Genetic and Physical Mapping of *Avr1a* in *Phytophthora sojae*

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Manuscript received August 16, 2001
Accepted for publication December 17, 2001

ABSTRACT

The interaction between soybean and the phytopathogenic oomycete *Phytophthora sojae* is controlled by host resistance (*Rps*) genes and pathogen avirulence (*Avr*) genes. We have mapped the *Avr1a* locus in F_2 populations derived from four different *P. sojae* races. Four RAPD and nine AFLP markers linked to *Avr1a* were initially identified. Nine markers were used to compare genetic linkage maps of the *Avr1a* locus in two distinct F_2 populations. Distorted segregation ratios favoring homozygous genotypes were noted in both crosses. Segregation analysis of all the markers in one F_2 population of 90 progeny generated a map of 113.2 cM encompassing *Avr1a*, with one marker cosegregating with the gene. The cosegregating DNA marker was used to isolate *P. sojae* BAC clones and construct a physical map covering 170 kb, from which additional DNA markers were developed. Three markers occurring within the BAC contig were mapped in an enlarged population of 486 F_2 progeny. *Avr1a* was localized to a 114-kb interval, and an average physical to genetic distance ratio of 391 kb/cM was calculated for this region. This work provides a basis for the positional cloning of *Avr1a*.

PHYTOPHTHORA sojae is an oomycete that causes root and stem rot on soybean. This disease is a serious and endemic problem in soybean-producing areas, especially in North America where it causes yield losses in excess of 10^9 kg/year (Wrather *et al.* 2001a,b). Disease control strategies have relied on developing resistant soybean cultivars through the identification and integration of soybean *Rps* (*r*esistance to *Phytophthora sojae*) genes. Thirteen different *Rps* genes, at seven loci, have been described thus far in soybean. Five of these genes segregate as alleles at the *Rps1* locus (*Rps1a*, *Rps1b*, *Rps1c*, *Rps1d*, *Rps1k*), while three segregate as alleles at the *Rps3* locus (*Rps3a*, *Rps3b*, *Rps3c*). The five remaining genes, *Rps2*, *Rps4*, *Rps5*, *Rps6*, and *Rps7*, are not allelic. Only the *Rps1* and *Rps7* loci are known to occur on the same linkage group (Anderson and Buzzell 1992; Cregan *et al.* 1999). Additional sources of many undescribed *Rps* genes in soybean germplasm have also been reported (Dorrance and Schmitthenner 2000). Although effective, disease control methods based on *Rps* genes impose heavy selective pressures and result in the emergence of new pathogen strains with novel virulence phenotypes.

To date, at least 53 different physiologic races or strains of *P. sojae* have been recorded, each distinguishable by its reaction against the known *Rps* genes (Ward 1990; Förster *et al.* 1994; Ryley *et al.* 1998). Genetic analysis of virulence in *P. sojae* has been difficult because this organism is diploid and homothallic and the germination of oospores is nonsynchronous. Nonetheless, Layton and Kuhn (1988) reported that avirulence against the *Rps1a* resistance gene was dominant to virulence in heterokaryons. More recently, fungicide-resistant mutants and DNA markers have been used to identify hybrids and to obtain F_2 populations from crosses between different races of *P. sojae* (Bhat 1991; Bhat and Schmitthenner 1993; Bhat *et al.* 1993; Whisson *et al.* 1994, 1995; Tyler *et al.* 1995). From such crosses it has been determined that avirulence against the *Rps1a*, *-1b*, *-1d*, *-1k*, *-3a*, *-4*, *-5*, and *-6* resistance genes is dominant to virulence and likely dominant in the cases of *Rps3b* and *-3c* (Whisson *et al.* 1994, 1995; Tyler *et al.* 1995; Gijzen *et al.* 1996b). Additionally, the segregation of avirulence against the *Rps1a*, *-1b*, *-1k*, *-3a*, *-4*, and *-6* resistance genes in F_2 populations has been shown to be consistent with that of single dominant genes, *Avr1a*, *-1b*, *-1d*, *-1k*, *-3a*, *-4*, and *-6*, respectively (Whisson *et al.* 1994, 1995; Tyler *et al.* 1995; Gijzen *et al.* 1996b). These segregation analyses also showed that *Avr1b* and *Avr1k* are closely linked, as are *Avr3a* and *Avr5*, and *Avr4* and *Avr6*.

Genetic inheritance of resistance and avirulence in the soybean-Phytophthora interaction fits the gene-for-gene concept proposed by Flor (1956). This model may be used to predict the outcome for many plant-

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pathogen and plant-pest interactions. Isolation and cloning of plant resistance genes and pathogen avirulence genes have validated many of the original tenets of Flor's theory and provided new insight into the molecular mechanisms that govern plant-pathogen interactions. Numerous *Avr* genes that have been isolated from bacterial, viral, and fungal plant pathogens encode putative proteins with diverse sequence and structural characteristics (Leach and White 1996; De Wit and Joosten 1999). No race-specific avirulence gene product has been described in *P. sojae* or any other oomycete pathogen, but small cysteine-rich elicitor proteins, termed elicitins, have been shown to act as important determinants of host range in *P. infestans* (Kamoun *et al.* 1993, 1998).

Cloning and characterizing *P. sojae* avirulence genes will aid in deciphering the molecular events involved in *P. sojae* pathogenesis and in understanding plant-pathogen interactions in general. As a first step toward the map-based cloning of *Avr1a*, we have identified randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers that are linked closely to this locus. These DNA-based markers were mapped in two independent F_2 populations, and a high-density genetic map of the *Avr1a* region has been constructed. The DNA fragments corresponding to the RAPD and AFLP markers were cloned and used as probes in Southern blot analysis of *P. sojae* genomic DNA and for screening a *P. sojae* bacterial artificial chromosome (BAC) library to construct a physical map of the region.

MATERIALS AND METHODS

***Phytophthora sojae* isolates, mapping populations, and virulence assays:** *P. sojae* race 1 (48FPA18, avirulent on *Rps1a*) and race 3 (25MEX4, virulent on *Rps1a*) were pure-breeding single-oospore isolates from the Ohio Agricultural Research and Development Center, Wooster, OH (Bhat 1991; Bhat and Schmitthenner 1993); race 2 (P6497, avirulent on *Rps1a*) and race 7 (P7064, virulent on *Rps1a*) isolates were from the Phytophthora culture collection at the University of California, Riverside, CA (Förster *et al.* 1994). All other isolates were from the Phytophthora species collection at Agriculture and Agri-Food Canada, London, ON. Methods for the generation and identification of hybrid F_1 progeny and segregating F_2 populations have been described (Bhat 1991; Bhat and Schmitthenner 1993; Tyler *et al.* 1995; MacGregor 2000). A summary of the mapping populations is provided in Table 1.

Virulence assays were performed on cv. Harosoy (*Rps7*) and cv. Harosoy 63 (*Rps1a*, *Rps7*) soybean plants by hypocotyl inoculation of 5–10 plants with zoospores (Ward *et al.* 1979; Gijzen *et al.* 1996a) or a mycelial slurry (Haas and Buzzell 1976). All inoculations were performed at least twice. In the vast majority of cases the virulence phenotype for an individual culture was consistent and uniform for all plants tested. However, escapes were noted and cultures were considered virulent if more than half of the inoculated seedlings were susceptible to infection and avirulent if more than half were resistant.

Extraction and purification of DNA samples: Mycelium (200–500 mg) grown in liquid synthetic medium (Hoitink and Schmitthenner 1969) was ground to a fine powder in liquid N_2. The powder was transferred to 2–3 ml of DNA extraction buffer (Tyler *et al.* 1995), and DNA was extracted using standard phenol extraction procedures.

For small-scale extraction of DNA, batches of mycelium were grown in 500 μl of liquid minimal medium in 1.5-ml microfuge tubes. The tubes were inoculated with tufts of mycelium grown on solidified minimal medium. The cultures were incubated at 25° for 4 days and mycelium was collected by centrifugation at 20,000 × *g* for 10 min. Mycelium was resuspended in 500 μl of DNA extraction buffer and subjected to two rounds of freezing at −80° followed by quick thawing at 55° to break the cells. Debris was removed by centrifugation at 20,000 × *g* for 10 min and the supernatant was transferred to clean tubes. After treatment with RNase A (1 μl of 10 μg/ml), the DNA was isolated by precipitation with isopropanol and washed once with 70% ethanol. Samples were resuspended in 50 μl of 10 mm Tris-HCl, 0.1 mm EDTA, pH 8.0 and stored at 4°.

RAPD analysis: RAPD (Welsh and McClelland 1990; Williams *et al.* 1990) reactions were performed as per Kasuga *et al.* (1997), except that reactions contained 1.0 ng of DNA and 1.0 unit *Taq* DNA polymerase. Typically, 15 μl of each reaction was analyzed by electrophoresis through 1.2% agarose gels. RAPD markers were named according to the corresponding primer. For example, RAPD359 was amplified using UBC primer UBC359. Sequences for all primers mapped as DNA markers in this study are shown in Table 2.

Pools of DNA for RAPD bulked segregant analysis (Michelmore *et al.* 1991) were prepared by combining equal amounts of DNA from 10 virulent or avirulent individuals, respectively, chosen at random. Bulked segregant analysis was performed using 1.0 ng of pooled DNA per reaction.

AFLP analysis: AFLP reactions (Life Technologies, AFLP Analysis System II; Vos *et al.* 1995) were performed according to instructions provided by the manufacturer for the *Eco*RI/*Mse*I enzyme combination or according to Kasuga *et al.* (1997) for the *Hin*dIII/*Mse*I enzyme combination, with the following modifications. Restriction digests were carried out for 4 hr to ensure complete digestion, and all ligations were performed at 12° overnight using nonbiotinylated adapters. Preamplification reactions for the *Hin*dIII/*Mse*I enzyme combination were performed using *Hin*dIII and *Mse*I primers each with no selective nucleotides ($H + 0/M + 0$). Selective amplifications were performed with *Eco*RI primers having one or two selective nucleotides ($E + 1$ or $E + 2$) and *Mse*I primers having three selective nucleotides (M + 3) or with *Hin*dIII primers with one ($H + 1$) and *Mse*I primers with three ($M + 3$) selective nucleotides. Reaction products were resolved in polyacrylamide sequencing gels (6%). Samples were run at 70 W for 3–4 hr, after which the gels were dried under vacuum (gel dryer model 583; Bio-Rad, Richmond, CA) for 2 hr at 80° without fixing and exposed to film (Kodak BioMax MR) for 4–5 days. The amplification products were scored by visual inspection of the films. The AFLP markers were named according to the combination of selective amplification primers used to amplify the markers. For example, AFLP marker ETM-CAC was amplified with the *Eco*RI primer with a single T selective nucleotide and the *Mse*I primer with the three selective nucleotides CAC.

Cleaved amplified polymorphic DNA analysis: Polymorphisms mapped by the cleaved amplified polymorphic DNA analysis (CAP) technique (Koniecyzn and Ausubel 1993; Jarvis *et al.* 1994) were identified by comparing race 2-derived BAC end sequences to genomic DNA sequence from race 7. Race 7 genomic DNA regions were amplified using PCR primers designed from 10-J14-1 and 10-B21-7 BAC end sequences and cloned into a plasmid vector (pGEM-T Easy; Promega,

TABLE 1

Summary of *P. sojae* genetic crosses and segregation of *Avr1a* phenotype

Cross	Parent 1 (avirulent)	Parent 2 (virulent)	F_1[a]	F_2 population[b]					
				Total	Scored for *Avr1a*	Avirulent	Virulent	χ^2	Probability
R1/R3	48FPA18	25MEX4	Avirulent	72	72	54	18	0.00	1.00
R2/R7-BT	P6497	P7064	Avirulent	90	90	62	28	1.79	0.18
R2/R7-TM	P6497	P7064	Avirulent	192	109	93	16	6.19	0.01*
R2/R7-TMA	P6497	P7064	Avirulent	204	—	—	—	—	—

[a] Each of the R2/R7 populations was created from the same F_1 individual.
[b] Asterisk indicates that the segregation ratio is significantly different from Mendelian prediction ($P < 0.05$). Virulence phenotypes were not determined (—) for cross R2/R7-TMA.

Madison, WI) for sequencing and comparative analysis. To follow the segregation of these markers, genomic DNA from each F_2 progeny was PCR amplified and subjected to restriction enzyme digestion.

For CAP markers, 5 μl of the PCR reactions were digested overnight with 2–4 units of the appropriate restriction enzyme (10 μl total volume) and the digestion products were resolved through 2.0–2.5% agarose gels. The gels were stained with ethidium bromide and the markers were scored visually.

BAC library screening and contig construction: A *P. sojae* BAC library (pBeloBAC11 cloning vector) constructed from race 2 (P6497) contained 14,500 clones with an average insert size of 40 kb (B. Tyler, unpublished data). For hybridization screening, a 384-pin replicator (Nunc) was used to inoculate Luria-Bertani medium/chloramphenicol (12.5 μg/ml) agar plates, and colony lifts were performed with Hybond-N nylon membranes (Amersham Pharmacia Biotech, Braunschweig, Germany) using the methods suggested by the manufacturer. Prehybridization was in 0.25 M Na_2HPO_4, 7% SDS for 1 hr at 65°. Probes were hybridized in fresh solution overnight at 65°. Following hybridization, the blots were washed at 68° twice each with 25 mM Na_2HPO_4, 5% SDS and then 25 mM Na_2HPO_4, 1% SDS for 30 min per wash. Blots were stripped after use with a boiling solution of 0.1× SSC, 0.5% SDS.

The probe corresponding to marker CAP-CGA-3/4 was generated by PCR amplification (Table 2). The probe (607 bp) corresponding to the T7 end of BAC clone 10-J14-1 was amplified from 10-J14-1 using the forward primer 5′-TTCTCACCA GATCTCAGGCAGT-3′ and the reverse primer 5′-ATTTGG CGTCCCTCTTG-3′, designed from BAC end sequencing.

Linkage analysis: Chi-square analysis was used to determine significant 3:1 or 1:2:1 segregation of *Avr1a* and all DNA markers and to confirm the absence of independent assortment between markers and *Avr1a*. The segregation data, regardless of significance, was analyzed using the Mapmaker 3.0 computer program (Haldane mapping function; LOD 3.0; $R_F = 0.3$; Lander *et al.* 1987). Maps were drawn using the Mappit program, version 1.3 (L. Gianfranceschi and B. Koller, Swiss Federal Institute of Technology, http://www.pa.ipw.agrl.ethz.ch/).

Cloning RAPD and AFLP markers: The polymorphic RAPD or AFLP markers were cut from agarose or acrylamide gels, respectively. Agarose gel slices were spun through glass wool in a 1-ml pipette tip to remove the agarose. Slices of dried acrylamide gels were soaked in 100 μl TE buffer for at least 1 hr at room temperature followed by vigorous vortexing. One-microliter aliquots were used as template to amplify markers for cloning into a plasmid vector (pGEM-T Easy, Promega). *Escherichia coli* cells (XL1 Blue MRF′; Stratagene, La Jolla, CA) were transformed by electroporation using 2 μl of the ligation reaction. Automated cycle sequencing of plasmid DNA was carried out using dye-labeled terminators (377; Applied Biosystems, Foster City, CA), and sequences were edited and assembled into contiguous stretches using a software program (Lasergene, DNAStar).

RESULTS

***Avr1a* segregates as a dominant allele at a single locus:** Progeny resulting from two independent crosses segregating for virulence against *Rps1a* were analyzed in this study. A population of 72 F_2 progeny resulting from a race 1 (avirulent)/race 3 (virulent) cross (R1/R3) and 90 F_2 progeny from a race 2 (avirulent)/race 7 (virulent) cross (R2/R7-BT) provided a basis for comparative mapping and linkage analysis of *Avr1a* in different *P. sojae* isolates. Both F_1 hybrids resulting from these crosses were avirulent. Neither of the F_2 segregation ratios from these crosses differed significantly from 3:1 ($P > 0.05$), as shown in Table 1.

To increase the size of the R2/R7 mapping population for higher-resolution mapping, the F_1 used to generate the R2/R7-BT F_2 population was selfed on two separate occasions to generate two additional sets of R2/R7 F_2 progeny, designated R2/R7-TM and R2/R7-TMA. Of an estimated 45,000 oospores spread onto germination plates from each isolation, ~500 (1.1%) germinated. In total, the R2/R7-TM population consisted of 192 F_2 individuals, and virulence phenoytpes were determined for 109 of these progeny. Data in Table 1 show that the segregation ratio of avirulence to virulence in the R2/R7-TM population differed slightly from 3:1 ($P = 0.01$). However, when the data from the R2/R7-BT and R2/R7-TM F_2 populations were combined, the segregation ratio of avirulence to virulence (155:44) gave a good fit to the expected 3:1 segregation ratio ($P = 0.35$). The R2/R7-TMA population, containing 204 F_2 progeny, was not analyzed for virulence phenotypes.

Identical RAPD markers are linked to *Avr1a* in independent crosses: A collection of 600 arbitrary decanucleotide primers were tested for ability to amplify

TABLE 2

PCR primers and probes used to identify DNA markers linked to *Avr1a*

Marker name	Marker type[a]	Primers for identifying marker[b]	Marker size (bp)[c]
10B21T7-2/5A	CAP	F, TCGTCGCCGTATCTTAGG	R2, 520/215
		R, TCCCCAGCGAGAACAAC	R7, 724
CAP-CGA-3/4	CAP	F, TGCAGAGTTCAGTCCAACAA	R2, 383/227
		R, TGCCAGAATTGAGTGGTTAA	R7, 227/226/166
10F1/3R2	CAP	F, AGCCCAGCGCTGAATAC	R2, 1551/321
		R, AACCCGTTTGATGTACAGTGA	R7, 1872
ECMCTT	AFLP	E, GACTGCGTACCAATTCC	405
		M, GATGAGTCCTGAGTAACTT	
EGMCAC	AFLP	E, GACTGCGTACCAATTCG	323
		M, GATGAGTCCTGAGTAACAC	
EGMCAG	AFLP	E, GACTGCGTACCAATTCG	306
		M, GATGAGTCCTGAGTAACAG	
ETMCAA	AFLP	E, GACTGCGTACCAATTCT	357
		M, GATGAGTCCTGAGTAACAA	
ETMCAC	AFLP	E, GACTGCGTACCAATTCT	259
		M, GATGAGTCCTGAGTAACAC	
HAMACT	AFLP	H, AGACTGCGTACCAGCTTA	177
		M, GACGATGAGTCCTGAGTAAACT	
HAMCCT	AFLP	H, AGACTGCGTACCAGCTTA	~300
		M, GACGATGAGTCCTGAGTAACCT	
HAMCGA	AFLP	H, AGACTGCGTACCAGCTTA	344
		M, GACGATGAGTCCTGAGTAACGA	
HAMGAG	AFLP	H, AGACTGCGTACCAGCTTA	512
		M, GACGATGAGTCCTGAGTAAGAG	
RAPD77	RAPD	GAGCACCAGG	1566
RAPD359	RAPD	AGGCAGACCT	R2, 1731
			R7, 2025
RAPD431	RAPD	CTGCGGGTCA	454
RAPD437	RAPD	AGTCCGCTGC	723

[a] CAP, cleaved amplified polymorphism; PCR, polymerase chain reaction; AFLP, amplified fragment length polymorphism; RAPD, random amplified polymorphic DNA. Restriction enzymes used for generating CAPs markers: 10B21T7-2/5A, *Mse*I; CGA-3/4, *Alw*NI; 10F1/3R2, *Nco*I.

[b] F, forward primer; R, reverse primer; E, primer for *Eco*RI adapter; H, primer for *Hin*dIII adapter; M, primer for *Mse*I adapter. All sequences 5′ to 3′.

[c] Sizes of digestion products for CAPs markers shown for both race 2 and race 7; undigested race 2 CAP product 10B21T7-2/5A has an 11-bp insert relative to race 7; undigested race 2 CAP product CGA-3/4 has a 9-bp deletion relative to race 7.

polymorphic DNA sequences distinguishing the race 1 and race 3 parents. More than half (354 of 600) of these primers produced amplification products that could be resolved and visualized in agarose gels. In total, ~12% of the estimated 2100 sequences amplified were polymorphic between the race 1 and race 3 parental isolates. From this screening, 130 primers amplified dominant markers, DNA products present in one race but not the other. These dominant markers were equally distributed between the two parents: 63 were specific for race 1, and 67 were specific for race 3. A further 62 primers amplified polymorphic sequences in both parents, possibly representing codominant markers, although these sequences may not be allelic.

Bulked segregant analysis (Michelmore *et al.* 1991; Churchill *et al.* 1993) was carried out using the 125 primers that amplified polymorphic sequences in the race 1 parent. Four dominant and two codominant RAPD markers, (RAPD77, RAPD359, RAPD431, RAPD-433, RAPD437, and RAPD512) were able to distinguish between DNA bulks from avirulent and virulent F_2 progeny from the R1/R3 cross and were therefore considered to be putatively linked to the *Avr1a* locus. The segregation of each of these markers in the R1/R3 F_2 population was scored and a linkage test was performed. Four of the six RAPD markers were found to be linked to *Avr1a*, and all mapped to one side of the gene, as shown in Figure 1. Three of the markers, RAPD77, RAPD359, and RAPD431, cosegregated. Chi-square analysis of the segregation data of *Avr1a* and each RAPD marker also indicated these loci did not assort independently.

To determine whether these RAPD markers were also segregating in the R2/R7-BT cross, the race 2 and race 7 parents were tested and found to produce polymorphisms identical to the race 1 and race 3 isolates. Cloning and sequencing the polymorphic fragments showed

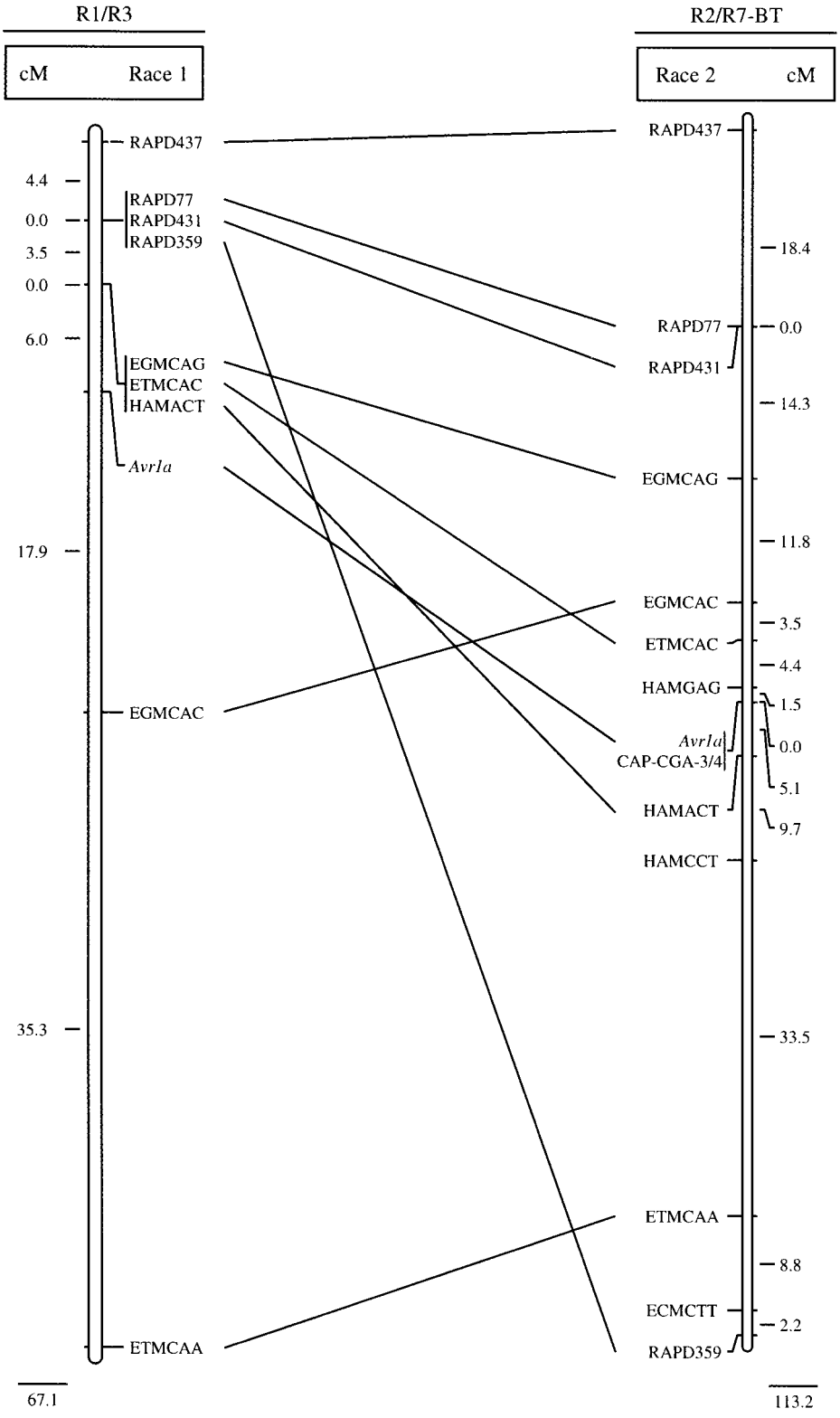
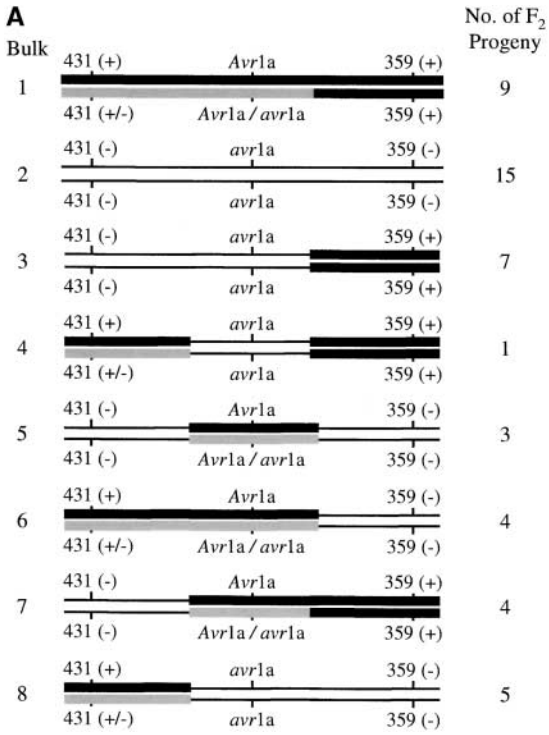


FIGURE 1.—A comparison of high-density genetic linkage maps of the *Avr1a* locus in *P. sojae* race 1 and race 2. A population of 72 F_2 progeny from a cross between race 1 and race 3 (R1/R3) was used to map 10 markers: 4 RAPD markers, 5 AFLP markers, and *Avr1a*. The distance spanned by the markers in race 1 is 67.1 cM. A population of 90 F_2 progeny from a cross between race 2 and race 7 (R2/R7-BT) was used to map 14 markers: 4 RAPD markers, 8 AFLP markers, 1 CAP marker, and *Avr1a*. The dominant AFLP marker HAMCGA was mapped as its corresponding codominant CAP marker, CAP-CGA-3/4. The total distance spanned by the markers in race 2 is 113.2 cM. The genetic distances in centimorgans (Haldane) are shown.

that each of the markers was distinct and that corresponding markers present in race 1 and race 3 were identical to those in race 2 and race 7, respectively. Segregation analysis of these RAPD markers in the R2/R7-BT F_2 population indicated that they were also linked to *Avr1a* in this cross. However, while three of the RAPD markers, RAPD77, RAPD431, and RAPD437, mapped to one side of *Avr1a*, RAPD359 mapped to the flanking side. The RAPD77 and RAPD431 markers cosegregated in this cross, as they did in the R1/R3 cross.

AFLP analysis generates markers closely linked to *Avr1a*: To identify more markers and to produce a high-



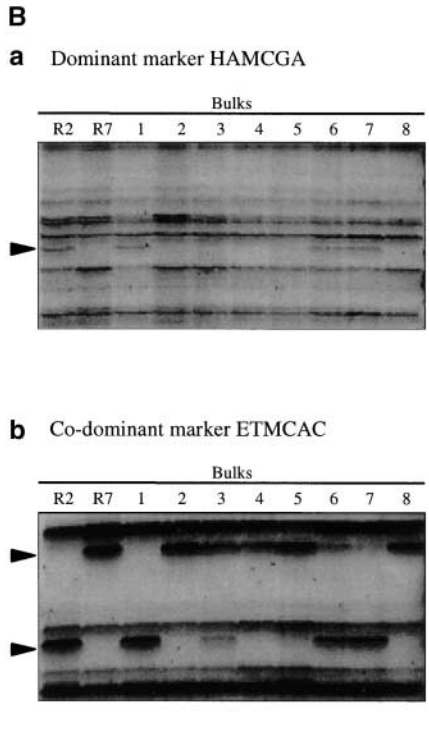


FIGURE 2.—Design of DNA bulks used to identify AFLP markers closely linked to *Avr1a* in *P. sojae* race 2. (A) Schematic representation of the genotypes of recombinant F_2 DNA bulks. Thick lines represent chromosome fragments of race 2; thin lines represent chromosome fragments of race 7. Gray lines indicate that the chromosomal fragment may be of either race. The total number of progeny within each bulk class is also shown. Bulk 1 is homozygous for race 2-specific RAPD359(+/+) alleles and either homozygous or heterozygous for *Avr1a* and RAPD431(+/+ or +/−) alleles; bulk 2 is homozygous for *avr1a*, race 7-specific RAPD359(−/−) alleles, and RAPD431(−/−) alleles. Bulks 3, 4, 5, 6, 7, and 8 consist of F_2 progeny that are recombinant for*Avr1a* and either one or both of the RAPD markers. (B) Examples of AFLP markers identified using the DNA bulks. Two classes of AFLP markers were identified: (a) dominant markers and (b) codominant markers. Lanes are labeled as follows: R2, race 2 (*Avr1a*); R7, race 7 (*avr1a*); 1, bulk 1; 2, bulk 2; 3, bulk 3; 4, bulk 4; 5, bulk 5; 6, bulk 6; 7, bulk 7; 8, bulk 8. Arrowheads indicate the polymorphic bands.

density linkage map around *Avr1a*, we resorted to AFLP analysis (Vos *et al.* 1995). A series of eight bulks from the R2/R7-BT F_2 population were designed, based on the segregation of *Avr1a* and two flanking RAPD markers, as shown in Figure 2. Bulk 2 was constructed using 10 of the 15 F_2 progeny chosen at random, while all other bulks were composed of equal amounts of all progeny available. All the bulks except bulk 3 can be accounted for by crossing over between *Avr1a* and either one or both of RAPD431 and RAPD359. Bulk 3 can be accounted for either by double nonsister chromatid crossover or by gene conversion at the RAPD359 locus after crossing over between *Avr1a* and RAPD359.

The identification of AFLP markers closely linked to *Avr1a* was accomplished in two steps. The first step was to screen primer combinations with the race 2 and race 7 parents and then to screen those primers able to distinguish the parents against the bulked samples. However, virtually all of the initial primer combinations (139 of 140) were found to amplify polymorphic sequences between the parents. Subsequently, primer screening was performed using the first two of the eight bulks. Bulk 1 was composed of avirulent F_2 progeny that were also positive for RAPD431 (+/+ or +/−, because this is a dominant marker) and homozygous for the race 2-specific allele of RAPD359 (+/+), as identified by analysis of the F_2 progeny. Bulk 2 was composed of virulent F_2 progeny that were negative for RAPD431 (−/−) and homozygous for the race 7-specific allele of RAPD359 (−/−). In all, 44 of the 336 (13%) selective primer combinations tested amplified polymorphic sequences that were able to distinguish between bulk 1 and bulk 2. On average, 55 DNA sequences were amplified per $E + 1/M + 3$ primer combination and 23 per $E + 2/M + 3$ combination. An average of 145 DNA sequences were amplified per $H + 1/M + 3$ primer combination. Approximately 2.5% of the estimated 40,000 loci examined were polymorphic.

In the second step, the 44 primer combinations identified from the bulk 1 and bulk 2 comparative analysis were retested against all eight bulks to assess their reproducibility and to determine whether the polymorphic sequences were closely linked to *Avr1a*. In the final analysis, nine of these AFLP markers were scored and mapped in the R2/R7-BT population and five in the R1/R3 population.

Genetic linkage maps of the *Avr1a* locus are comparable in race 1 and race 2: To compare the *Avr1a* genetic region in race 1 with that in race 2, the segregation of four RAPD and five AFLP markers was followed in each of the two populations and the data were assembled into the most probable genetic maps by linkage analysis software. Figure 1 shows that the two populations produced maps with conserved, but not identical, order and spacing of markers relative to *Avr1a*. The total genetic distance spanned by the markers was similar in both

TABLE 3

Summary of segregation data analyzed for *Avr1a* mapping

Marker	Phenotype[a]	Population[b]			
		R1/R3	R2/R7-BT	R2/R7-TM	R2/R7-TMA
RAPD437	D	53:19	56:34*	—	—
RAPD77	D	51:21	62:28	—	—
RAPD431	D	51:21	62:28	—	—
RFLP431	CD	48:3:21*	—	—	—
EGMCAG	D	52:20	59:31*	—	—
EGMCAC	D	52:20	62:28	—	—
ETMCAC	CD	50:2:20*	43:17:30*	—	—
HAMGAG	D	—	63:27	—	—
10F1/3R2	CD	—	43:18:29*	58:102:32*	67:87:50*
CAP-CGA-3/4	CD	NP	43:19:28*	58:102:32*	67:87:50*
Avr1a	D	54:18	62:28	93:16*	—
10B21T7-2/5A	CD	—	43:19:28*	57:103:32*	66:88:50*
HAMACT	D	52:20	59:31*	—	—
HAMCCT	D	—	62:28	—	—
ETMCAA	D	38:34	63:27	—	—
ECMCTT	D	—	62:28	—	—
RAPD359	CD	48:3:21*	11:53:26*	—	—
RFLP359	CD	48:3:21*	—	—	—
RFLP433[c]	CD	24:18:26	—	—	—
RFLP512	CD	23:20:29	—	—	—

[a] D, dominant; CD, codominant.

[b] Dominant marker scored as marker present:marker absent; codominant markers scored as homozygous avirulent-linked marker:heterozygous:homozygous virulent-linked marker. NP, not polymorphic; —, not determined. *, the segregation ratio is significantly different from Mendelian prediction ($P < 0.05$).

[c] No data for four F_2 progeny.

races, 67.1 cM in race 1 and 113.2 cM in race 2. The most significant difference between the two maps was the relative position of RAPD359. This marker is clustered with two other markers in race 1 but mapped to another location on the opposite side of *Avr1a* in race 2.

It was possible to produce a linkage map of higher density and resolution from the R2/R7-BT cross because both more progeny and segregating markers were available for this cross. Figure 1 also shows the results of a linkage analysis of the *Avr1a* region in race 2 constructed using 14 markers: 11 dominant markers (3 RAPD, 7 AFLP, and *Avr1a*) and 3 codominant markers (1 RAPD, 1 AFLP, and 1 CAP). All of the AFLP markers mapped closer to *Avr1a* than either of the two RAPD markers used to design the eight F_2 bulks, illustrating the effectiveness of bulked segregant analysis. The dominant AFLP marker HAMCGA was converted to a codominant CAP marker CAP-CGA-3/4 for mapping analysis (as described below). The markers encompass a total genetic distance of 113.2 cM, with the marker CAP-CGA-3/4 cosegregating with *Avr1a*.

Codominant DNA markers linked to *Avr1a* show distorted segregation patterns: Table 3 summarizes segregation data for the DNA markers linked to *Avr1a* in the two mapping populations. Codominant markers were verified by sequence analysis of polymorphic fragments. In the R1/R3 progeny, only one dominant marker deviated from Mendelian segregation ratios but both codominant markers were skewed in favor of homozygous genotypes. To further explore this deficiency of heterozygous genotypes, the dominant marker RAPD431 and the race 1-specific amplification product of the codominant marker RAPD359 were used as DNA probes in Southern blot analysis of the R1/R3 population. This effectively converts the RAPD markers into RFLP markers. As shown in Table 3, both RFLP markers displayed highly skewed segregation ratios in favor of homozygous genotypes, especially for alleles associated with the avirulent phenotype. Two additional RAPD markers identified through bulked segregant analysis, RAPD433 and RAPD512, were also converted to codominant RFLP markers to determine whether the skewed ratios of the linked markers were related to their proximity to *Avr1a*. Segregation analysis showed that RAPD433 and RAPD512 fall on the same linkage group with *Avr1a* only when default linkage threshold values are increased. Thus, these markers are distally located from *Avr1a* or occur on a separate chromosome. Results show that the segregation ratios of RFLPs corresponding to RAPD433 and RAPD512 also do not fit expected Mendelian ratios, although the deviation from expected values was less than that observed for the *Avr1a*-linked markers.

Data from the R2/R7 progeny indicate that closely linked, codominant markers also show distorted segregation patterns in this cross, although not as severe as in the R1/R3 cross. Furthermore, the level of distortion differed for each of the three F_2 populations analyzed. The greatest distortion was observed in the R2/R7-BT population, while only a slight distortion was observed in the R2/R7-TM and R2/R7-TMA populations.

Two marker sequences show similarity to known protein sequences: Each of the cloned RAPD and AFLP markers was sequenced, and the DNA sequences were compared to other publicly available DNA and protein sequences at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the National Center for Genome Resources (http://www.ncgr.org/), including databases of *P. sojae* and *P. infestans* expressed sequence tags from the Phytophthora Genome Initiative (Kamoun *et al.* 1999; Qutob *et al.* 2000; Waugh *et al.* 2000). No nucleotide similarities were identified, but translation of the DNA sequences revealed that AFLP marker EGMCAG showed strong similarity ($E = 10^{-23}$) to a necrosis-inducing protein from *P. parasitica* (GenBank accession no. AAK19753), while RAPD431 was similar ($E = 10^{-9}$) to *Bremia lactucae* heat-shock protein 70 (GenBank accession no. P16394). Using the cloned sequences as probes in Southern analysis revealed all of the sequences tested represent single or low copy sequences in the *P. sojae* genome (data not shown).

Markers cosegregating with *Avr1a* occur on an 170-kb BAC contig: To construct a physical map of the *Avr1a* region, markers closely linked to the gene were used as probes to screen a BAC library of *P. sojae* race 2 genomic DNA. A single BAC clone, 10-J14-1, was isolated using the AFLP marker HAMCGA as a probe. Fingerprinting of random BAC clones suggested that 10-J14-1, overlapped with clones 3-M5-1, 17-C2-1, and 20-G5-1 (B. Tyler, unpublished data). This contig was confirmed by restriction mapping of the clones with the enzymes *Ase*I, *Dra*I, and *Not*I. Chromosome walking, using a probe derived from the T7 end of clone 10-J14-1, identified four new BAC clones, 31-A20-18, 23-H5-10, 36-M2-1, and 10-B21-7 (Figure 3). Overlapping regions were determined by restriction mapping and hybridization experiments, resulting in a physical contig of 170 kb. Besides the original marker used to screen the BAC library (CAP-CGA-3/4), none of the other markers shown in Figure 1 hybridized to any of the BAC clones in the extended contig. Thus, two new markers (10F1/3R2 and 10B21T7-2/5A) were developed along the contig for fine mapping of *Avr1a*.

The marker 10B21T7-2/5A was developed from a 735-bp region corresponding to the T7 end of BAC clone 10-B21-7. This site contained an 11-bp insert with a unique *Mse*I restriction site that was absent in the corresponding region of race 7. Similarly, the marker 10F1/3R2 was derived from 1872 bp of sequence correspond-

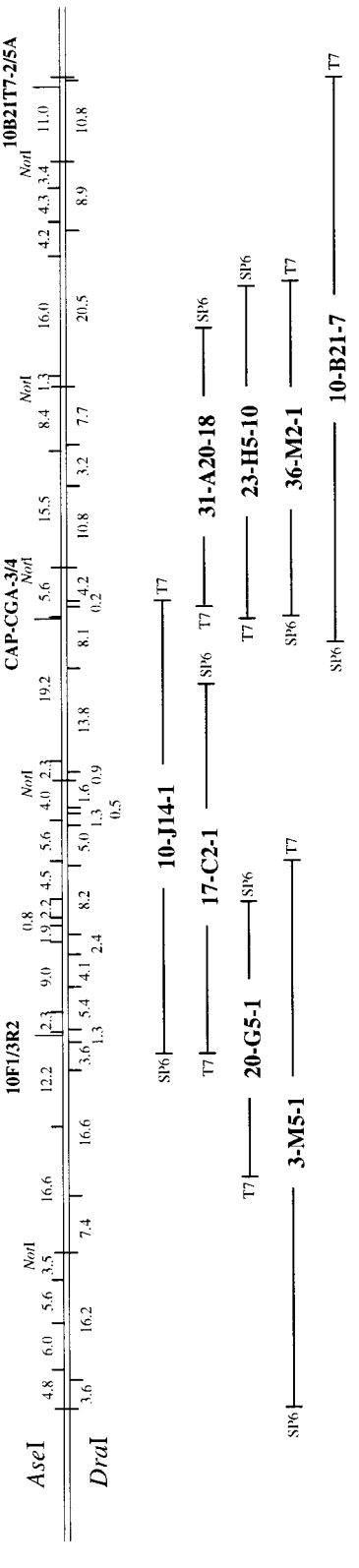


Figure 3.—A 170-kb contig of BAC clones cosegregating with the *Avr1a* avirulence gene in *P. sojae* race 2. The size of fragments generated with the restriction enzymes is indicated in kilobases. The orientation of each BAC clone within the cloning vector is indicated by identifying SP6 and T7 ends. The physical positions of three DNA markers (10F1/3R2, CAP-CGA-3/4, and 10B21T7-2/5A) used for genetic mapping are also shown.

ing to the SP6 end of BAC clone 10-J14-1. Four single nucleotide polymorphisms (SNPs) were found in this region that distinguished race 2 from race 7. A single SNP, within a unique *Nco*I site, was selected and mapped as a codominant marker, 10F1/3R2. Finally, to convert the AFLP marker HAMCGA to CAP marker CAP-CGA-3/4, a region of the SP6 end of 10-B21-7 that included the site of the AFLP marker was analyzed for polymorphisms. In 610 bp of sequence, a 9-bp deletion in race 2 relative to race 7, as well as two SNPs, was discovered. One of the SNPs occurred within an *Alw*NI site, affording the CAP marker CAP-CGA-3/4. Sequence comparisons between race 2 and race 7, corresponding to the SP6 end of BAC 3-M5-1, did not yield polymorphic bases and this site was not mapped.

The *Avr1a* locus is delimited by two flanking markers: To orient the physical map and estimate physical to genetic distance in the region, the three markers occurring on the BAC contig were mapped in all 486 F_2 progeny derived from the R2/R7 crosses. Three recombinants were identified from this analysis, one from each of the three sets of F_2 progeny: two individuals recombinant between CAP-CGA-3/4 and 10B21T7-2/5A and a single recombinant between CAP-CGA-3/4 and 10F1/3R2. Two of the three recombinant progeny were avirulent on *Rps1a* and, hence, F_3 progeny were generated from these F_2 individuals to determine whether they were homozygous or heterozygous for *Avr1a*. This analysis showed that the *Avr1a* genotype cosegregated with that for CAP-CGA-3/4, but not with 10B21T7-2/5A or 10F1/3R2. These results indicate that *Avr1a* occurs within the 114-kb region flanked by 10F1/3R2 and 10B21T7-2/5A. The recombination frequencies of the DNA markers also provide a ratio of physical distance to genetic distance of 437 kb/cM between 10F1/3R2 and CAP-CGA-3/4 and a ratio of 345 kb/cM between CAP-CGA-3/4 and 10B21T7-2/5A, yielding an average of 391 kb/cM for this region.

DISCUSSION

In this study we followed the segregation of avirulence against the *Rps1a* resistance gene in F_2 populations of *P. sojae* and identified molecular markers linked to this trait. We carried out RAPD analysis, using 600 arbitrary decanucleotide primers, and AFLP analysis, using 336 primer combinations, to identify molecular markers linked to the *Avr1a* avirulence gene. The AFLP procedure generated 60-fold more loci per primer pair and is therefore superior to the RAPD assay as a method for marker identification. In total, more than 42,000 loci were screened.

Segregation patterns for most of the dominant RAPD and AFLP markers fit expected Mendelian ratios except for the AFLP marker ETMCAA. This marker fit a 3:1 segregation in the R2/R7-BT population whereas in the R1/R3 cross the segregation was near 1:1. Similar results have been previously reported in other crosses of *P. sojae* (Tyler *et al.* 1995; Whisson *et al.* 1995). For example, Whisson *et al.* (1995) described RAPD markers that segregated normally in a race 7/race 25 cross but showed distorted segregation ratios in a race 1/race 7 cross.

By following the segregation of codominant markers and by converting dominant markers into RFLP probes that reveal heterozygotes, we showed that genetic segregation patterns may be far from normal, despite that most dominant markers segregated normally. Codominant markers displayed skewed segregation ratios that favored homozygous genotypes in the R1/R3 and R2/R7-BT progeny, but not in the R2/R7-TM or R2/R7-TMA progeny. It seems that time spent in culture may influence segregation ratios. The R1/R3 and R2/R7-BT progeny were both initially constructed several years before this study and their F_2s were propagated over extended periods prior to scoring for virulence and DNA markers, whereas the R2/R7-TM and R2/R7-TMA populations were studied immediately upon isolation. Although it is not clear what is causing this overrepresentation of homozygous individuals in the R1/R3 and R2/R7-BT progeny, gene conversion or mitotic crossing over are possible explanations. Homozygosity may also result from inbreeding within the F_2 populations during culture and propagation since this organism is homothallic. Biased segregation ratios that favor particular allele combinations could also arise from selective pressures. Regardless of the cause(s), these distorted segregations do not present obstacles for mapping studies. In fact, screening for DNA markers linked to discrete traits is more efficient using pools of homozygous individuals.

A comparison of genetic linkage maps of the *Avr1a* locus in race 1 and race 2 demonstrated that marker order is generally conserved, although some rearrangements are evident. Southern blot analysis has shown that each of the RAPD and AFLP markers used as probes represent single or low copy sequences in each of the four *P. sojae* races used in this study. This result was somewhat surprising because repetitive sequences are prevalent in *P. sojae* and may comprise 50% or more of the total genome (Mao and Tyler 1991). That all of the markers are single or low copy sequences may indicate that the *Avr1a* region has a relatively low content of repetitive DNA sequences. The hybridization patterns obtained with the marker-probes also suggest that the area encompassing the markers is conserved, but not identical, in these four races (data not shown).

A genome linkage map of *P. sojae*, consisting of 257 markers (22 RFLP, 228 RAPD, and 7 avirulence genes) and composed of 10 major and 12 minor linkage groups has been proposed by Whisson *et al.* (1995). In their analysis, *Avr1a* was located toward the end of a large linkage group that also included *Avr1b* and *Avr1k*, and a total genome size of 1600 cM was suggested. A physical

genome size for *P. sojae* of 62 Mb has been estimated by quantitative fluorescence microscopy (Rutherford and Ward 1985) and by reassociation kinetics (Mao and Tyler 1991). More recently, Feulgen image analysis resulted in a size estimation of 91 Mb (Voglmayr and Greilhuber 1998). These values may be used to predict an average relationship of genetic to physical distance of 38–56 kb/cM for *P. sojae*. A similar study in *P. infestans* (Van Der Lee *et al.* 1997) was used to estimate a ratio of 200 kb/cM in that species and six *Avr* genes have been mapped in *P. infestans* using AFLP markers (Van Der Lee *et al.* 2001). From our *P. sojae* crosses, we calculate an average ratio of 391 kb/cM in the vicinity of *Avr1a*.

In summary, we have produced a high-resolution genetic map of the *Avr1a* locus in *P. sojae* and constructed a 170-kb physical contig of the region encompassing the gene. Cloning and characterization of *P. sojae* avirulence genes will aid in deciphering the molecular events involved in *P. sojae* pathogenesis and in the understanding of plant-pathogen interactions in general. Identifying molecular markers linked to avirulence genes also provides new tools for detection, diagnosis, and for studies of evolution and population genetics of this agronomically important pathogen.

We thank Aldona Gaidauskas-Scott for technical assistance; Dr. Ed Ward for comments and suggestions; and Pearl Campbell, Sandra Millar, Heather Schneider, and Ida van Grinsven for DNA sequencing. Research was supported in part by grants from The Noble Foundation (Ardmore, OK) and by the Ontario Soybean Growers (Chatham, ON).

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Communicating editor: P. J. Pukkila