. Nevertheless, we feel that RAPDs are potent molecular markers, not only for fingerprinting purposes, but also for the construction of linkage maps. Primers can be easily and rapidly obtained, and RAPDs are particularly useful for associating markers with specific genes when NILs are available.

SSRs or microsatellites are ideal genetic markers in that they are highly abundant and highly polymorphic (Tautz, 1989). The few published plant SSR studies suggest that SSR markers are potentially as powerful in plant systems as they are in mammalian systems. In this study, we tested a SSR marker closely linked to the Rps1-c gene with agarose gel electrophoresis without the use of radioisotope. The ease in screening for SSRs should further facilitate their use in

practical plant breeding settings. SSRs are codominant markers and can detect heterozygous individuals in a segregating population.

AFLPs detect about the same level of polymorphism as RFLPs, but many more fragments are observed for each reaction. In this study, we screened 25 primer combinations. Each primer combination, on average, produced approximately 40 bands, and only one primer combination identified a polymorphism that was linked to the Rps1-c gene. AFLPs are dominant markers and did not detect the heterozygous loci.

A long-term goal of this project is to use molecular markers as anchors for physical mapping and as a starting point for chromosome walking or landing. Therefore, we will need to estimate genetic distance relative to physical distance between markers closely linked to the Rps1-c gene. If the physical distances are within the range of insert size of BAC or YAC vectors, then the markers can be used for map-based cloning of the gene. The successful cloning of this gene will increase our understanding of the mechanism by which soybean can be resistant to Phytophthora this gene and may allow us to quickly transfer this resistance gene to other elite soybean cultivars.

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