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Phytophthora sojae Avirulence Genes, RAPD, and RFLP Markers Used to Construct a Detailed Genetic Linkage Map

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Two crosses between different races of *Phytophthora sojae* were established using one race as a common parent in both crosses. F_2 populations comprising over 200 individuals were generated for each cross. A subset of 53 F_2 individuals from each cross was selected at random for genetic analysis of virulence/avirulence and molecular markers, and finally the construction of a detailed genetic linkage map. The linkage map developed for *P. sojae* is based on a total of 257 markers (22 RFLP, 228 RAPD, and 7 avirulence genes). The linkage map comprises 10 major and 12 minor linkage groups covering a total of 830.5 cM. Close linkage was observed between *Avr*4 and *Avr*6 (0.0 cM), *Avr*1b and *Avr*1k (0.0 cM), and *Avr*3a and *Avr*5 (4.6 cM). Coupling phase linkage of RFLP and RAPD markers to all seven avirulence genes was identified at the minimum and maximum distances of 0.0 and 14.7 cM, respectively.

Additional keywords: fungi, gene-for-gene, linkage mapping, Oomycetes, *Phytophthora megasperma* f.sp. *glycinea*, virulence/avirulence.

Plant pathogens frequently exhibit the phenomenon of cultivar specificity in which certain strains of the pathogen are able to overcome the resistance genes in certain cultivars of the host plant. The interaction between the two components of cultivar specificity, the host and the pathogen, appear to be based on a gene-for-gene system as first described by Flor (1956). Flor proposed that for every dominant resistance gene present in the host plant, there was a corresponding dominant gene for avirulence in the pathogen. A gene-for-gene interaction has been hypothesized for many plant/pathogen interactions such as *Phytophthora infestans*/potato, *Melampsora lini*/flax, and *Xanthomonas campestris*/capsicum (Day 1974; Thompson and Burdon 1992). The Oomycete plant pathogen *Phytophthora sojae* (syn. *Phytophthora megasperma* f. sp. *glycinea*) is host specific and causes Phytophthora root and stem rot of soybean (*Glycine max*). *P. sojae* is homothallic (self fertile), producing sexually derived oospores in single culture (Hansen and Maxwell 1991). The vegetative growth stage is diploid as in other Oomycetes (Shaw 1983).

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At present, control of *P. sojae* relies largely on the introduction of genes conferring resistance to *P. sojae* (*Rps* genes) into commercial soybean cultivars using conventional plant breeding. The genetics of resistance to *P. sojae* has been extensively studied in soybean (Ward 1990). Thirteen dominant *Rps* genes have been identified at seven loci. Five alleles have been identified at the *Rps*1 locus, three at the *Rps*3 locus, whereas only one allele has been identified at each of the following loci: *Rps*2, 4, 5, 6, and 7.

The deployment of resistance genes has not been entirely successful due to the ability of *P. sojae* to develop new races that overcome the resistance genes (Schmitthenner 1985). At least 37 races of *P. sojae* are known to exist worldwide (Förster et al. 1994). In Australia, seven described races; 1, 2, 4, 10, 13, 15, 25, and three undescribed races are known to occur (Ryley et al. 1991; M. J. Ryley, personal communication).

In contrast to the genetics of resistance in soybean, the genetics of virulence/avirulence in the pathogen was considered intractable due to the homothallic nature of *P. sojae*. Recently, occasional in vitro outcrossing in *P. sojae* has been reported and was subsequently used to produce F_2 populations in which both avirulence genes and molecular markers were shown to segregate in a Mendelian fashion (Tyler et al. 1995; Whisson et al. 1994). Avirulence towards seven *Rps* genes (*Rps*1a, 1b, 1c, 1d, 1k, 3a, and 5) was shown to be dominant and semi-dominant towards *Rps*3b, 3c, 4, and 6 (Tyler et al. 1995; Whisson et al. 1994).

Linkage among a large number of molecular markers allows the construction of a genetic linkage map and the genetic localization of avirulence genes. The molecular markers of choice for construction of a genetic linkage map are restriction fragment length polymorphisms (RFLPs) which are typically codominant. Many genetic maps based on RFLPs have been constructed in animal and plant systems (Eppig 1993; Kesseli et al. 1994; NIH/CEPH Collaborative Mapping Group 1992; Tanksley et al. 1992). However, RFLPs are labor intensive and typically require the use of radioisotopes for detection.

The recent development of markers based on the polymerase chain reaction (PCR), such as random amplified polymorphic DNA (RAPD) (Williams et al. 1990), has allowed construction of detailed genetic linkage maps in relatively short time spans (Dietrich et al. 1994; Grattapaglia and

Sederoff 1994; Postlethwait et al. 1994). However, PCR-based markers such as RAPDs are typically dominant, yielding a low information content from a single RAPD marker compared to a single RFLP marker. RAPD markers may appear codominant when linked in repulsion phase (functional codominance; Williams et al. 1990). However, RAPD markers are only codominant when arising from small insertions or deletions between priming sites (true codominance; Grattapaglia and Sederoff 1994). Since the amount of linkage information arising from dominant markers linked in repulsion phase is negligible with small progeny sizes, a frequent approach to mapping using dominant markers is to construct a linkage map for markers derived from each parent in the cross and then superimpose these maps over a framework, or scaffold, of codominant markers (Echt et al. 1993; Kesseli et al. 1994; Grattapaglia and Sederoff 1994). Dominant markers close to genetic characters of interest may be converted to codominant markers through sequence characterization to yield SCAR (sequence characterized amplified region; Kesseli et al. 1994) markers or may be used directly as probes to yield RFLPs (Williams et al. 1990).

Despite the relative ease of use of some recently developed molecular marker technologies, few genetic linkage maps of plant pathogens involving avirulence genes have been constructed (Brown and Simpson 1994; Smith and Leong 1994). The aims of the current investigation were to: (i) study the inheritance of virulence/avirulence in *P. sojae*, (ii) construct a genetic linkage map of *P. sojae* based on two F_2 populations, (iii) place the avirulence genes on the map, and (iv) identify molecular markers closely linked to avirulence genes. Close linkage of molecular markers to avirulence genes may form the starting point for chromosome walking towards avirulence genes and their ultimate cloning (Hulbert et al. 1988; Young 1990). Cloned and characterized avirulence genes will enable a better understanding of the interactions between plants and pathogens at the molecular level.

RESULTS

Virulence testing of F_1 hybrids and F_2 populations.

Generation of F_1 and F_2 progeny from a cross between race 7 and race 1 (race 7/1 cross) has been described by Whisson et al. (1994). A second F_2 population was constructed from a cross between race 7 and race 25 (race 7/25 cross). Four F_1 hybrids were identified from the race 7/25 cross, all of which exhibited avirulence towards resistance genes *Rps*1b, 1k, 3a, 4, 5, and 6, and virulence towards resistance genes *Rps*1a, 1c, and 7. Fifty-three F_2 individuals from the race 7/1 cross and 53 F_2 individuals from the race 7/25 cross were selected at random for genetic analysis of virulence/avirulence. Segregation ratios of avirulence genes from the race 7/1 cross have been published previously (Whisson et al. 1994) and are included in Table 1. From the race 7/25 cross, avirulence towards resistance genes *Rps*1b, 1k, 3a, 4, 5, and 6 segregated as dominant alleles at single loci (Table 1). Virulence towards resistance gene *Rps*1c was observed to be dominant, but no segregation was observed in the F_2 population. All F_2 individuals from the race 7/25 cross exhibited virulence towards resistance genes *Rps*1a and 7. None of the 53 randomly selected F_2 individuals from either cross were noticeably less, or more, aggressive than the three parental strains used to construct the crosses.

RAPD markers.

Among the 400 decanucleotide primers tested for the ability to generate polymorphisms between the parental isolates, 27 primers generated no products or very weakly amplified products and were not used in any subsequent analysis. The remaining primers generated moderate to intense DNA fragment patterns on ethidium bromide stained agarose gels. Of these primers, 224 generated no polymorphic DNA fragments between the parental isolates, 87 generated a single polymorphic fragment, and 62 generated multiple polymorphic fragments; a final average of 0.8 RAPDs per primer screened was observed.

Size variant RAPD markers.

Size variant RAPD markers mapping to a single locus, that is, fragments generated from both parental isolates but of slightly differing size, were identified in *P. sojae*. To be scored as size variant (i.e., codominant) alleles, the markers were required to fit two criteria. Firstly, no recombinant genotypes in repulsion phase (absence of both alleles) were identified in the F_2 population and, secondly, the marker must

Table 1. Data obtained for segregating avirulence genes from two different F_2 populations of *Phytophthora sojae*

Resistance gene	US7 (R7)	UQ244 (R1)	F_1	F_2 ratio (A:V)	Expected ratio	χ^2	Probability
*Rps*1a	V	A	A	38:15	3:1	0.31	$0.50 < P < 0.70$
*Rps*3a	V	A	A	30:23[a]	3:1	9.57	$0.001 < P < 0.005$
*Rps*5	V	A	A	30:23[a]	3:1	9.57	$0.001 < P < 0.005$
*Rps*6	V	A	A	44:8[b]	3:1	2.56	$0.10 < P < 0.20$
*Rps*7	V	V	V	0:53	0:1	0.00	1.00
	US7 (R7)	**UQ1200 (R25)**					
*Rps*1a	V	V	V	0:53	0:1	0.00	1.00
*Rps*1b	A	V	A	39:14	3:1	0.06	$0.80 < P < 0.90$
*Rps*1c	A	V	V	0:53[a]	1:3	17.67	$P < 0.001$
*Rps*1k	A	V	A	39:14	3:1	0.06	$0.80 < P < 0.90$
*Rps*3a	V	A	A	46:7[a]	3:1	3.93	$0.025 < P < 0.05$
*Rps*4	V	A	A	45:8	3:1	2.77	$0.05 < P < 0.10$
*Rps*5	V	A	A	46:7[a]	3:1	3.93	$0.025 < P < 0.05$
*Rps*6	V	A	A	45:8	3:1	2.77	$0.05 < P < 0.10$
*Rps*7	V	V	V	0:53	0:1	0.00	1.00

[a] Deviation from expected Mendelian segregation ratio ($P<0.05$).
[b] F_2 population size was 52 individuals for this gene.

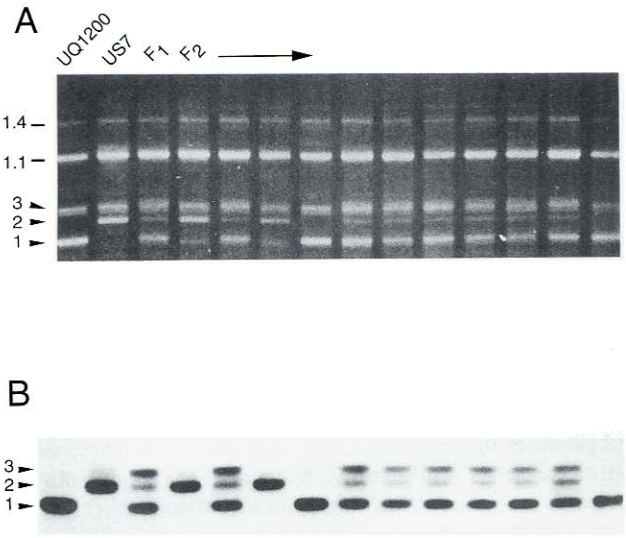

Fig. 1. A, Inheritance and segregation of size variant RAPD marker 161C from parental isolates UQ1200 and US7 (lanes 1 and 2) among the F_1 and a subset of F_2 individuals of *Phytophthora sojae* (lanes 3, and 4 to 14 respectively). The large polymorphic DNA fragment (fragment 2) from US7 was isolated from a duplicate gel and used as a probe to a Southern blot made from this gel. **B,** Both polymorphic fragments were inherited in the F_1 hybrid and segregate as a codominant marker (fragments 1 and 2) in the F_2 population. A DNA fragment of greater molecular size (fragment 3), comigrating with another fragment of similar size in (A), was observed after Southern analysis in all individuals possessing both alleles of the marker. DNA fragment size calibrations are indicated as base pairs $\times 10^3$.

be amplified by a single primer. In this study 33 size variant RAPD markers were detected. Verification to determine the sequence homology of both alleles of size variant RAPD markers was performed by Southern analysis of RAPD gel blots using one eluted fragment (putative allele) as a probe (Fig. 1). The four most extreme cases (with the greatest size difference between putative alleles) were tested in this manner, and in each case, the two putative allelic fragments gave strong hybridization signals, consistent with codominant alleles at a locus. DNA fragments of greater molecular size, not identified in either of the two parents or any F_2 individuals possessing only a single allele of the marker, were observed in heterozygous F_1 and F_2 individuals (Fig. 1) for 17 size variant RAPD markers. The molecular basis of these DNA fragments was not investigated.

Linkage analysis and map construction.

Linkage analysis was initially performed on data arising from the race 7/1 cross and was based on 267 genetic markers. This included 11 codominant RFLP, 11 dominant RFLP, 241 RAPD (208 dominant and 33 size variant), and 4 avirulence markers. At the threshold LOD score of 3.0, and recombination fraction less than 0.3, linkage was observed among 254 markers while 13 RAPD markers remained unlinked. From this initial map, a total of 89 RAPD, 2 RFLP scaffold markers, and 3 avirulence genes from the major linkage groups of the race 7/1 cross were analyzed in the race 7/25 cross to allow the introgression of *Avr*1b, 1k, and 4.

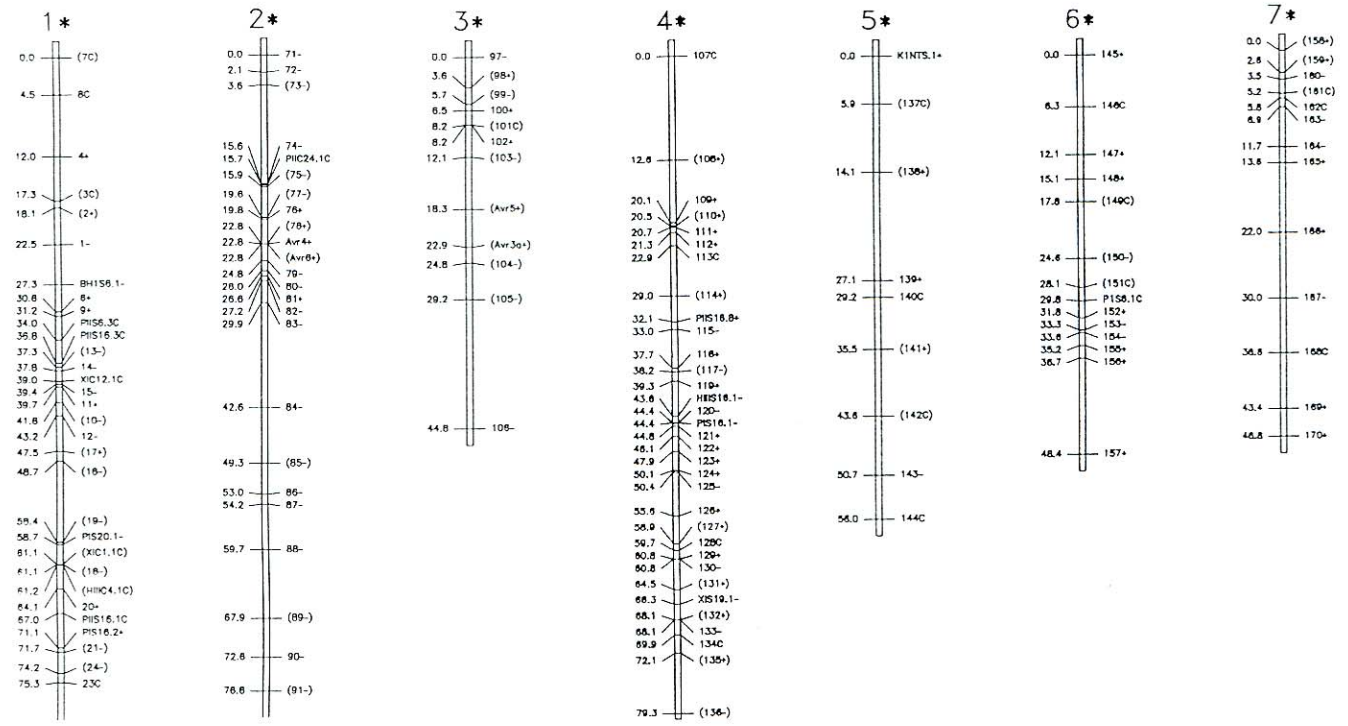

Fig. 2. The genetic linkage map of *Phytophthora sojae*. RFLP markers are represented by a lettered code and RAPD markers are represented by numbers. Distances between markers (cM) are indicated at the left and markers are indicated to the right. Phase of linkage is indicated by "+" or "–" notation at the right of marker names; codominant markers are indicated as "C" to the right of the marker names. Markers in parenthesis were used as scaffold markers in both crosses for introgressing new avirulence genes. All seven avirulence genes are located in linkage groups 1, 2, and 3. *Avr*1b and 1k, and *Avr*1a are located in group 1 at 92.7 and 145.3 cM, respectively. *Avr*4 and 6 are both located in group 2 at 22.8 cM. *Avr*3a and 5 are located in group 3 at 18.3 cM and 22.9 cM, respectively. Due to the degree of marker saturation in linkage group 1 and F_2 population size, six markers were unable to be placed accurately in linkage group 1 and have been shown below this linkage group. *(continued on next page)*

Thus, a total of 257 markers from both crosses showed linkage and were used to construct the final linkage map. Linkage distances between scaffold markers were similar in both crosses. Of the 208 dominant (RFLP and RAPD) markers used to construct the linkage map, the race 1 parent carried the dominant allele of 108 markers and the race 7 parent carried the dominant allele of 100 markers. Ten major linkage groups (groups 1 to 10; Fig. 2) with more than five markers and longer than 20 cM were identified together with 12 minor linkage groups (groups 11 to 17, and a to e; Fig. 2). The total genome coverage represented by all linkage groups is 830.5 cM. Based on the method of Postlethwait et al. (1994; assuming 5.8 cM for each telomere, 13 chromosomes, and 22 gaps in the map at 30 cM per gap), we estimate the total genome size to be approximately 1,600 cM. The average spacing of markers for major linkage groups varies from 2.0 (group 1) to 7.0 cM (group 5) and the largest marker interval is 15.6 cM (group 3) (Table 2).

Thirteen RAPD markers remain unlinked at LOD 3.0 for both crosses and represent a proportion of 4.8 % of all markers. These markers show linkage to the major linkage groups at a lower LOD score (1.5) but did not contribute any additional significant information with respect to genome coverage and so were not included in the final map version.

Distorted segregation ratios ($P < 0.05$) were noted for 46 markers in the race 7/1 cross. Fifteen of these markers are located in one area of linkage group 1 bounded by RFLP marker PIIS16.3C (36.8 cM) and RAPD marker 23C (75.3 cM). Another nine markers which exhibit aberrant segregation are located in an area of linkage group 2 bounded by RFLP marker PIIC24.1C (15.7 cM) and RAPD marker 87- (54.2 cM; Fig. 2). A selection of markers from both of these linkage regions did not show significant deviation from Mendelian 3:1 segregation ratios in the race 7/25 cross. The remaining 22 markers exhibiting aberrant segregation in the race 7/1 cross were scattered through the other linkage groups; 14 of these markers were located in the major linkage groups. Five of these markers located in the major linkage groups were tested in the race 7/25 cross and all five did not show any significant deviation from a 3:1 segregation ratio.

*Avr*4 cosegregated with *Avr*6 in all cases in the F_2 population of the race 7/25 cross and was located in linkage group 2 (22.8 cM; Fig. 2) and cosegregated in all cases with RAPD marker 78+ in coupling phase. *Avr*1b cosegregated with *Avr*1k in all cases in the F_2 population of the race 7/25 cross, and both are located in linkage group 1 (92.7 cM; Fig. 2) with the nearest marker (RAPD 27-) at a distance of 7.3 cM in coupling phase from both genes. *Avr*3a cosegregated with

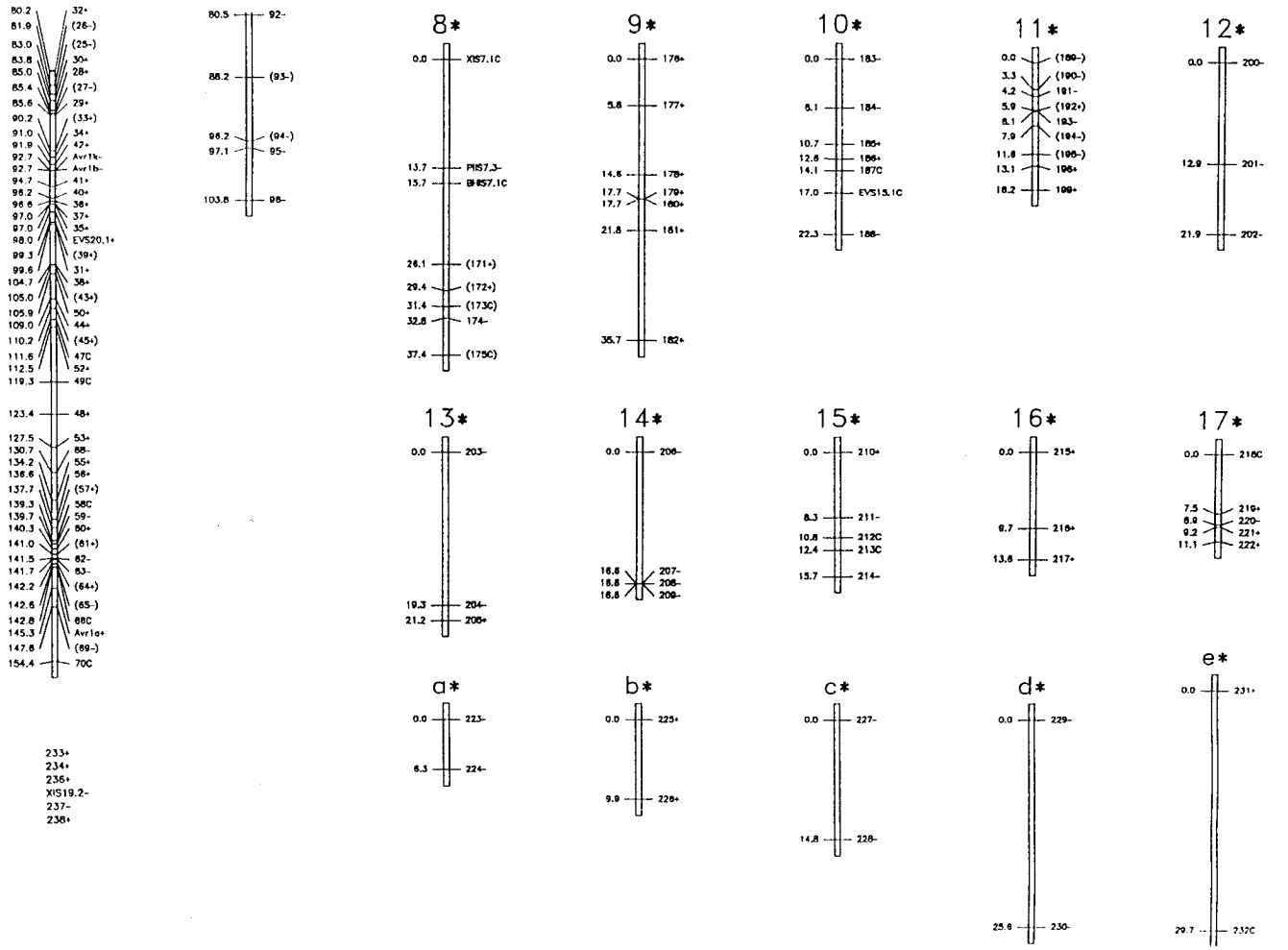

Fig. 2. *(continued from preceding page)*

*Avr*5 in all cases in the race 7/25 cross and, with consideration of data from the race 7/1 cross for these genes (cosegregation in 49 out of 53 cases), were located 4.6 cM from each other in linkage group 3. The nearest marker (RAPD 101C) in coupling phase to *Avr*3a and *Avr*5 is 10.1 cM away.

DISCUSSION

In this report we have presented the first detailed genetic linkage map for the plant pathogen *Phytophthora sojae,* based on two different genetic crosses using 22 RFLP, 228 RAPD, and 7 avirulence genes. This initial genetic linkage map of *P. sojae* comprises 10 major linkage groups and 12 minor linkage groups covering a total of 830.5 cM. The haploid chromosome count in *P. sojae* is in the order of 10 to 13 chromosomes (Sansome and Brasier 1974) covering approximately 62 Mb (Mao and Tyler 1991). However, the chromosome count is uncertain due to the small size of *P. sojae* chromosomes which are difficult to resolve using light microscopy. Pulsed field gel electrophoresis has been used to separate a maximum of eight chromosome-sized DNA bands for *P. sojae* (Judelson et al. 1993), two of which stained intensely with ethidium bromide and may represent doublets or triplets of comigrating chromosomal sized DNA. Presumably, most of the major linkage groups presented herein represent chromosomes and most of the minor linkage groups and unlinked markers will be incorporated into major groups in the map as more markers are added as was observed for the linkage map of lettuce (Kesseli et al. 1994). It is also possible that some of the minor linkage groups may represent chromosomes. This may only be resolved by identifying single copy RFLPs in each linkage group followed by physical mapping of linkage groups to chromosomes, genomic DNA cleaved by rare cutting restriction endonucleases, or contiguous large cloned DNA fragments. Therefore, the linkage map presented herein represents a "working map" for introgressing new avirulence characters by constructing crosses with one or more common reference isolates such as those used in this study.

Table 2. Length of individual linkage groups, number of markers, average spacing between markers and largest genetic intervals for linkage groups of *Phytophthora sojae*

Linkage group	Length (cM)	Number of markers	Average spacing (cM)	Largest distance (cM)
1	154.4	77	2.0	9.7
2	103.8	29	3.6	12.7
3	44.8	12	4.1	15.6
4	79.3	33	2.5	12.6
5	56.0	9	7.0	13.1
6	48.4	14	3.7	11.6
7	46.8	13	3.9	8.4
8	37.4	8	5.3	13.7
9	35.7	7	6.0	13.9
10	22.3	7	3.2	6.1
11	16.2	9	2.0	3.7
12	21.9	3	11.0	12.9
13	21.2	3	10.6	19.3
14	16.6	4	5.5	16.6
15	15.7	5	3.9	8.3
16	13.6	3	4.5	9.7
17	11.1	5	2.8	7.5
a	6.3	2	6.3	6.3
b	9.9	2	9.9	9.9
c	14.8	2	14.8	14.8
d	25.6	2	25.6	25.6
e	29.7	2	29.7	29.7

Since *P. sojae* is homothallic and produces sexually derived oospores in infected soybean tissue, it may be assumed that individuals in a population would be near completely homozygous. RFLP analysis of the U.S. and Australian populations of *P. sojae* (Förster et al. 1994; A. Drenth, unpublished data) detected no evidence for heterozygosity. Segregation ratios for markers in the crosses described herein also show no evidence for heterozygosity. Additionally, isolate US7 was allowed to self fertilize and the selfed progeny were screened with RFLP probes (S.C. Whisson, unpublished data). Again, no evidence for heterozygosity was observed. Tyler et al. (1995) also observed no evidence for heterozygosity in their crosses.

RFLPs are the marker type of choice for constructing genetic linkage maps. The paucity of codominant RFLPs in the *P. sojae* genetic map is partially overcome by the presence of codominant RAPD markers, which were observed at a proportion of 13.8% of all RAPD markers. The frequency of detection of codominant RAPD markers in this study is greater than in some plant systems (Grattapaglia and Sederoff 1994; Kesseli et al. 1994), but similar to that detected by Postlethwait et al. (1994) in the zebrafish. Size variant RAPD markers as described by Grattapaglia and Sederoff (1994) are true codominant markers, as was confirmed by Southern blot analysis (Fig. 1), and differ from "functionally codominant" pairs of markers (linked in repulsion) as described by Williams et al. (1990).

The three new avirulence characters (*Avr*1b, 1k, and 4), segregating in the race 7/25 cross, were all dominant genes at single loci. However, data for *Avr*3a, 5, and 6 from this cross were interpreted differently than for the race 7/1 cross. In an earlier report (Whisson et al. 1994), segregation of *Avr*3a and 5 (Table 1) was interpreted as suggesting the presence of two independent complementary dominant genes to effect avirulence in each case. The simplest explanation of such a system would be if the pathogen component determining avirulence was the end product of a multi-step biosynthetic pathway (Ilott et al. 1989). However, segregation ratios for both *Avr*3a and 5 from the race 7/25 cross were 46:7, more consistent with a single dominant allele for each gene. For linkage analysis, the data for *Avr*3a and 5 from both crosses were treated as dominant genes at single loci, and it was assumed that the observed deviation from the expected segregation ratio for *Avr*3a and 5 in the race 7/1 cross had arisen by chance. Whisson et al. (1994) also reported linkage of a RAPD marker (104-, group 3) in repulsion phase to both *Avr*3a and 5. No significant linkage in repulsion phase was observed between RAPD marker 104- and *Avr*3a and 5 in the race 7/25 cross but by using a codominant (size variant) RAPD marker (101C), located approximately 10 cM from *Avr*3a and 5 as an anchor locus, RAPD 104- was placed in repulsion at a similar distance (1.9 and 6.5 cM, respectively) from *Avr*3a and 5 confirming our earlier results (Whisson et al. 1994).

*Avr*6 has been reported as a codominant gene at a single locus by Whisson et al. (1994). In the race 7/25 cross described in this paper, *Avr*6 segregated as a dominant gene at a single

locus. A similar result was found by Gijzen and coworkers (personal communication) when studying segregation of *Avr*4 and 6 using zoospore inoculation of etiolated hypocotyls. Intermediate phenotypes were noted in the F_2 of the race 7/25 cross when tested on *Rps*6, but when re-tested, were often avirulent. The phenotypes of some of these individuals when re-tested, ranged from fully avirulent through intermediate to fully virulent and required several re-tests before consistency was achieved. Data for the intermediate and virulent progeny generated from the race 7/1 cross were re-tested as for the 7/25 cross and classified as virulent or avirulent (Table 1).

Virulence towards *Rps*1c was observed to be a dominant trait but did not segregate in our race 7/25 cross. This may be due to linkage to a lethal function or chromosome mismatching events during crossing over as a result of a deletion, insertion or translocation. It may be possible to map this trait in a further cross involving race 25 and another race avirulent on *Rps*1c.

Linkage mapping in *P. sojae* from our crosses has been scaled back since a high proportion of new markers do not yield any further map information and typically map to linkage groups which are already close to saturation. Linkage group 1 contains the highest proportion of markers and at the highest degree of saturation possible for our limited F_2 population sizes. Most of the variation existing between parental strains appears to originate from one linkage group (group 1) which has several clusters of closely linked markers. To make the map of group 1 comprehensible in Figure 2, several markers were excluded from regions of high marker density as they did not contribute any further information to the map. The highest density region surrounds RAPD marker 35+ (97 cM on group 1) where eight markers were observed to cosegregate. Typically, these densely marked regions of group 1 contain more markers than can accurately be ordered with our F_2 population sizes. This may be overcome by expanding the progeny size to increase the accuracy of marker placement in centiMorgan distances and hence map order.

The generation of a genetic linkage map for *P. sojae* which incorporates seven segregating avirulence genes and 250 molecular markers will permit the rapid mapping of further avirulence genes from additional crosses. Avirulence genes *Avr*1c, 1d, 2, 3c, and 7 still remain to be mapped from the current crosses (*Avr*2 and 3c) or additional crosses in which *Avr*1c, 1d, and 7 segregate. Problems with availability of differential seed in Australia have prevented us from mapping *Avr*3c. *Rps*2 is typically expressed as field resistance and requires a quantitative assay such as described by Irwin and Langdon (1982).

Our genetic linkage map incorporates seven segregating avirulence characters, all of which are linked in coupling phase to DNA markers at a minimum distance of 0.0 cM and a maximum map distance of 14.7 cM. Any of these marker/avirulence gene linkages may serve as a starting point for bulked segregant analysis as described by Michelmore et al. (1991) and finally chromosome walking strategies towards cloning avirulence genes. The small genome size of *P. sojae* (62 Mb) (Mao and Tyler 1991) also makes map-based cloning in this organism a feasible prospect. Cloned and characterized avirulence genes will greatly assist in the understanding of plant/pathogen recognition, the evolution of virulence, and may be useful as novel resistance genes (De Wit 1992) against *P. sojae* in soybean.

MATERIALS AND METHODS

P. sojae isolates.

The three parental isolates of *P. sojae* used in this investigation were UQ244, UQ1200, and US7 from the culture collection of the Cooperative Research Centre for Tropical Plant Pathology, University of Queensland, Australia. UQ244 is an Australian isolate belonging to race 1 of *P. sojae* and only able to overcome resistance gene *Rps*7. UQ1200 represents the first record of race 25 of *P. sojae* in Australia (M. J. Ryley, personal communication) and is able to overcome resistance genes *Rps*1a, 1b, 1c, 1k, and 7. Isolate US7, belonging to race 7, was isolated from soybean in the United States and is able to overcome resistance genes *Rps*1a, 2, 3a, 3c, 4, 5, 6, and 7. UQ1200 showed identical background genotype to UQ244 when compared using 40 individual RAPD primers (S.C. Whisson, unpublished data). Isolate US7 is readily distinguished from UQ244 and UQ1200 by RAPD and RFLP markers (Whisson et al. 1992; S.C. Whisson, unpublished data).

Hybrid isolation and generation of an F_2 population.

A description of the preparation of an F_2 population from the race 7 × race 1 cross (race 7/1 cross) has been published elsewhere (Whisson et al. 1994). The second cross used in this investigation involved race 7 and race 25 (race 7/25 cross). Among 200 single oospore derived progeny from the pairing, screened as described for the race 7/1 cross (Whisson et al. 1994), four hybrids were identified and verified. One F_1 hybrid (344Z1), derived from a single zoospore, was allowed to self fertilize and produce an F_2 population of 234 individuals.

DNA extraction and RAPD analysis of F_2 individuals.

DNA was extracted from mycelia of the parents, F_1, and 53 randomly selected F_2 individuals from each cross using the method of Panabiéres et al. (1989) with the modifications described by Whisson et al. (1992). RAPD analysis was conducted using decanucleotide primers (Operon Technologies, Alameda, CA; primer kits A, B, C, D, E, F, G, H, I, M, P, Q, R, T, W, Z, AC, AP, AR, and AS). Each RAPD reaction of total volume 25 µl comprised 250 µM each dNTPs, 25 ng primer, 4 mM $MgCl_2$, 30 ng *P. sojae* DNA, 1.6 units of *Tth* Plus DNA polymerase (Biotech International, Australia), 2.5 µL of 10× reaction buffer supplied by the enzyme manufacturer, and ultrapure water. Thermocycling was carried out for 40 cycles of 1 min at 94°C, 1 min at 37°C, and 2 min at 72°C. The final cycle was followed by an extension step at 72°C for 7 min. Amplified DNA fragments were size fractionated on 1.5 % agarose gels in 1× TBE buffer (Sambrook et al. 1989) at 6 V/cm, stained with ethidium bromide, and visualized using UV light.

RFLP probes and DNA hybridization.

DNA samples (5 µg) from the parental strains were cleaved with restriction endonucleases (New England Biolabs, Beverly, MA) *Xho*I, *Pvu*II, *Hin*dIII, *Eco*RI, *Kpn*I, *Eco*RV, *Pst*I, *Bam*HI, *Bst*BI, *Sma*I, *Dra*I, and *Bsp*DI; each restriction endonuclease had a six-base recognition sequence. Restricted DNA fragments were separated on 0.8% agarose gels before transfer to Hybond N^+ membrane (Amersham, Australia) as

described by Whisson et al. (1992). Hybridizations were carried out at 65°C in 15 ml of hybridization solution (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM disodium ethylenediaminetetraacetic acid, 7 % sodium dodecyl sulfate, pH 7.2) for 12 to 16 h. Stringency washes and stripping of blots for reuse were performed as described by Whisson et al. (1992).

Fourteen low copy cDNA and 14 moderately repetitive genomic DNA clones were screened for the ability to detect RFLPs between the parental isolates. From the screened probes, four low copy clones and seven moderately repetitive clones were used to generate 11 codominant markers and 11 dominant markers. For moderately repetitive probes, polymorphisms were regarded as dominant except where the alternative allele could be identified unambiguously.

Virulence/avirulence testing.

Soybean cultivars Harosoy 63 (*Rps*1a, *Rps*7), Sanga (*Rps*1b), Wells II (*Rps*1c), Harosoy OX682 (*Rps*1c, *Rps*7), Harosoy 1572 (*Rps*1k, *Rps*7), PI86972-1 (*Rps*3a), L85-2352 (*Rps*4), Harosoy 5272 (*Rps*5, *Rps*7), Altona (*Rps*6), and Harosoy (*Rps*7) were used as differentials to evaluate the virulence spectrum of parental, F_1, and F_2 individuals of *P. sojae*. *Rps*4 was not tested in the race 7/1 cross as differential seed was not available at that time. *Rps*3c was also not tested in either cross due to unavailability in Australia of seed of an appropriate differential cultivar. Virulence was tested using the hypocotyl inoculation technique described by Ryley et al. (1991), in which for each individual tested, at least 10 plants of each soybean cultivar were inoculated. Seedlings were assessed 4 days postinoculation, and were rated as susceptible (hypocotyl collapsed) or resistant (no lesion). Cultures were classified as avirulent if at least 9 out of 10 plants were resistant, and virulent if at least 9 out of 10 plants were killed. Cultures which killed between 10 and 90% of the plants tested were classed as giving a mixed response, and were re-tested until the reaction was able to be classified as consistently virulent or avirulent. Unequivocal results were obtained on the second re-testing for all *Rps* genes except *Rps*6 which, in some cases, required re-testing on several further occasions. All F_2 individuals exhibiting a virulent phenotype were verified by repeat testing; avirulent individuals were not re-tested since experience showed that these results were always unequivocal and appropriate susceptible controls (*Rps*7) were included.

Linkage analysis and map construction.

All avirulence, RFLP, and RAPD markers were assessed for significant 3:1 or 1:2:1 segregation using a χ^2 test (α = 0.05) and were subjected to two point linkage analysis using the computer program Mapmaker version 3.0 (Whitehead Institute, Cambridge, MA; Lander et al. 1987) to provide map distances in centiMorgan (cM) and LOD scores for linked markers. All markers were included, irrespective of significance of segregation. Linkage groups were identified using the Mapmaker program set at the default settings of LOD 3.0 and maximum distance of 30 cM (recombination fraction of 0.3). Ordering of markers was carried out using the Joinmap 1.1 mapping program (Kosambi mapping function) designed by Stam (1993) which is a statistically more accurate program for ordering dominant markers (Säll and Nilsson 1994). Marker orders were determined at a lower LOD score (Linklod = 2.5, Maplod = 0.05) than for linkage detection. New avirulence markers from the race 7/25 cross were quickly located by screening scaffold markers on each major linkage group from the race 7/1 cross spaced every 10 to 20 cM. In all, 254 molecular and avirulence markers from the race 7/1 cross and 94 scaffold markers and three new avirulence markers from the race 7/25 cross were integrated to form one genetic linkage map using Joinmap. No statistical tests for significant clustering of markers were performed. Output from Joinmap was converted to figures using the companion graphics program Drawmap 1.0 (van Ooijen 1993).

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