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Inheritance of Avirulence Factors and Restriction Fragment Length Polymorphism Markers in Outcrosses of the Oomycete *Phytophthora sojae*

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F_1 hybrids from crosses of three *P. sojae* isolates (P6497, P7064, and P7076) were identified by random amplified polymorphic DNA (RAPD) markers specific for each parent. The race types of the isolates were similar to races 2, 7, and 19, respectively. The hybrid nature of the progeny was confirmed by restriction fragment length polymorphisms (RFLPs). Selected F_1 progeny were selfed to produce F_2 progeny. RAPD and RFLP markers segregated in regular Mendelian fashion among one set of F_2 progeny (from P6497 × P7064), but considerable bias in the transmission of markers was observed among F_2 progeny from the second cross (P6497 × P7076). Specific avirulence in the pathogen against soybean resistance genes (*Rps* genes) was confirmed to be dominant in six cases tested (*Rps1a, Rps1b, Rps1c, Rps1d, Rps1k,* and *Rps3a*) and appeared to be dominant or semidominant in four others (*Rps3b, Rps3c, Rps4,* and *Rps6*). The segregation of avirulence among the F_2 progeny supported the presence of single dominant avirulence genes (*Avr1a, Avr1b,* and *Avr3a*) in the pathogen, matching three *Rps* genes (*Rps1a, Rps1b,* and *Rps3a*), and was consistent with the possible presence of two others (matching *Rps1k* and *Rps1d*). Genetic linkage was observed between some pairs of RFLP markers and between an RFLP marker and *Avr1b*.

Additional keywords: genetic mapping; *Phytophthora megasperma* f. sp. *glycinea*.

Phytophthora sojae Kaufmann & Gerdemann (syn. *P. megasperma* Drechs. f. sp. *glycinea* T. Kuan & D. C. Erwin) is a necrotroph which causes damping-off of soybean seedlings and a root and stem rot of mature plants (Schmitthenner 1989). *P. sojae* is an oomycete, belonging to a group of organisms which have classically been included among the fungi, but which in fact are closely related to chromophyte algae, such as diatoms (Förster *et al.* 1990). Like most oomycetes, *P. sojae* is diploid (Long and Keen 1977). The genetics of resistance in soybean against *P. sojae* have been well characterized (Anderson and Buzzell 1992; Buzzell and Anderson 1992; Buzzell *et al.* 1987). Thirteen single dominant resistance genes (*Rps* genes) have been identified at seven loci (Anderson and Buzzell 1992; Buzzell and Anderson 1992; Buzzell *et al.* 1987). Five genes (or alleles) are clustered at one locus (*Rps1a, Rps1b, Rps1c, Rps1d,* and *Rps1k*), and three are clustered at a second locus (*Rps3a, Rps3b,* and *Rps3c*). *Rps7* is closely linked to but separable from the *Rps1* locus (Anderson and Buzzell 1992), while the remaining genes—*Rps4, Rps5,* and *Rps6*—appear to be unlinked. *Rps2* confers a different level of resistance, expressed most clearly in the field (Kilen *et al.* 1974). Several additional resistance genes are less well characterized (Rennie *et al.* 1992). Most of the *Rps* genes have been introduced into one or both of two isolines based on the cultivars Harosoy and Williams (Buzzell *et al.* 1987).

At least 37 *P. sojae* races with different reactions against these resistance genes have been described (Förster *et al.* 1994; Layton *et al.* 1986; Schmitthenner *et al.* 1994; Wagner and Wilkinson 1992; S. Abney, personal communication). The first race described, race 1, is avirulent against all the *Rps* genes except *Rps7*. Races isolated later are virulent against different sets of *Rps* genes. Till recently, little has been known about the genetic basis of race-specific virulence against different *Rps* genes. In a variety of plant-pathogen interactions the outcome of an infection is determined by a gene-for-gene interaction between matched pairs of single dominant genes in the pathogen (avirulence genes) and in the plant host (resistance genes) (De Wit 1992; Keen 1990). When matching resistance and avirulence genes are present, the plant is resistant, and the interaction is incompatible. Otherwise, the infection is successful, and the interaction is compatible. Although the genetics of resistance to *P. sojae* are consistent with a gene-for-gene interaction, there has been little genetic analysis of the pathogen to test for the presence of avirulence genes which match *Rps* genes. Genetic analysis of *P. sojae* has been hampered by the fact that the organism is homothallic and produces oospores prolifically by selfing. Layton and Kuhn (1988) used drug resistance markers to produce heterokaryons between race 1 and race 3 strains by protoplast fusion. The heterokaryons were avirulent against *Rps1a*, like the race 1 parent, suggesting that avirulence against *Rps1a* is dominant (Layton and Kuhn 1988). Long and Keen (1977) and Bhat and Schmitthenner (1993) used auxotrophic markers and drug resistance markers, respectively, to identify F_1 hybrid progeny from crosses of different races. In the latter study, segregation of avirulence against *Rps1a* was observed among the F_2 progeny (Bhat *et al.* 1993), consistent with the

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presence of a single dominant avirulence gene. Whisson *et al.* (1994) recently reported evidence of single dominant avirulence genes against *Rps1a, Rps3a, Rps5,* and *Rps6,* using a cross in which an F_1 hybrid was identified by random amplified polymorphic DNA (RAPD) markers (Williams *et al.* 1990) specific for each parent.

We have identified F_1 hybrids from crosses of isolates with race types similar to race 2, race 7, and race 19, also using RAPDs. We have also obtained F_2 progeny by selfing selected F_1 progeny, to analyze the genetic segregation of molecular markers (restriction fragment length polymorphisms [RFLPs] and RAPDs) and to analyze the genetic basis of avirulence against six *Rps* genes. A preliminary report of some of these results was presented by Förster *et al.* (1994).

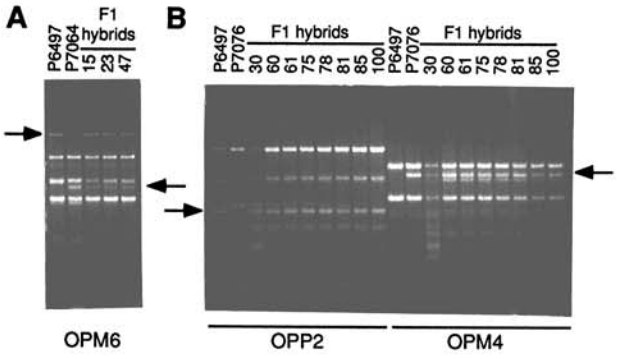

Fig. 1. Detection of F_1 hybrids of *Phytophthora sojae* isolates by random amplified polymorphic DNA (RAPD) markers. **A,** Three F_1 hybrids from P6497 × P7064. Primer OPM6 amplifies two RAPDs (arrows), one specific for P6497 and one specific for P7064 (primer OPP15 was used for screening the progeny, and OPM6 was used for confirmation). **B,** Eight F_1 hybrids from P6497 × P7076. Primer OPP2 amplifies a RAPD (the arrow on the left) specific for P6497, while primer OPM4 amplifies a RAPD (the arrow on the right) specific for P7076. In both crosses, the F_1 hybrids display both parent-specific RAPDs.

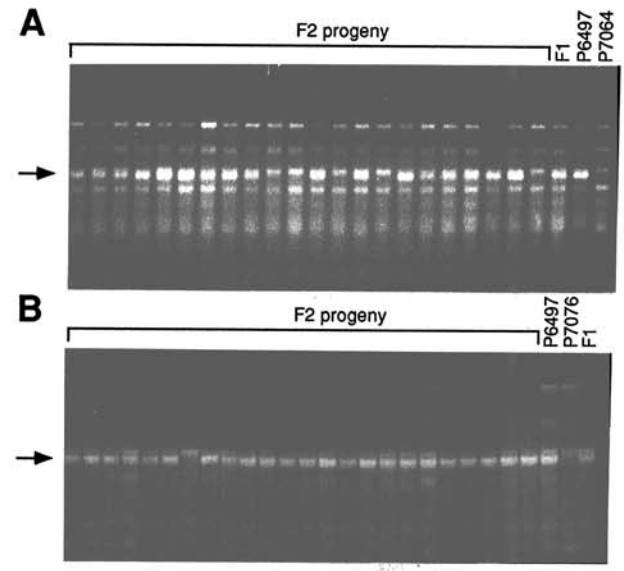

Fig. 2. Segregation of random amplified polymorphic DNA (RAPD) markers among F_2 progeny of hybrids of *Phytophthora sojae* isolates P6497 × P7064 (**A**) and P6497 × P7076 (**B**). Primer OPP2, which amplifies a RAPD (arrow, the lower band of the doublet) specific to P6497, was used in both cases. F_1 indicates the hybrids which were selfed in each case to produce the F_2 progeny, namely, P6497/P7064-47-1 (**A**) and P6497/P7076-62-2 (**B**).

RESULTS

Identification of F_1 hybrids.

In order to identify F_1 hybrid progeny, we used RAPD markers (Williams *et al.* 1990) specific to the parent strains to screen random F_1 progeny (Francis and St Clair 1993; Whisson *et al.* 1994). We screened 113 F_1 progeny from P6497 × P7064 and 88 from P6497 × P7076. The great majority of progeny from P6497 × P7064 resembled P6497 and presumably resulted from selfs of P6497. Only two resembled P7064. Four progeny exhibited markers from both parents and therefore were candidate F_1 hybrids (markers from three of these are shown in Fig. 1A). In the second cross, the majority of progeny resembled P7076. Eleven potential hybrids were obtained from this cross (markers from eight of these are shown in Fig. 1B). All the potential hybrids contained all of the RAPD markers specific to both parents. To confirm that the hybrids were not heterokaryons or mixed cultures, six single-zoospore derivatives were obtained from each potential hybrid, and the derivatives were rescreened with the RAPD markers. All potential hybrids transmitted both parental markers to their zoospore progeny. Since more than 99% of zoospores are mononucleate (Erwin *et al.* 1983), this observation indicated that none of the potential hybrids were heterokaryons or mixed cultures.

Specific virulence of F_1 hybrids.

At least two F_1 progeny from each cross were tested against cultivars containing each of the *Rps* genes (Table 1). All four F_1 progeny from P6497 × P7064 were tested against cultivars containing no *Rps* genes or containing *Rps1a, Rps1b,* or *Rps7,* while 10 F_1 progeny from P6497 × P7076 were tested

Table 1. Avirulence phenotypes of parental isolates of *Phytophthora sojae* and their F_1 progeny[a]

	Parental isolate and race type			F_1 progeny of:[c]	
Rps gene[b]	P6497 race 2*	P7064 race 7	P7076 race 19*	P6497 × P7064	P6497 × P7076
None	V	V	V	V (2, 4)	V (2, 10)
Rps1a	A	V	V	A (3, 4)	A (3, 10)
Rps1b	V	A	V	A (2, 4)	…[d]
Rps1c	A	A	V	A (1, 2)	A (2, 2)
Rps1d	A	A	V	…	A (2, 2)
Rps1k	A	A	V	A (1, 2)	A (2, 2)
Rps3a	A	V	A	A (2, 2)	…
Rps3b	A	A	V	…	A (3, 2)
Rps3c	A	V	A	A (1, 2)	…
Rps4	A	V	A	I (1, 2)	…
Rps6	A	V	A	I (1, 2)	…
Rps7	V	V	(V)[e]	V (1, 4)	V (1, 10)

[a] Phenotype of parents and progeny on resistant and susceptible soybean cultivars inoculated with the pathogen. A = avirulent; V = virulent; I = intermediate.

[b] *Rps* resistance genes present in soybean cultivars inoculated with the *P. sojae* parents and progeny.

[c] Phenotype followed by the number of different resistant cultivars tested and the number of F_1 individuals tested.

[d] Not tested.

[e] Virulent reaction not characteristic of race 19.

against cultivars containing no *Rps* genes or containing *Rps1a* or *Rps7*. In addition, two F_1 progeny (the same two) from each cross were tested against cultivars carrying other *Rps* genes: *Rps1c, Rps1k, Rps3a, Rps3c, Rps4,* or *Rps6* for F_1 progeny of P6497 × P7064, and *Rps1c, Rps1d, Rps1k,* or *Rps3b* for F_1 progeny of P6497 × P7076.

For each *Rps* gene except *Rps4* and *Rps6*, the F_1 progeny were all avirulent if one or both of the parents were avirulent (Table 1). In cultivars carrying *Rps4* and *Rps6*, the tested progeny produced intermediate responses reproducibly. When both parents were virulent against a particular *Rps* gene, as in the case of *Rps7*, the F_1 progeny were also virulent. All of the F_1 progeny tested were virulent against cultivars lacking *Rps* genes, indicating that none of the progeny had lost basic pathogenicity. In all cases tested, there were no consistent differences in the reactions of different F_1 progeny. These results are consistent with avirulence being a dominant trait against all *Rps* genes except *Rps4* and *Rps6*, with which it could be semidominant.

Segregation of RFLP and RAPD markers in the F_2 progeny.

In order to confirm that markers in these crosses showed regular Mendelian inheritance, we selfed single-zoospore derivatives of an F_1 individual from each cross and scored the F_2 progeny for segregation of RAPDs and RFLPs. Field isolates

Table 2. Segregation of random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers among the F_2 progeny of *Phytophthora sojae* isolates

	Genotypes[b]							
	Parental isolate			F_1	Segregation in F_2 progeny (number and class)[d]			Goodness of fit (χ^2)[e]
Marker[a]	P6497	P7064	P7076	progeny[c]				
Cross 1 (P6497 × P7064)								
RAPDs								
OPP2	P–	MM	...	PM	18 P–	...	5 MM	0.13
OPP15	P–	MM	...	PM	34 P–	...	9 MM	0.38
RFLPs								
68H3P3	FF	SS	...	FS	7 FF	12 FS	4 SS	0.83
68P5	MM	P–	...	PM	17 P–	...	6 MM	0.01
82P1	SS	FF	...	FS	9 FF	25 FS	12 SS	0.74
82P2	P–	MM	...	PM	30 P–	...	15 MM	1.67
82P3	FF	SS	...	FS	17 FF	20 FS	8 SS	4.16
83P1	FF	SS	...	FS	16 FF	20 FS	12 SS	2.00
83P2	P–	MM	...	PM	39 P–	...	9 MM	1.00
83H1	FF	SS	...	FS	16 FF	16 FS	14 SS	4.43
83H2	P–	MM	...	PM	39 P–	...	9 MM	1.00
121P1	FF	SS	...	FS	13 FF	21 FS	13 SS	0.53
128P2	FF	SS	...	FS	10 FF	21 FS	15 SS	1.43
128P3	P–	MM	...	PM	31 P–	...	17 MM	3.78
151P1	SS	FF	...	FS	11 FF	26 FS	8 SS	1.49
151P2	MM	P–	...	PM	39 P–	...	7 MM	2.35
206H1	FF	SS	...	FS	11 FF	24 FS	11 SS	0.09
267P1	FF	SS	...	FS	2 FF	30 FS	15 SS	10.79*[f]
284H1	FF	SS	...	FS	2 FF	29 FS	16 SS	10.91*[f]
285R1	FF	SS	...	FS	12 FF	20 FS	13 SS	0.60
285R2	FF	SS	...	FS	12 FF	24 FS	11 SS	0.06
Cross 2 (P6497 × P7076)								
RAPDs								
OPA13	FF	...	SS	FS	7 FF	11 FS	6 SS	0.25
OPP2	P–	...	MM	PM	23 P–	...	1 MM	8.49*
OPM4	MM	...	P–	PM	5 MM	...	19 P–	0.22
RFLPs								
8H1P2	SS	...	FF	FS	23 FF	1 FS	0 SS	64.3*
40H2	P–	...	MM	PM	3 P–	...	21 MM	50.0*
68H4	P–	...	MM	PM	2 P–	...	21 MM	53.9*
87H1	P–	...	MM	PM	3 P–	...	21 MM	50.0*
121P1	FF	...	MM	F–	22 F–	...	0 MM	7.33*

[a] RAPD primers were from Operon Technologies. RFLP hybridization probes were described by Förster *et al.* (1994).

[b] Genotypes inferred from RAPD and RFLP phenotypes and, in the case of F_1 progeny, from the phenotypes of the F_2 progeny. P = band present; M = band missing; F = fast allele; S = slow allele; F– = genotype FF or FM; P– = genotype PP or PM.

[c] RAPD phenotypes were identical in all four F_1 progeny from cross 1 and in all 11 F_1 progeny from cross 2 for each given primer. Where the phenotypes of the F_2 progeny were used to infer that the F_1 genotype was PM, only F_2 progeny from one F_1 individual from each cross were analyzed (F6497/7064-47 from cross 1 and F6497/7076-62 from cross 2).

[d] Only F_2 progeny from one F_1 individual from each cross were analyzed (F6497/7064-47 from cross 1 and F6497/7076-62 from cross 2). The number of F_2 progeny of each genotype is given. Each marker was scored against 24 or 48 F_2 progeny except where technical problems prevented unambiguous scoring.

[e] Goodness of fit (χ^2) of the ratios of the different genotype classes in F_2 progeny to those expected for single dominant markers (3:1) or for single semidominant loci (1:2:1). Asterisks (*) denote χ^2 values in excess of 95% significance levels (3.84 for two classes and 5.99 for three classes).

[f] F_2 genotype ratios for 267P1 and 284H1 fit the ratios expected if the region carrying these two closely linked loci is present in three copies in the F_1 individual which was selfed (see text). If the third copy is translocated to another chromosome, the expected ratio is 4:11:1, and the χ^2 values are 1.36 and 2.18, respectively. If the region is located on a trisomic chromosome, the expected ratio is 9:26:1, and the χ^2 values are 1.69 and 2.58, respectively.

of *P. sojae* appear to be extensively homozygous (Förster *et al.* 1994). Therefore we expected that most F_1 progeny would be genetically identical and that most genetic information would be obtained from the phenotypes of the F_2 progeny. We scored 47 F_2 progeny from P6497 × P7064 and 24 F_2 progeny from P6497 × P7076, representing oospores from each cross which had germinated at different times after harvesting.

In the cross P6497 × P7064, the RAPD marker was present in 18 progeny scored with primer OPP2 and missing in five progeny (Fig. 2A), very close to the 3:1 ratio expected for a dominant marker. In the cross P6497 × P7076, this RAPD marker was present in 23 progeny and missing in one (Fig. 2B), which is significantly different from the expected ratio. One other RAPD tested among the progeny of P6497 × P7064 and the two other RAPDs tested among progeny of the cross P6497 × P7076 showed regular Mendelian segregation (Table 2).

The progeny were also analyzed by the use of RFLP markers characterized in our previous analysis of field isolates (Förster *et al.* 1994). Figure 3 shows the segregation in the two crosses of RFLPs detected by probe p121. Isolates P6497 and P7064 exhibit a size polymorphism (121P1, alleles F and S, respectively) in the largest *Pst*I fragment detected by p121,

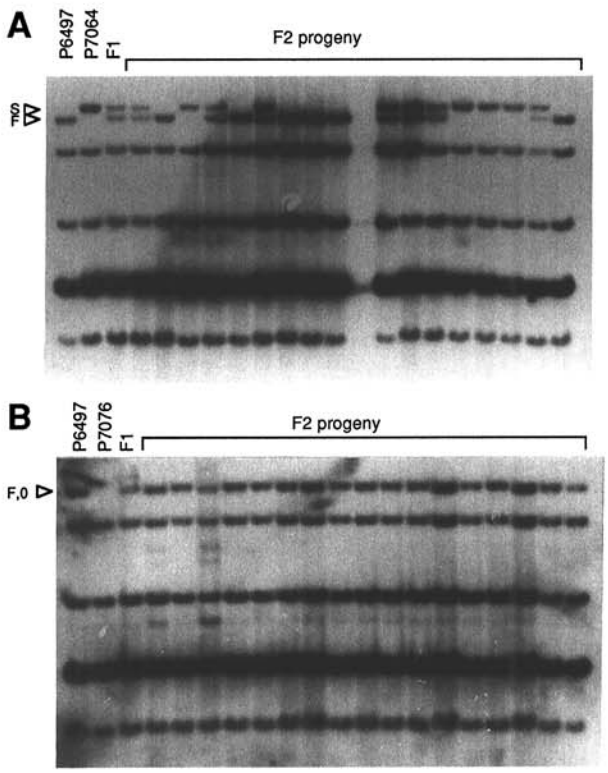

Fig. 3. Segregation of restriction fragment length polymorphism (RFLP) markers among representative F_2 progeny of hybrids of *Phytophthora sojae* isolates P6497 × P7064 (**A**) and P6497 × P7076 (**B**). In both cases, the DNAs were digested with *Pst*I, and the hybridization probe was p121. This probe detects the codominant F and S alleles (arrows) of RFLP 121P1 in P6497 and P7064, respectively (**A**), and the dominant F and recessive O alleles of 121P1 in P6497 and P7076, respectively (**B**). O is the same as M in Table 2. F_1 indicates the same hybrids described in Figure 2. In **B,** none of the F_2 progeny shown display the OO genotype. The mark crossing the top of the lanes containing P6497 and P7076 is a hybridization artifact.

while the fragment is completely missing (or comigrates with another fragment) in P7076 (121P1, allele M). As expected, F_1 hybrid F6497/7064-47-1 was heterozygous for alleles F and S, and the F_2 progeny obtained from it contained the genotypes FF, FS, and SS in a ratio of approximately 1:2:1 (Fig. 3A). F_1 hybrid F6497/7076-61-2 exhibited the F allele, as expected, since the M allele is recessive (Fig. 3B). However, all of its F_2 progeny tested also exhibited the F allele, whereas 25% would be expected to exhibit the M allele. Sixteen other RFLPs segregated in a regular Mendelian fashion in the cross P6497 × P7064, while two did not (Table 2). An additional seven RFLP markers (not shown) segregated in a regular Mendelian fashion in this cross. However of the five RFLPs scored among progeny of P6497 × P7076, including 121P1, none showed normal segregation (Table 2).

Segregation of specific virulence among the F_2 progeny.

In order to test the hypothesis that avirulence is a dominant trait and is determined by single genes, the F_2 progeny from the two crosses were scored for specific virulence against eight *Rps* genes (Table 3). In both crosses, the two parents and all of the F_1 and F_2 progeny tested were virulent on cultivars carrying *Rps7* and on cultivars carrying no *Rps* genes. Therefore there was no evidence for loss of virulence during the crosses due either to segregation of recessive avirulence genes or to other types of genetic changes.

In the cross P6497 × P7064, significant numbers of the F_2 progeny were virulent against *Rps1a, Rps1b,* and *Rps3a,* whereas the parental F_1 hybrid was avirulent against these *Rps* genes. Therefore avirulence against these three *Rps* genes is clearly dominant. The ratios of avirulent to virulent progeny were 2.6:1, 2.5:1 and 2.8:1, respectively (disregarding intermediate phenotypes; see Materials and Methods), which are not significantly different from 3:1, the ratio expected for a single dominant avirulence gene. All of the segregation ratios differed significantly from 15:1 (expected in the case of two independent unlinked avirulence genes) and from 9:7 (expected in the case of two unlinked avirulence genes acting together). The F_2 progeny were not tested against cultivars containing *Rps3c, Rps4,* or *Rps6.*

In the cross P6497 × P7076, segregation of avirulence against *Rps1d* fit a 3:1 ratio well, while segregation of avirulence against *Rps1k* fit a 9:7 ratio or a 3:1 ratio. However, the segregation ratios of avirulent to virulent progeny were highly skewed for *Rps1a* and *Rps1c* (0.33:1 and 0.26:1, respectively). For *Rps3b,* only one of 22 progeny showed consistent virulence against the resistant cultivars, which is a ratio similar to 15:1. However, since several molecular markers showed very biased transmission in this cross, the significance of the above ratios is questionable (see Discussion).

Genetic linkage among markers.

In the cross P6497 × P7064, which showed regular segregation of markers, 26 RFLP markers (including the 19 shown in Table 2), two RAPD markers, and three avirulence genes were analyzed for linkage by the program Mapmaker Macintosh 1.0 (Lincoln and Lander 1987; Proctor *et al.* 1990). Four pairs of RFLP markers (68P5 and 82P1; 267P1 and 284H1; 8P5 and 83H1H3P3; and 40H2 and 83H2P2) were found to be linked, with a log of the odds (LOD) score of 3.0 or better, and one group of three linked RFLP markers (8H2,

8P4, and 40H1) was found. The genetic distances ranged from 0 to 11 cM. In addition, RFLP 121P1 was found to be linked to the gene responsible for avirulence against *Rps1b* (*Avr1b*) at a distance of 11 cM. From this observed frequency of linked markers, a preliminary estimate of 1,430 cM was made for the total size of the genetic map of *P. sojae,* by the method of Hulbert *et al.* (1988). None of the genes responsible for the avirulence phenotypes scored (*Rps1a, Rps1b,* and *Rps3a* in P6497 × P7064 and *Rps1a, Rps1c, Rps1d, Rps1k,* and *Rps3b* in P6497 × P7076) showed any evidence of linkage to one another.

DISCUSSION

Outcrossing of *Phytophthora sojae.*

Most *Phytophthora* species are heterothallic, requiring strains of different mating types, A1 and A2, to conduct genetic crosses (Erwin *et al.* 1983). This has facilitated genetic analysis in several heterothallic species, such as *P. infestans* and *P. parasitica.* In contrast, some species, such as *P. sojae,* are homothallic, and individual strains readily form oospores by themselves. Homothallic species have several potential advantages for genetic analysis. For example, recessive lethal mutations should be rare, and strains should be substantially pure-breeding, as a result of repeated selfing. However, it is necessary to use genetic markers to identify F_1 hybrids among the progeny of outcrosses in these species.

Here, we used molecular markers (RAPDs) to identify F_1 hybrids produced when oospores are produced by a mixed culture of two *P. sojae* strains (Francis and St Clair 1993; Whisson *et al.* 1994). RFLP markers have also been used to verify the hybrid nature of progeny from crosses between *P. parasitica* isolates (Förster and Coffey 1990). Long and Keen (1977) used auxotrophic markers to select F_1 hybrids of *P. sojae,* while Bhat and Schmitthenner (1993) used drug resistance mutations. Although direct selection of hybrids can potentially be applied to greater numbers of progeny, molecular markers do not require mutagenesis of the parent strains, with the attendant risk of introducing additional deleterious mutations.

The parent isolates are extensively homozygous.

Since *P. sojae* is homothallic and has an active sexual cycle in the field (Schmitthenner 1989), it could be predicted that individual isolates would be extensively homozygous, as a results of repeated selfing. An analysis of the distribution of RFLP markers among field isolates supported this prediction (Förster *et al.* 1994). The segregation of RAPD and RFLP markers among the F_1 and F_2 progeny analyzed in this study also indicates that the three parental isolates are extensively homozygous. In the first of the two crosses analyzed, P6497 (race 2*) × P7064 (race 7), the two parents were homozygous, and the F_1 hybrids were heterozygous for all 15 of the codominant RFLP markers analyzed. Similarly, in the second cross, although fewer markers were scored, no evidence for heterozygosity was observed. These observations, however, do not rule out that some isolates, including the ones studied here, may be heterozygous at some loci. In fact, if outcrossing occurs in the field (Förster *et al.* 1994), then some isolates that are substantially heterozygous should occasionally be recovered.

Evidence for simple diploid genetics in *P. sojae.*

Polyploidy and aneuploidy are quite common in *P. infestans* and in several other species of *Phytophthora* (Erwin *et al.* 1983; Sansome and Brasier 1974; Therrien *et al.* 1989). Therefore we used molecular markers to establish whether genetic segregation in the *P. sojae* crosses is regular or biased as a result of polyploidy, aneuploidy, or lethal mutations. In the cross P6497 (race 2*) × P7064 (race 7), all but two of the 28 molecular markers showed 3:1 or 1:2:1 segregation, consistent with the F_1 hybrid F6497/7064-47 and its parents being simple diploids. Two closely linked semidominant RFLP markers (267P1 and 284H1), however, showed segregation that closely matched ratios expected if the region carrying the loci were present in three copies in the F_1 hybrid and in the P7064 parent: either 4:11:1, expected if one copy of the region was translocated to another chromosome, or 9:26:1, expected if the entire chromosome were tripled (i.e., trisomy). This hypothesis was supported by close inspection of the relative hybridization intensities of the bands detected among the progeny by the single-copy probe, which revealed at least three classes of heterozygotes having different allele ratios (four classes would be expected, with ratios of 2:1, 1:1, 1:2, and 1:3). Therefore, while both P6497 and P7064 appear to be substantially clean diploids, there appears to be one chromosome or a region of one chromosome for which P7064 may be aneuploid.

In the cross P6497 × P7076, two of the three RAPD markers analyzed and all of the five RFLP markers analyzed showed very biased segregation; three markers showed a seven- to 10-fold excess of the recessive allele. Bias occurred in favor of P6497 alleles at some loci and in favor of P7076 alleles at other loci. Avirulence against *Rps1a, Rps3a,* and *Rps3b* also showed biased segregation. Since both crosses share the common parent P6497, the biased segregation appears to be caused by the genome of P7076 or its interaction with that of P6497. The mechanisms responsible for the biased segregation are presently unknown. They could include the presence of balanced lethal mutations, the presence of chromosomal rearrangements, or the occurrence of high-frequency mitotic crossing-over or gene conversion in the F_1 hybrids. The bias does not appear to be entirely due to polyploidy or aneuploidy in P7076, since if it were, then bias would have occurred only in favor of P7076 alleles, and strong bias in favor of recessive alleles would be unlikely. Since F_2 progeny were analyzed from only one F_1 hybrid individual from this cross, it remains possible that the biased segregation is peculiar to this individual, and that the parent isolates and other F_1 progeny are normal. Whisson *et al.* (1994) and Bhat and Schmitthenner (1993) reported mostly regular segregation of markers.

Avirulence against six *Rps* genes is dominant.

The two pairs of parental isolates differed in specific virulence against a total of 10 *Rps* genes. The F_1 progeny from the two crosses were fully avirulent against eight of these *Rps* genes (*Rps1a, Rps1b, Rps1c, Rps1d, Rps1k, Rps3a, Rps3b,* and *Rps3c*) and partially avirulent against the two others (*Rps4* and *Rps6*). The avirulence phenotypes of the F_1 progeny were specific to particular *Rps* genes, as all the progeny were fully virulent against cultivars having no *Rps* genes and those having *Rps7.* Therefore the F_1 data suggest that specific

avirulence is dominant or semidominant to virulence in the case of all 10 *Rps* genes. The dominance of avirulence against *Rps1a, Rps1b, Rps1c, Rps1d, Rps1k,* and *Rps3a* was confirmed by the presence of significant numbers (18% or more) of virulent individuals among the F_2 progeny obtained by selfing avirulent F_1 progeny.

In the case of *Rps3b,* only one of 24 F_2 individuals was virulent. Since bias in transmission is probably responsible for this ratio, or possibly the presence of two unlinked genes (see below), avirulence against *Rps3b* is probably dominant. However, if the one virulent individual was consistently misscored or was otherwise aberrant, then avirulence against *Rps3b* might be recessive. Only two F_1 individuals were tested against the resistant cultivar carrying *Rps3c,* and the F_2 progeny were not tested for virulence against this gene. Therefore it remains possible that the avirulent F_1 progeny might have been obtained from the segregation of a recessive avirulence gene in the initial cross. For *Rps4* and *Rps6* also, only two F_1 individuals were tested, so analysis of the F_2 progeny will be required to demonstrate that the intermediate phenotypes are due to a single locus and not to multiple genes or modifier genes. Whisson *et al.* (1994) obtained F_2 data supporting the presence of a codominant avirulence gene matching *Rps6.* In the case of *Rps7,* while it is theoretically possible that one or both parents as well as the F_1 progeny could have been heterozygous for a recessive avirulence gene, this is unlikely, because of the absence of any avirulent progeny in the F_2 generation of either cross.

Evidence for at least three single avirulence genes.

In the cross P6497 (race 2*) × P7064 (race 7), in which the molecular markers showed regular segregation, avirulence against *Rps1a, Rps1b,* and *Rps3a* segregated in a ratio not significantly different from 3:1, indicating that a single locus determines avirulence against these three *Rps* genes. Although we cannot rule out the presence of multiple tightly linked genes at each locus, the simplest hypothesis is that there is a single dominant avirulence gene at each locus, as has been found in several other bacterial and fungal pathogens (De Wit 1992; Joosten *et al.* 1994; Keen 1990; Valent and Chumley 1991). We have designated these presumptive avirulence genes *Avr1a, Avr1b,* and *Avr3a,* respectively, following the system employed for the oomycete *Bremia lactucae* (Michelmore *et al.* 1984). Whisson *et al.* (1994) adopted the same nomenclature. The existence of a single gene for avirulence against *Rps1b* is also supported by the linkage of an RFLP to *Avr1b* (11 cM, LOD score of 5.05).

Avirulence against *Rps1d, Rps1k,* and *Rps3b* segregated in ratios similar to 3:1, 3:1 or 9:7, and 15:1, respectively, in the second cross. Thus one gene might be responsible for avirulence against *Rps1d,* one or two for avirulence against *Rps1k,* and two for avirulence against *Rps3b.* However, given the extremely biased transmission ratios in this cross for most of the molecular markers and for avirulence against *Rps1a* and *Rps1c,* no firm interpretation can be drawn from these data as to the number of genes involved. The biased segregation in the second cross emphasizes the importance of verifying regular segregation of molecular markers in any cross of *P. sojae* (or any other organism) before inferring the genetic basis of a trait from segregation ratios alone.

Layton and Kuhn (1988), using heterokaryons, demonstrated that avirulence against *Rps1a* is dominant. Our data confirm this finding. Bhat and Schmitthenner (1993) crossed isolates of race 1 and race 3 (which differ only in virulence against *Rps1a*) and used drug resistance markers to select the F_1 hybrids. Bhat et al (1993) observed 3:1 segregation, consistent with a single dominant avirulence gene (i.e., *Avr1a*) against *Rps1a.* However, on the basis of the presence of a small number of nonpathogenic progeny and progeny which had lost avirulence against *Rps1k,* these authors proposed a

Table 3. Segregation of specific virulence among F_2 progeny of *Phytophthora sojae* isolates

	Virulence phenotypes[b]										
	Parental isolate			F_1 progeny	Segregation in F_2 progeny (number and class)[c]			Goodness of fit (χ^2) of F_2 ratios to:[d]		
Rps gene[a]	P6497	P7064	P7076					3:1	9:7	15:1
Cross 1 (P6497 × P7064)										
None	V	V	...	V	72 V	0 A	...			
Rps1a	A	V	...	A	49 A	19 V	4 I	0.31	6.91*	55*
Rps1b	V	A	...	A	47 A	19 V	6 I	0.51	6.00*	57*
Rps3a	A	V	...	A	47 A	17 V	8 I	0.08	7.68*	45*
Rps7	V	V	...	V	72 V	0 A	...	24*	56*	4.8*
Cross 2 (P6497 × P7076)										
None	V	...	V	V	24 V	0 A	...			
Rps1a	A	...	V	A	5 A	15 V	4 I	26.7*	7.94*	161*
Rps1c	A	...	V	A	5 A	19 V	...	38*	12.2*	217*
Rps1d	A	...	V	A	18 A	5 V	1 I	0.13	4.53*	9.4*
Rps1k	A	...	V	A	13 A	8 V	3 I	1.92	0.27	36*
Rps3b	A	...	V	A	21 A	1 V	2 I	4.91*	13.7*	0.1
Rps7	V	...	V	V	24 V	0 A	...	8.00*	18.7*	1.6

[a] *Rps* resistance genes present in soybean cultivars inoculated with the *P. sojae* parents and progeny.

[b] Phenotype of parents and progeny in resistant and susceptible soybean cultivars inoculated with the pathogen. A = avirulent; V = virulent; I = intermediate.

[c] Only F_2 progeny from one F_1 individual from each cross were analyzed (F6497/7064-47 from cross 1 and F6497/7076-62 from cross 2). The number of F_2 progeny of each phenotype is given.

[d] Goodness of fit (χ^2) of the ratios of the different phenotype classes to those expected for single dominant avirulence genes (3:1), for two dominant avirulence genes acting together (9:7), or for two dominant avirulence genes acting independently (15:1), except in the case of *Rps7,* in which the null hypothesis was that the avirulence genes were recessive. Asterisks (*) denote χ^2 values in excess of 95% significance levels (3.84). The minority of F_2 progeny with intermediate phenotypes were not included in the significance tests (see Materials and Methods).

more complex hypothesis, involving dominant genes for race 1–specific virulence and race 3–specific virulence, with the race 1 gene epistatic to the race 3 gene. Our cross did not involve isolates of races 1 and 3, but instead isolates of races 2 and 7, yet we still observed the same segregation for avirulence against *Rps1a* (3:1) as did Bhat *et al.* (1993). Moreover, both we and Whisson *et al.* (1994) observed independent 3:1 segregation of avirulence against at least five different *Rps* genes (*Rps1a, Rps1b, Rps3a, Rps5,* and *Rps6*). Therefore it is much more likely that race types in *P. sojae* are determined by independent dominant avirulence genes, as in other pathogens (Day 1974; Keen 1990), rather than by interacting dominant race-specific virulence genes. The minority phenotypes observed by Bhat *et al.* (1993) probably resulted from genetic variation unrelated to the gene (*Avr1a*) conferring avirulence. Whisson *et al.* (1994) also obtained evidence for a single avirulence gene (*Avr1a*) matching *Rps1a.* However, they observed 9:7 segregation for avirulence matching *Rps3a* and *Rps5,* suggesting either slightly biased segregation (at least one of their RAPD markers segregated in a ratio of 9:7) or else the presence of two cooperating avirulence genes.

Other oomycetes also display gene-for-gene interactions. Segregation of avirulence has also been observed in crosses between different races of *P. infestans,* with the conclusion that some dominant and some possibly recessive avirulence genes were present (Spielman *et al.* 1989, 1990). However, the conclusions were limited by poor fertility and biased transmission in the F_2 generations and in backcrosses. In the oomycete *Bremia lactucae,* which causes lettuce downy mildew, a number of single dominant avirulence genes have been identified, which segregate in regular Mendelian fashion (Hulbert *et al.* 1988; Michelmore *et al.* 1984).

Outcrossing in the field.

We previously presented evidence that *P. sojae* undergoes outcrossing in the field and suggested that the consequent reassortment of avirulence genes is an important mechanism by which new physiological races arise (Förster *et al.* 1994). The data presented here, together with those of Bhat and Schmitthenner (1993) and Whisson *et al.* (1994), firmly establish that this homothallic pathogen is capable of fertile outcrosses in the laboratory when two isolates are cultured together. Since the pathogen selfs frequently in the field, it is likely that outcrosses will occur whenever two distinct isolates infect the same plant. Layton and Kuhn (1990) showed that heterokaryons can form when two isolates infect one plant; selfing of a heterokaryon would readily produce an outcross (Long and Keen 1977). The independent segregation of avirulence genes in the laboratory crosses described here and by Whisson *et al.* (1994) also clearly predicts that reassortment of avirulence phenotypes will occur when outcrosses occur in the field, resulting in the generation of large numbers of new race types among the progeny. This will need to be considered when developing molecular probes to characterize race types and when breeding soybeans for resistance to *P. sojae.*

MATERIALS AND METHODS

***P. sojae* strains and crosses.**

Potential parent isolates P6497 (race 2*), P7064 (race 7), P7069 (race 12), P7076 (race 19*), P7079 (race 22), and P7082 (race 26) were chosen because the strains produce abundant oospores and show extensive DNA sequence polymorphisms in RFLP analysis (Förster *et al.* 1994). F_1 progeny from two crosses, P6497 × P7064 and P6497 × P7076, were selected for analysis because a total of 10 potential avirulence genes would segregate in either or both of these two crosses. Isolate P6497, which in the past was scored as race 2, gave variable intermediate reactions against *Rps5* (instead of an avirulent reaction) when it was used for crosses. As a result, reactions against *Rps5* were not included in this study. Isolate P7076, which was originally scored as race 19, proved to be virulent against *Rps7* when it was used for crosses. For convenience, we refer to these isolates here as race 2* and race 19*, rather than designating them as new races.

Isolates were maintained on solid V8 juice medium (Ribeiro 1978). Crosses were conducted by plating a mixture of homogenized mycelia from each parent (for outcrosses) or from just one strain (for selfs) on solid uncleared V8 juice medium and incubating them for 5–6 weeks at 25° C in the dark. Oospores were then harvested, sonicated to kill mycelia, and placed in a solution of 10 mM alanine at room temperature under blue light (Westinghouse 15W, F15T8/B) until germination was observed. After 3, 4, and 5 days, germinating oospores were transferred individually to solid V8 juice medium by microdissection, to reduce the possibility of obtaining mixed cultures or heterokaryons. Since *P. sojae* is homothallic, it was straightforward to obtain a large number of F_2 oospores by selfing. The germination frequency of the F_2 oospores was at least as high as that of oospores produced by selfing the parents, indicating that the F_1 hybrids retained full fertility.

To obtain single-zoospore derivatives, zoospore suspensions were produced as previously described (Judelson *et al.* 1993). Drops of diluted suspensions containing zero to five zoospores were placed on plates containing solid V8 juice medium. After 24 h of growth at 25° C, drops containing single germinating zoospores were identified under a microscope and marked. After 2 days of further growth, small colonies derived from single zoospores were transferred to individual plates.

Extraction of DNA for RAPD and RFLP analysis.

Small amounts of DNA for RAPD analysis (miniprep DNA) were obtained as follows. A small block (approximately 5 mm^3) of solid V8 juice medium infested with *P. sojae* was transferred to 5 ml of liquid V8 juice medium containing pimaricin (200 g/ml), ampicillin (1.25 mg/ml), rifampicin (100 g/ml), and *p*-chloronitrobenzene (1.33 mg/ml) in the wells of a 6 × 15-ml tissue culture dish. After 3–7 days of growth at 25° C, a piece of mycelium 10–20 mm^3 was torn from the mycelia growing at the edge of the agar block (carefully, to avoid taking any agar) and placed in a 1.5-ml microfuge tube. The mycelium was centrifuged for 5 min at 15,000 rpm, and excess medium was removed. Then 150 µl of extraction buffer (200 mM Tris HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, and 2% sodium dodecyl sulfate), 150 µl of dry 0.5-mm glass beads, and 150 µl of phenol saturated with extraction buffer were added to the mycelium. The mixture was vortexed or shaken in a paint mixer for 2 min and then centrifuged for 10 min at room temperature. The aqueous phase was transferred to a microfuge tube containing 120 µl of chloro-

form/isoamyl alcohol (49:1), vortexed, and then centrifuged for 2 min. The aqueous phase was transferred to a microfuge tube containing 5 μg of RNase A and incubated at 37° C for 15 min; then 0.6 vol of isopropanol was added, and the mixture was frozen at −80° C for 10 min to precipitate the DNA. The DNA was recovered by centrifugation, washed briefly with 200 μl of 80% ethanol, dried, and dissolved in 50 μl of a solution containing 10 mM Tris HCl, pH 7.4, and 0.1 mM EDTA. Normally, 1 μl of the DNA solution was used per 25 μl of RAPD reaction.

RAPDs.

To identify RAPD markers specific to the parents, we screened 60 commercially available RAPD primers (sets A, M, and P from Operon Technologies), using purified nuclear DNAs from P6497, P7064, and P7076. Candidate polymorphisms were checked for reproducibility with several preparations of miniprep DNAs from these parents. Eleven polymorphisms that distinguish P6497 and P7064 were obtained, detected with seven primers, while five polymorphisms that distinguish P6497 and P7076 were obtained, detected by four primers. The levels of polymorphism observed (4.6 and 2.1%, respectively) were lower than those observed with the use of RFLPs (10 and 5.7%, respectively [Förster *et al.* 1994]), probably because we were very conservative in identifying polymorphisms which could be reproducibly scored. To identify F_1 hybrids from the cross P6497 × P7064, we used primer OPP15, which detects two RAPDs, one present in P6497 and one present in P7064. To identify F_1 hybrids from P6497 × P7076, we used primer OPP2, which detects a RAPD specific to P6497, and primer OPM4, which detects a RAPD specific to P7076. Potential hybrids were retested with the other RAPD markers specific to the two parents.

RAPD reactions (25 μl) contained 10 mM Tris HCl, pH 8.2; 50 mM KCl; 1.9 mM $MgSO_4$; 100 M (each) dATP, dCTP, dGTP, and dTTP; 400 nM primer (Operon Technologies); gelatin (100 g/ml); DNA (approximately 60 ng/ml); and Taq DNA polymerase (40 units per milliliter). Reactions were incubated in a Perkin-Elmer DNA Thermal Cycler 480 for 2 min at 94° C; then for 40 cycles of 1 min at 94° C, 1 min at 35° C, and 2 min at 72° C; and finally for 5 min at 72° C. The reaction products were resolved by electrophoresis through 2% agarose gels.

RFLPs.

RFLP probes and extraction and hybridization of DNA for RFLP analysis were as previously described (Förster *et al.* 1994).

Specific virulence tests.

The specific virulence of parent and progeny strains was determined by inoculating seedlings of a standard set of soybean cultivars carrying particular *Rps* resistance genes, namely, Williams and HARO(1-7)1 (no *Rps* genes); L75-6141 and Asgrow 1937 (*Rps1a*); Harosoy 63 (*Rps1a* and *Rps7*); L77-1863 and HARO13 (*Rps1b*); L75-3735 (*Rps1c*); HARO1472 (*Rps1c* and *Rps7*); PI 103091 and HARO16 (*Rps1d*); Williams 82 (*Rps1k*); HARO1572 (*Rps1k* and *Rps7*), L83-570 (*Rps3a*); HARO3272 (*Rps3a* and *Rps7*); PRX146-47, L89-1541, and L91-8347 (*Rps3b*); PRX147-48 (*Rps3c*); L85-2352 (*Rps4*); L85-3059 (*Rps5*); HARO5272 (*Rps5* and *Rps7*); Altona and L89-1581 (*Rps6*); HARO6272 (*Rps6* and *Rps7*); and Harosoy (*Rps7*). When virulence against a particular *Rps* gene was tested, two or three different cultivars carrying that gene were tested in the same experiment. Every test of F_2 progeny included the relevant parental and F_1 *P. sojae* strains and the susceptible soybean cultivar Williams as controls. Seedlings were raised in the greenhouse and then, at the age of 7–12 days, were placed in constant light in a growth chamber at 25° C for 24 h prior to inoculation. For each combination of a *P. sojae* strain and a soybean cultivar, five or six seedlings were inoculated with small pieces of infested V8 juice agar inserted in small slits in the hypocotyl. The wounds were not sealed, and the plants were not covered with plastic bags, as such treatments often resulted in false susceptible responses. After 5 days at 25° C in the light, individual seedlings were scored as susceptible (total collapse), resistant (continued strong growth), or intermediate (slowly spreading black lesions, but continued growth of the plant). Pathogen-cultivar interactions were scored as compatible if no more than one out of five or six seedlings were resistant or two were intermediate. They were scored as incompatible if no more than one seedling was susceptible or if two were intermediate. In other cases, the interaction was scored as intermediate. All tests were carried out at least twice, at least a month apart. Tests showing inconsistent or unexpected results were repeated a total of three or four times. Isolates or progeny which repeatedly gave inconsistent results were scored as intermediate. Progeny which tested as intermediate tended toward an avirulent phenotype and might actually be avirulent, since our inoculation assay more frequently produces false virulent reactions. However, they were excluded from statistical analysis, because of uncertainty in the correct scoring. The minority of this class of progeny suggests they are simply scoring artifacts or reflect minor gene effects.

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