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Inheritance and mapping of 11 avirulence genes in *Phytophthora sojae*

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Abstract

Two new crosses involving four races (races 7, 16, 17, and 25) of the soybean root and stem rot pathogen *Phytophthora sojae* were established (7/16 cross; 17/25 cross). An F_2 population derived from each cross was used to determine the genetic basis of avirulence towards 11 different resistance genes in soybean. Avirulence was found to be dominant and determined by a single locus for *Avr*1b, 1d, 1k, 3b, 4, and 6, as expected for a simple gene-for-gene model. We also observed several cases of segregation, inconsistent with a single dominant gene being solely responsible for avirulence, which suggests that the genetic background of the different crosses can affect avirulence. *Avr*4 and 6 cosegregated in both the 7/16 and 17/25 crosses and, in the 7/16 cross, *Avr*1b and 1k were closely linked. Information from segregating RAPD, RFLP, and AFLP markers screened on F_2 progeny from the two new crosses and two crosses described previously (a total of 212 F_2 individuals, 53 from each cross) were used to construct an integrated genetic linkage map of *P. sojae*. This revised genetic linkage map consists of 386 markers comprising 35 RFLP, 236 RAPD, and 105 AFLP markers, as well as 10 avirulence genes. The map is composed of 21 major linkage groups and seven minor linkage groups covering a total map distance of 1640.4 cM.

Keywords: Gene-for-gene; Oomycetes; Genetics; Virulence; Genetic linkage mapping; *Phytophthora megasperma* f. sp. *glycinea*; AFLP; RFLP; RAPD

1. Introduction

Phytophthora sojae Kaufmann and Gerdemann (syn. *Phytophthora megasperma* f. sp. *glycinea*) is the causal agent of Phytophthora root and stem rot of soybean (*Glycine max*). It is a soil-borne, homothallic (self-fertile) oomycete, with a diploid somatic stage, and is host specific to soybean. The pathogen is largely controlled by deployment of resistant soybean cultivars (Schmitthenner et al., 1994). However, resistance in soybean is often rapidly overcome by the occurrence of new races of the pathogen (Schmitthenner, 1985). Diversity studies and genetic analysis of different *P. sojae* races in North America by Förster et al. (1994) suggested that new races arise by mutation and possibly infrequent outcrossing in the field. A detailed study by Drenth et al. (1996) revealed that the *P. sojae* population in Australia was genetically uniform and suggested that new races arose by mutation from a common genetic background.

A genetic basis for the interaction between resistance in the host plant and virulence in the pathogen was first hypothesised by Flor (1942) and Oort (1944). They hypothesised a gene-for-gene interaction in which for every gene conditioning avirulence in the pathogen, a corresponding gene conferring resistance exists in the host. The gene-for-gene theory has often been used to explain the interaction between hosts and their pathogens (Barrett, 1985; Day, 1974; Thompson and Burdon, 1992). Only in a few systems, however, have both the pathogen and the host been characterised genetically

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(Hutcheson, 1998; Laugè and de Wit, 1998). The first such example involving an oomycete was the lettuce/*Bremia lactucae* host pathogen system. A detailed study of the genetics of avirulence for the heterothallic *B. lactucae* (Ilott et al., 1989) showed that 13 single dominant avirulence genes in the pathogen match 13 dominant resistance genes (*Dm*) in lettuce (Farrara et al., 1987; Ilott et al., 1989; Michelmore et al., 1984). Linkage maps have been made for both *B. lactucae* (Hulbert et al., 1988) and lettuce (Kesseli et al., 1994). Similar studies have also been carried out for the *P. infestans*/*potato* interaction (Al-Kherb et al., 1995; El-Kharbotly et al., 1994, 1996; Van der Lee et al., 2001). The genetics of resistance to *P. sojae* in soybean has also attracted considerable attention (Ward, 1990) and 13 dominant resistance (*Rps*) genes at seven different loci have been identified (Diers et al., 1992). For the *Rps*1 locus, five alleles (*Rps*1a, 1b, 1c, 1d, and 1k) are known to confer resistance to various races of *P. sojae* and, for the *Rps*3 locus, three resistance alleles (*Rps*3a, 3b, and 3c) have been identified. Single resistance alleles are only known for the remaining loci, *Rps*2, 4, 5, 6, and 7.

Genetic studies on homothallic oomycetes such as *P. sojae* only became possible when molecular markers enabled the detection of low levels of outcrossing among divergent genotypes (Tyler et al., 1995; Whisson et al., 1994, 1995). The detection of F_1 hybrids has allowed the generation of F_2 mapping populations and enabled detailed studies of the genetic basis of avirulence. On the basis of two crosses (race 7 and race 1; race 7 and race 25), Whisson et al. (1995) reported that the avirulence genes *Avr*1a, 1b, 1k, 3a, 4, 5, and 6 acted as dominant alleles at single loci. Using independent crosses, Tyler et al. (1995) and Gijzen et al. (1996) confirmed that *Avr*1a, 1b, 3a, 4, and 6 were dominant alleles at single locus. The first genetic linkage map of *P. sojae* was constructed using 53 progeny from each of the two crosses (cross 7/1 and cross 7/25), described by Whisson et al. (1995), and consisted of seven avirulence genes, 228 random amplified polymorphic DNA (RAPD) markers, and 22 RFLP markers. In the 7/25 cross, *Avr*1b and 1k cosegregated and were assigned to linkage group 1, and *Avr*4 and 6 cosegregated and were assigned to linkage group 2. *Avr*3a and 5 cosegregated in the 7/25 cross but segregated in 4 out of 53 progeny in the 7/1 cross and were located 4.6 cM apart on linkage group 3. The final map based on 257 markers consisted of 10 major linkage groups containing five or more markers and 12 minor linkage groups. The map covered 830.5 cM of the *P. sojae* genome.

The purpose of this investigation was to construct two additional crosses that enable genetic analysis of four additional avirulence genes and place them on a refined and expanded genetic linkage map of *P. sojae*. The specific aims were to: (i) confirm the genetics of previously examined avirulence genes (*Avr*1a, 1b, 1k, 3a, 4, 5, and 6); (ii) determine the genetics of the previously uncharacterised avirulence genes: *Avr*1c, 1d, 3b, and 3c; (iii) place these additional avirulence genes on the linkage map; and (iv) include additional RFLP and AFLP markers to expand the genetic linkage map and assist in the incorporation of previously unlinked markers and avirulence genes. It was expected that this new information on the genetics of avirulence genes in *P. sojae* would (a) provide a further test of the universality of the gene-for-gene hypothesis, (b) provide further evidence for the potential contribution of outcrossing to the production of new races, and (c) assist in our understanding of the coevolution of soybean with *P. sojae*. For example, three closely linked pairs of avirulence genes were observed previously (Whisson et al., 1995), and linkage might occur with the four additional avirulence genes included in this study. A comprehensive genetic linkage map for *P. sojae* assembled from avirulence phenotypes and closely associated molecular markers will provide a vital starting point for cloning and characterising avirulence genes. Cloned and characterised avirulence genes are basic tools for efforts directed towards revealing the nature and functions of factors in plant pathogens that induce or overcome resistance in the host.

2. Materials and methods

2.1. Phytophthora sojae isolates

Five different *P. sojae* isolates were used: UQ2990, UQ244, UQ1200, UQ1689, and UQ1690 from the culture collection of the Cooperative Research Centre for Tropical Plant Pathology, the University of Queensland, Australia. Table 1 provides a description of the virulence spectrum and origin of the *P. sojae* isolates used in this and previous studies. Inconsistencies exist with regard to the definition of the virulence spectrum for race 16. Ward (1990) described race 16 as virulent against *Rps*1b, 1c, 1k, and 5. Other virulence spectra described for "race 16" include *Rps*1b, 1c, 1k, and 3a (Buzzell and Anderson, 1992) and *Rps*1b, 1c, 1k, 3a, and 3b (Förster et al., 1994). A repeated testing of isolate UQ1689 revealed that it was virulent toward *Rps*1b, 1c, 1k, and 7 and for this investigation it shall be referred to as race 16.

2.2. Generation of F_1 hybrids and F_2 populations

In addition to the two existing crosses (7/1 cross and 7/25 cross; Whisson et al., 1994, 1995), two new crosses were constructed for this investigation. The third cross was between race 7 and race 16 (7/16 cross) and the fourth between race 17 and race 25 (17/25 cross). Table 2 lists the crosses constructed and the avirulence genes that may potentially segregate in each cross. The

Table 1
Race, virulence spectrum, origin, and source of isolates of *P. sojae* used in this investigation

Parental isolate	Race	Resistance genes												Origin	Source
		1a	1b	1c	1d	1k	3a	3b	3c	4	5	6	7		
UQ2990	7	V	A	A	A	A	V	A	V	V	V	V	V	USA	J. Paxton
UQ244	1	A	A	A	A	A	A	A	A	A	A	A	V	Australia	M. Ryley
UQ1200	25	V	V	V	A	V	A	A	A	A	A	A	V	Australia	M. Ryley
UQ1689[a]	16	A	V	V	A	V	A	A	A	A	A	A	V	Canada	M. Gijzen
UQ1690	17	A	V	A	V	A	V	V	V	V	V	V	V	Canada	M. Gijzen

[a] Race designation for this isolate is different to designated race 16 as defined by Ward (1990).

Table 2
List of crosses of *P. sojae* and potential segregating avirulence genes

Cross	Isolate		Potential segregating avirulence genes
	Parent 1	Parent 2	
7/1[a]	UQ2990	UQ244	**1a**, **3a**, 3c, 4, 5, 6
7/25[a]	UQ2990	UQ1200	**1b**, 1c, **1k**, **3a**, 3c, **4**, **5**, **6**
7/16	UQ2990	UQ1689	**1a**, **1b**, **1c**, **1k**, **3a**, **3c**, **4**, **5**, **6**
17/25	UQ1690	UQ1200	1a, 1c, **1d**, 1k, 3a, **3b**, 3c, 4, 5, 6

Segregation data for Avr genes printed in bold were used to construct the genetic linkage map.
[a] Crosses by Whisson et al. (1995).

procedures for oospore isolation, identification of F_1 hybrids, and generation of an F_2 population have been described previously by Whisson et al. (1994).

2.3. DNA extraction and RAPD analysis

DNA was extracted from the parental isolates, F_1 hybrids, and a subset of 53 randomly chosen F_2 individuals from each of the 7/16 and 17/25 crosses as described previously by Whisson et al. (1992). DNA concentration was determined using fluorometry and its integrity was verified by gel electrophoresis. RAPD markers were generated between the five parental races using decanucleotide primers (Operon Technologies, Alameda, CA; primer kits A-I, L-M, O-T, V-X, Z, AC, and AM-AZ). Selected primers, which produced polymorphisms in the 7/1 cross and 7/25 cross, were also used for RAPD analysis of the 7/16 and 17/25 crosses. Primers, which produced polymorphic markers between the parental isolates, were screened against the F_1 hybrid and the 53 F_2 progeny for use in linkage analysis. RAPD protocols and analysis of amplified products were conducted as described by Whisson et al. (1995). Primer sequences of all RAPD primers (markers designated by the prefix OP) can be found at the Operon Technologies website (www.operon.com/store/merkits.php).

2.4. RFLP probes and DNA hybridisation

Restriction endonuclease digestion, transfer to nylon membrane, hybridisation, and autoradiography were carried out as described by Whisson et al. (1995). In addition to the RFLP probes used by Whisson et al. (1995), 22 additional probes were used to detect RFLPs in the 7/1 cross and 7/25 cross. Of these RFLP probes, seven were homologous to genes of known or suspected function that had been isolated from various *Phytophthora* species (Table 3). Ten of the RFLP probes originated from randomly selected cloned segments of *P. sojae* DNA inserted in the cosmid vector Supercos 1 (Stratagene, La Jolla, CA).

2.5. AFLP analysis

AFLP markers were prepared and detected as described by Vos et al. (1995) using the Gibco-BRL Life Technologies AFLP Core Reagent and Analysis System II, which is modified for the small genomes of some plants. To accommodate the smaller genome of *P. sojae*, modifications were made to manufacturer's instructions as outlined below.

Table 3
Probes with known function used in this investigation and their source

Gene	Source organism	References	Marker name	Linkage group[a]
Ribosomal non-transcribed spacer	*P. sojae*	Whisson et al., 1993	KINts+	6
Non-host elicitor	*P. sojae*	Sacks et al., 1995	BDIEL.2+	21
HSP70 homolog	*P. cinnamomi*	Lehnen and Hardham, unpublished	EVBipC	6
Peripheral vesicle protein	*P. cinnamomi*	Marshall et al., 2001	PILpv 18C	8
Elicitin INF1	*P. infestans*	Kamoun et al., 1997	PIFB7C	3
Ubiquitin	*P. infestans*	Pieterse et al., 1991	PIUbi.1C	11
			PIUbi.2C	11
Translation elongation factor EF1α	*P. infestans*	Pieterse et al., 1993	KTefC	7

[a] Linkage groups as defined in Fig. 1.

Digestion of genomic DNA and ligation of adaptors to DNA fragments. Genomic DNA from the parental isolates, F_1 hybrid, and the 53 F_2 progeny was digested using the restriction endonucleases *Eco*RI and *Mse*I in the following manner. Each reaction contained 5 μl of 5× reaction buffer [50 mM Tris–HCl (pH 7.5), 50 mM Mg-acetate, and 250 mM K-acetate], 2 μl *Eco*RI/*Mse*I (1.25 U/μl each), and 250 ng genomic DNA and distilled water was added to make a total volume of 25 μl. All reactions were incubated at 37 °C for 8 h. The *Eco*RI and *Mse*I adaptors were then ligated to the resultant DNA fragments by adding to the digested genomic DNA 24 μl adaptor/ligation solution (GIBCO-BRL Life Technologies AFLP Core Reagent Kit) and 1 μl T4 DNA ligase (1 U/μl). The reactions were incubated at 18 °C overnight. A 1:10 dilution was then performed using $T_{10}E_{0.1}$ buffer [10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA].

Pre-amplification reactions. Each pre-amplification reaction contained 5 μl diluted digested/ligated template DNA, 5 μl of 10× PCR buffer, and 40 μl pre-amp primer mix GIBCO-BRL Life Technologies AFLP Small Genome Primer Kit) and 1 μl DNA polymerase (5U/μl). The thermocycling sequence was as follows: one cycle of 94 °C for 15 s, 65 °C for 15 s, and 72 °C for 30 s. The annealing temperature was lowered stepwise by 0.7 °C for each step of the following 12 cycles. This was proceeded by 23 cycles of 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 30 s. Reactions were diluted (1:50) using $T_{10}E_{0.1}$ buffer and stored at −20 °C.

Primer labelling. The *Eco*RI primers were radiolabelled with [γ-^{33}P]ATP. Each *Eco*RI primer had a single selective nucleotide (E + A, E + C, E + T, and E + G). For a single reaction in the selective PCR, the following was needed for the labelled primer: 1 μl *Eco*RI primer (50 ng/μl), 1 μl T4 polynucleotide kinase (PNK) buffer, 1 μl [γ-^{33}P] ATP, 1.8 μl distilled water, and 0.2 μl T4 PNK (10 U/μl). The contents were incubated at 37 °C for 1 h and then at 70 °C for 10 min.

Selective amplification. For the final selective PCR, each reaction contained 0.5 μl labelled *Eco*RI primer 5′-GACTGCGTACCAATTCN-3′, 4.5 μl *Mse*I primer 5′-GATGAGTCCTGAGTAAN-3′ (contains dNTP nucleotides), 5 μl diluted template DNA, 2 μl of 10× PCR buffer, 0.1 μl DNA polymerase (5 U/μl), and 7.9 μl distilled water with a total volume of 20 μl. The PCR amplification profile was the same as that used in the preamplification reactions.

Gel analysis. A 5% polyacrylamide gel (5% acrylamide:bis acrylamide 20:1; 7.5 M urea; 0.5× TBE buffer) was used to size fractionate the amplified DNA fragments. A BioRad sequencing gel system apparatus was used for gel analysis and 1× TBE buffer (100 mM Tris; 100 mM boric acid; and 2 mM EDTA) was used in both chambers as the electrophoresis buffer. The gel was run at 120 W for 2 h, then dried onto a sheet of Whatmann paper (No. 4), and placed into a cassette (Amersham, Australia) with X-ray film (Kodak Biomax MR). It was left to be exposed to the film for 1–2 days after which the film was developed. Polymorphic markers were then analysed and used in linkage analysis.

2.6. Virulence testing

The cultivars Harosoy 63 (*Rps*1a, *Rps*7) Harosoy 12 (*Rps*1a), Sanga (*Rps*1b), Harosoy 13 (*Rps*1b), Wells II (*Rps*1c), PI103091 (*Rps*1d), Kingwa (*Rps*1k), Harosoy 15 (*Rps*1k), PI86972-1 (*Rps*3a), L83-570 (*Rps*3a) L88-1479 (*Rps*3b) PRX146-36 (*Rps*3b) X571-291 (*Rps*3b) X572-373 (*Rps*3c), L85-2352 (*Rps*4), Harosoy 52 (*Rps*5), L85-3059 (*Rps*5), Altona (*Rps*6), and L89-1581 (*Rps*6) were used as differentials to determine the virulence profiles of the F_1 hybrids and the segregation within the F_2 population from the 7/16 cross and the 17/25 cross, Appropriate susceptible controls were used: Harosoy (l–7) (*rps*) and Ross (*rps*) for the 7/16 cross and Harosoy (*Rps*)7, Harosoy(l–7) (*rps*), and Ross (*rps*) for the 17/25 cross. For each cross, the parental isolates, F_1 hybrid, and 53 randomly chosen F_2 individuals were used in the virulence assays. The virulence assay performed was a simple hypocotyl inoculation test (Ryley et al., 1991) and scoring of the reactions was conducted according to Whisson et al. (1995). All virulence tests were repeated on at least two separate occasions.

2.7. Linkage analysis

A set of previously mapped markers from the existing linkage map of Whisson et al. (1995) was used as scaffold markers for the 7/16 cross to locate the new avirulence genes and any new molecular markers. Scaffold markers were spaced every 10–20 cM across the existing linkage groups. Bulked segregant analysis (Michelmore et al., 1991) was used for the 17/25 cross to identify markers that were linked to *Avr*1d and 3b. For each gene, two separate pools of DNA from F_2 progeny were made. The first pool contained DNA from five avirulent F_2 progeny and the second from five virulent F_2 progeny.

All RAPD and AFLP markers, including any new or unlinked markers and avirulence factors, were subjected to a χ^2 test to determine if segregation was significantly different from that expected for normal Mendelian segregation (3:1 or 1:2:1). All markers were then analysed by the computer program MAPMAKER version 3.0 (Whitehead Institute, Cambridge, MA; Lander et al., 1987) for two-point linkage analysis at a default setting of LOD 3.0 and maximum distance of 30 cM. Markers from all four crosses were ordered into integrated linkage groups using the JoinMap 1.7 (Stam, 1993) mapping program (Kosambi mapping function), which was completed at LOD scores of 3.0 (Linklod) and 1.0

(Maplod). A total of 386 markers were used to construct the final genetic linkage map. These included 211 pre-existing markers from the 7/1 cross and 7/25 cross, 165 new molecular markers (105 AFLP markers and 41 RAPD and 19 RFLP markers), and 10 avirulence markers. DrawMap 1.0 (van Ooijen, 1993) was used to graphically convert the output from JoinMap 1.7 into figures.

3. Results

3.1. Hybrid production and generation of F_2 populations

Three hundred single oospore cultures were isolated from the cross between race 7 and race 16 (7/16 cross). A screen of 100 of these cultures with RAPD primer OPD03 identified 36 F_1 hybrids, indicating an outcrossing frequency of 36%. A further eight RAPD primers confirmed the hybrid nature of these putative F_1 progeny. For the cross between race 17 and race 25 (17/25 cross), 300 single oospore cultures were screened using the RAPD primer OPQ04. One F_1 hybrid was identified and confirmed with an additional four RAPD primers, indicating an outcrossing frequency of 0.3%. A single F_1 hybrid from each cross was self-fertilised to generate two F_2 populations.

3.2. Genetics of virulence

The F_1 hybrid from the 17/25 cross and four F_1 hybrids from the 7/16 cross were tested for virulence on a differential set of soybean cultivars. All four F_1 hybrids from the 7/16 cross exhibited avirulence toward resistance genes *Rps*1b, 1k, 3c, 4, 5, and 6, and virulence towards resistance genes *Rps*1a, 1c, and 3a. The F_1 hybrid of the 17/25 cross was avirulent on *Rps*1a, 1d, 1k, 3a, 3b, 3c, 4, 5, and 6, and virulent on *Rps*1c. The parents, F_1 hybrid, and 53 randomly chosen F_2 individuals were screened against all *Rps* alleles. *Rps*1d and 3b for the 7/16 cross and *Rps*1b for the 17/25 cross were excluded for the F_2 tests, since the parents and the F_1 hybrids were considered homozygous avirulent and homozygous virulent, respectively. The F_2 population segregated in a 3:1 ratio with avirulence dominant for *Avr*1b, 1k, 4, 5, and 6 in the 7/16 cross, and for *Avr*1a, 1d, 3a, 3b, 4, 5, and 6 in the 17/25 cross, consistent with avirulence being controlled by a single, dominant allele for these genes (Table 4). For *Avr*3c in both crosses and *Avr*1k and *Avr*3b in the 17/25 cross, the F_2 segregation best fitted a 9:7 ratio with avirulence dominant, suggesting that avirulence was controlled by two dominant, independently segregating genes. However, the observed segregation ratio for *Avr*3b also fits a 3:1 ratio consistent with a dominant allele at a single locus. A 3:1 segregation ratio, with virulence dominant, was observed for *avr*1a (7/16 cross), *avr*1c (both crosses), and *avr*3a (7/16 cross), suggesting that the avirulence alleles at these loci were recessive. However, the segregation ratios for *Avr*1c (7/16 cross) and *Avr*3a (7/16 cross) also fitted a 3:13 segregation ratio, consistent with a dominant suppressor exerting epistatic control over a dominant avirulence gene. The parents, F_1 hybrid, and all F_2 individuals from both crosses were equally pathogenic on soybean cultivars containing no *Rps* genes.

3.3. Linkage analysis and map construction

From the original genetic linkage map of Whisson et al. (1995) based on the previously constructed 7/1 and 7/25 crosses, 74 RAPD markers that were also polymorphic in the new 7/16 cross were used to form a scaffold map spanning the major linkage groups. Nineteen new RFLP markers were also mapped using the previous 7/1 cross and the new 7/25 cross. The RFLP probes included genes of known and suspected functions cloned from various *Phytophthora* species (Table 3). However, none of these genes exhibited linkage to any of the *P. sojae* avirulence genes mapped in the 7/1, 7/16, or 7/25 crosses. Bulked segregant analysis (Michelmore et al., 1991), involving a further 380 RAPD primers and 64 AFLP primer combinations, identified markers in the 17/25 cross linked to *Avr*1d and 3b. Multi-point linkage analysis, performed using the JoinMap 1.7 mapping program, allowed integration of avirulence genes and molecular markers from all four crosses into a single detailed genetic linkage map (Fig. 1). Segregation data from the new 7/16 cross showed no linkage of *Avr*1a, 1c, 3a, and 5 to any of the additional molecular markers acquired, and these avirulence loci were mapped using linked markers from the previous 7/1 and 7/25 crosses of Whisson et al. (1995). However, *Avr*1c failed to show linkage to any DNA marker. Two new avirulence genes analysed using the 17/25 cross (*Avr*1d and 3b) and one using the 7/16 cross (*Avr*3c) were included in the map. For both the 7/16 and 17/25 crosses, cosegregation was observed between *Avr*4 and 6. *Avr*1b and 1k cosegregated in 52 of the 53 F_2 individuals in the 7/16 cross, and. *Avr*3a and 5 cosegregated for 51 of the 53 F_2 individuals in the 17/25 cross. A total of 386 markers (312 dominant and 74 codominant), consisting of 35 RFLP, 236 RAPD, and 105 AFLP markers, and 10 avirulence genes were used to construct the final map (Fig. 1), which covers a distance of 1640.4 cM. Based on the method of Postlethwait et al. (1994) and assuming 5.8 cM for each telomere, 13 chromosomes (Sansome and Brasier, 1974), and 23 gaps in the map at 30 cM per gap, the total genome size was estimated to be 2590 cM. Table 4 lists the map location of each avirulence gene and its distance in cM to the closest linked molecular marker in coupling phase.

The final map (Fig. 1) consisted of 21 major linkage groups containing five or more markers (groups 1–21) and 7 minor linkage groups (groups a–g) (Table 5).

Table 4
Data obtained for segregating avirulence genes from crosses 7/16 and 17/25 of *P. sojae*

Rps	Cross	P1	P2	F_1	F_2 ratio (A:V)	χ^2	Probability	Linkage group and position	Closest marker[a]
1a	7/16	V	A	V	19:34 (1:3)	3.33	0.07	1 (87.1 cM)	OPAS5.1+ (6.8 cM)
					(7:9)	1.10	0.30		
	17/25	A	V	A	37:16 (3:1)	0.76	0.38		
1b	7/16	A	V	A	42:11 (3:1)	0.51	0.48	2 (36.2 cM)	OPA20.3C (3.1 cM)
1c	7/16	A	V	V	8:45 (1:3)	2.77	0.10		
					(3:13)	0.46	0.50		
	17/25	A	V	V	19:34 (1:3)	3.33	0.07		
					(7:9)	1.10	0.30		
1d	17/25	V	A	A	36:17 (3:1)	1.42	0.23	b (0.0 cM)	OPD8.1+(15.1 cM)
1k	7/16	A	V	A	43:10 (3:1)	1.06	0.30	2 (37.1 cM)	OPA20.3C (2.2 cM)
	17/25	A	V	A	30:23 (3:1)	9.57	0.002		
					(9:7)	0.003	0.96		
3a	7/16	V	A	V	8:45 (1:3)	2.77	0.10	4 (24.6 cM)	OPC12.4+(14.4 cM)
					(3:13)	0.46	0.50		
	17/25	V	A	A	37:16 (3:1)	0.76	0.38		
3b	17/25	V	A	A	34:19 (3:1)	3.33	0.07	c (0.0 cM)	OPG11.1+21.6 cM)
					(9:7)	1.34	0.25		
3c	7/16	V	A	A	33:20 (3:1)	4.59	0.03	3 (0.0 cM)	OPM10.3C(1.3 cM)
					(9:7)	0.78	0.38		
	17/25	V	A	A	33:19 (3:1)	3.69	0.06		
					(9:7)	1.10	0.30		
4	7/16	V	A	A	43:10 (3:1)	1.06	0.30	3 (16.2 cM)	OPE7.1C(4.0 cM)
	17/25	V	A	A	36:16 (3:1)	0.92	0.34		
5	7/16	V	A	A	38:15 (3:1)	0.31	0.58	4 (20.0 cM)	OPC12.4+ (9.8 cM)
	17/25	V	A	A	39:14 (3:1)	0.06	0.81		
6	7/16	V	A	A	43:10 (3:1)	1.06	0.30	3 (16.2 cM)	OPE7.1C (4.0 cM)
	17/25	V	A	A	36:16 (3:1)	0.92	0.34		

[a] The distance is given in cM to the closest marker in coupling phase.

A number of original linkage groups published by Whisson et al. (1995) were split, as marker by marker analysis of the data revealed that some inconsistent linkages occurred close to the LOD score of 3.0 that was used for map construction. Separation of these linkage groups yielded a more accurate and robust map. New linkage groups, 1, 2, 13, and 20, were formed from linkage group 1, and new groups 5 and 10 from the original linkage group 5, both previously described by Whisson et al. (1995). A series of closely associated markers from the original group 1 were integrated into linkage group 3 through the inclusion of new codominant RFLP markers. RAPD markers used in this investigation from the previous crosses were reliably mapped in the 7/16 cross for the majority of linkage groups. However, some rearrangement of markers within linkage groups 1, 2, and 3 was observed. This was probably due to greater accuracy lent to linkage analysis through the incorporation of data from the new 7/16 cross and additional codominant RFLP markers. Minor linkage groups a, b, and e from Whisson et al. (1995) were removed from analysis here, as these groups contained RAPD markers that could not be reliably amplified in the 7/16 cross. The minor groups a–g described herein are not intended for comparison with the minor groups described by Whisson et al. (1995). Thirty-two RAPD markers out of 236 and 22 AFLP markers out of 105 from the 7/16 cross that were included in the map did not follow normal Mendelian segregation ratios. Five markers were clustered in linkage group 1 between OPZ18.C (32.7 cM) and OPP19.3C (58.3 cM) and another five markers in linkage group 7 between OPD 16.3C (28.3 cM) and OPQ6.2+ (44.3 cM). In linkage group 15, 12 markers bound by the markers E2MCTT8− (34.9 cM) and E3MCTT3− (119.7 cM) did not follow normal Mendelian segregation. The remaining markers exhibiting aberrant segregation were scattered throughout the other linkage groups.

4. Discussion

This report gives a detailed account of the genetic analysis of avirulence in *P. sojae* and an integrated genetic linkage map has been constructed, based on a total of 212 F_2 progeny from four different crosses, 53 from each of the two new crosses (7/16 and 17/25 crosses) and 53 from each of the 7/1 and 7/25 crosses described previously (Whisson et al., 1995). In this study, four previously uncharacterised avirulence genes (*Avr*1c, 1d, 3b, and 3c) have been genetically analysed. A total of 11 avirulence genes from *P. sojae* have now been

characterised genetically and 10 of these have been placed on the genetic linkage map that is presented in this report (Fig. 1). We were unable to find any molecular markers linked to *Avr*1c. Hence, three avirulence genes, *Avr*1c, 2, and 7, are still to be positioned on the linkage map. The current genetic linkage map consists of 386 markers comprising 376 molecular markers (302 dominant and 74 codominant markers) and 10 avirulence genes. The markers were assigned to 21 major linkage groups (groups 1–21) and 7 minor linkage groups (a–g). The complete linkage map covers a distance of 1640.4 cM, compared to the 830.5 cM coverage of the original map (Whisson et al., 1995). Based on the estimated genome size of 2590 cM, the linkage map presented herein represents 63% of the estimated genome size.

Several genes cloned from various *Phytophthora* species were used as probes in this investigation. Some of these genes, such as ubiquitin (Pieterse et al., 1991) and putative elicitors (Kamoun et al., 1997; Sacks et al., 1995; Table 3), have been shown to exhibit increased/decreased expression during infection. Other genes placed on the genetic linkage map, such as LPV18 (Marshall et al., 2001) and BIPBH5 (Lehnen and Hardham, unpublished), appear to have a role in the *Phytophthora* life cycle. Homologues for all these genes were found in the *P. sojae* genome but none showed linkage to any of the *P. sojae* avirulence genes.

4.1. Dominant avirulence allele at a single locus (Avr1b, 1d, 1k, 3b, 3c, 4, 5, and 6)

Segregation ratios fitting a typical 3:1 Mendelian pattern were observed for avirulence genes *Avr*1b, 1k, 4, and 6 in the 7/16 cross and *Avr*1d, 3b, 3c, 4, 5, and 6 in the 17/25 cross, consistent with avirulence being a dominant allele at a single locus (Table 4). The above results for *Avr*1b, 1d, 4, and 6 agree with those of Gijzen et al. (1996), Tyler et al. (1995), and Whisson et al. (1995). Segregation ratios for *Avr*1k, 3b, and 3c showed a better fit to a 9:7 ratio in some crosses. Genetic data for *Avr*1k were difficult to interpret in some crosses. The F_1 and F_2 progeny of the 7/16 cross gave phenotypes and segregation data that typified a dominant avirulence allele at a single locus. The F_1 from the 17/25 cross gave mixed reactions in which some inoculated hypocotyls collapsed, suggesting a susceptible reaction, whereas other hypocotyls gave a strong defence response in which the infection was contained, despite extensive tissue collapse typifying a hypersensitive response. We interpreted this F_1 response and segregation data from the 17/25 cross as avirulent against *Rps*1k, supported by data from the F_2 population, which segregated closest to a 9:7 ratio. This result could be explained by dominant avirulence, with an overestimate of the frequency of available alleles due to the severity of the hypersensitive response in some F2 genetic backgrounds, but is also consistent with avirulence controlled by two dominant, independently segregating genes at separate loci in the 17/25 genetic background. Tyler et al. (1995) also observed a 9:7 segregation ratio for *Avr*1k. A 9:7 segregation ratio was observed for *Avr*3b in the 17/25 cross, in contrast to Tyler et al. (1995) who reported a segregation ratio of 15:1 in another cross, consistent with two independent dominant genes controlling avirulence, although this result could be misleading due to the small (24 progeny) F_2 population size analysed in that study.

4.2. Segregation ratios inconsistent with a dominant avirulence allele at a single locus

Our investigations provided two other examples of segregation inconsistent with a single dominant allele determining avirulence. *Avr*1c gave a 1:3 segregation ratio for both the 7/16 and 17/25 crosses, with virulence being the dominant phenotype. However, *Avr*1a gave opposing reactions in each cross and was dominant with a 3:1 ratio in the 17/25 cross, but was recessive with a segregation ratio fitting 1:3 in the 7/16 cross. It is thus evident that the genetic background of the different *P. sojae* races used in the genetic crosses can clearly affect segregation ratios. For example, mutations at different loci could have produced different *Avr*1a phenotypes in the two crosses. Additional genes, such as a suppressor, could modulate a single dominant avirulence gene, e.g., for *Avr*1k (17/25 cross), *Avr*3b (17/25 cross), and *Avr*3c (both crosses). Other researchers have also observed segregation ratios that are consistent with multiple genes controlling avirulence within the F_2 populations of their crosses (Tyler et al., 1995; Whisson et al., 1994, 1995). Alternatively, *Avr* gene silencing may be an important factor in some genetic backgrounds (van West et al., 1999). Multiple locus models for avirulence were not confirmed by the genetic mapping relative to DNA markers in our study and linkage could only be found for the cross specific *Avr* loci listed in Table 4. Understanding these aberrant segregation ratios may be resolved by the construction of additional crosses, increasing progeny size of existing populations and analysis with additional molecular markers, and the cloning and molecular analysis of the dominant *Avr* genes.

4.3. Linkage of avirulence genes

*Avr*4 and 6 cosegregated in both new crosses and in the previous 7/25 cross of Whisson et al. (1995) and were located in group 3 with the nearest marker, OPE7.1C, 4.0 cM away (Table 4; Fig. 1). *Avr*3c also mapped to linkage group 3 and was located at one end (Fig. 1). *Avr*1b and 1k cosegregated in the 7/25 cross of Whisson et al. (1995), but were very closely linked in the 7/16

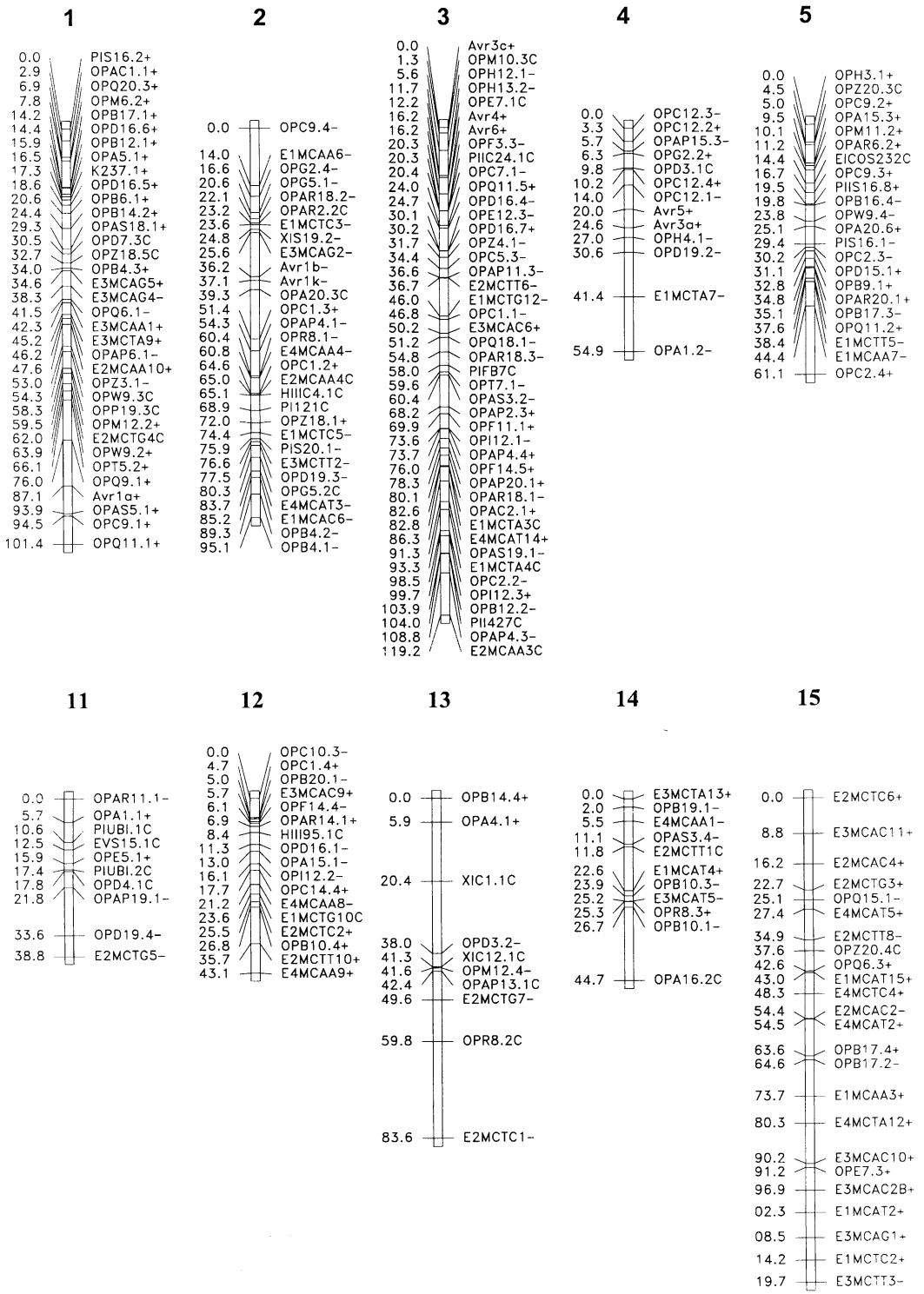

Fig. 1. Integrated genetic linkage map of *P. sojae*. The lettered codes represent three types of molecular markers. RAPD markers start with the letters 'OP' and the operon primer code while the numbers behind the decimal point start at the largest polymorphic band. RFLP markers are a lettered code and AFLP markers begin with the letter 'E' (*Eco*RI primer) and contain a four letter code starting with 'M' (*Mse*I primer). Distance in centimorgans (cM) is on the left of the linkage groups. The phase of the linkage of the markers has been given either a '+' or '−' notation by the right of the marker; any codominant marker is indicated by 'C' to the right of the marker. Ten avirulence (*Avr*) loci have been positioned on the major linkage groups 1, 2, 3, and 4 and the minor linkage groups, b and c.

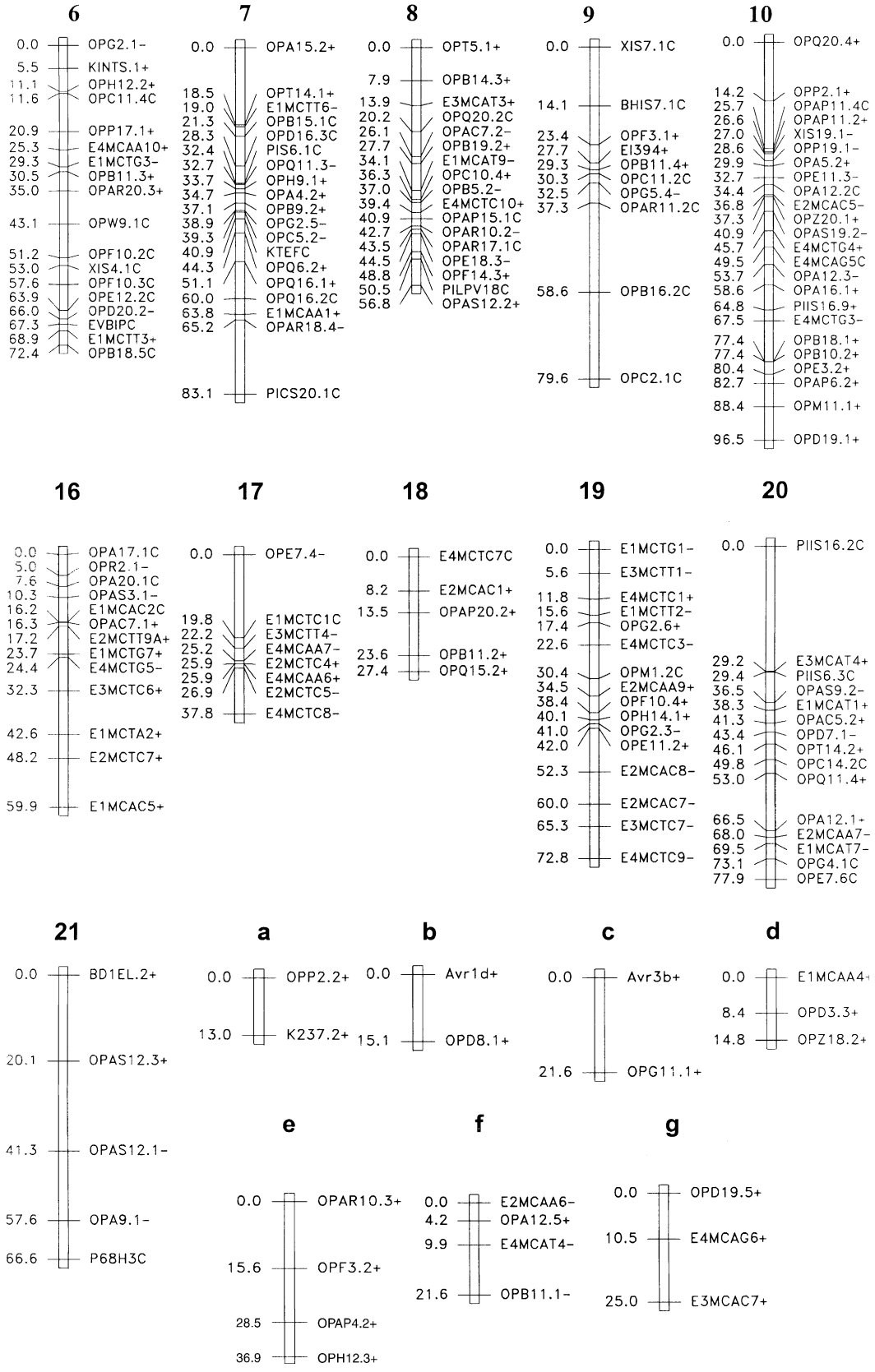

Fig. 1. (*continued*)

Table 5
Total genome coverage, total number of markers, length of individual linkage groups, number of markers per group, average spacing between markers, and largest genetic interval for each linkage group for *P. sojae*

Linkage group	Length (cM)	No. of markers	Average spacing (cM)	Largest interval (cM)
1	101.4	35	3.0	11.1
2	95.1	30	3.3	14.0
3	119.2	44	2.8	10.4
4	54.9	13	4.6	13.5
5	61.1	22	2.9	16.7
6	72.4	18	4.3	9.3
7	83.1	19	4.6	18.5
8	56.8	17	3.6	7.9
9	79.6	10	8.8	21.5
10	96.5	24	4.2	14.2
11	38.8	10	4.3	11.8
12	43.1	17	2.7	8.9
13	83.6	10	9.3	23.8
14	44.7	11	4.5	18.0
15	119.7	24	5.2	9.9
16	59.9	13	5.0	11.7
17	37.8	8	5.4	19.8
18	27.4	5	6.9	10.1
19	72.8	16	4.9	10.3
20	77.9	15	5.6	29.2
21	66.6	5	16.7	21.2
a	13.0	2	13.0	13.0
b	15.1	2	15.1	15.1
c	21.6	2	21.6	21.6
d	14.8	3	7.4	8.4
e	36.9	4	12.3	15.6
f	21.6	4	7.2	11.7
g	25.0	3	12.5	14.5
Total	1640.4	386	4.6	—

cross (Fig. 1) and were assigned to linkage group 2. A single F_2 individual from the 7/16 cross was virulent toward one *Rps* gene but avirulent on the other demonstrated that *Avr*1b and *Avr*1k were distinct but closely linked genes. Such an example was observed for *Avr*1b and 1k in the 7/16 cross. In contrast, when the number of F_2 individuals was increased to 99 in the 7/25 cross, *Avr*4 and 6 were still observed to cosegregate absolutely (S.C. Whisson, unpublished). In both examples cited above, certain races isolated from the field express avirulence toward one *Rps* gene but not the other (Ward, 1990). For example, seven races (races 2, 10, 11, 17, 23, 24, and 26) possessed *Avr*1k but not *Avr*1b (Schmitthenner et al., 1994); five races (races 6, 13, 23, 38, and 51) possessed *Avr* but not *Avr* (Henry and Kirkpatrick, 1995; Ryley et al., 1998; Schmitthenner et al., 1994; Ward, 1990). Linkage was also observed between *Avr*3a and 5 (linkage group 4 in Fig. 1), and between *Avr*3c and *Avr*4/6 (linkage group 3). However, no other avirulence genes exhibited linkage. Genomic regions defined by *Avr4–Avr6*, *Avr*1b–1k, and *Avr*3a–*Avr*5 may represent clusters of genes that are associated with virulence and perhaps other sets of related functions.

4.4. Outcrossing between different Phytophthora sojae races

Outcrossing rates between North American and Australian isolates were consistently lower than those among North American isolates. In this investigation, an outcrossing rate of 0.3% between a North American race 17 and an Australian race 25 was observed. Whisson et al. (1994, 1995) also observed relatively low rates of 2.8 and 2.0%, respectively, for their 7/1 and 7/25 crosses, representing isolates from Australia and North America. In contrast, the two North American isolates in the 7/16 cross produced an outcrossing rate of 36%. Tyler et al. (1995) observed outcrossing rates of 12.5% (race 2 and race 19) and 3.5% (race 2 and race 7) among North American isolates. It is unclear whether there is a genetic basis for this difference in outcrossing rates among isolates of different geographic origins or if different experimental conditions are the sole cause of these differences. However, it is of interest to note that the North American *P. sojae* populations have a much higher level of genetic diversity than the Australian *P. sojae* population (Drenth et al., 1996; Förster et al., 1994). It is possible that there are few, if any, genetic barriers to prevent outcrossing among different races in North America. Therefore, it is of considerable practical importance to study the possible occurrence and the level of outcrossing among races in field situations in North America, as outcrossing may be a mechanism that leads to the appearance of new races as hypothesised by Förster et al. (1994).

In this paper, we present genetic data on 11 of the 13 avirulence genes that are currently known in *P. sojae*. This has enabled us to construct a detailed integrated genetic linkage map of the oomycete *P. sojae* that incorporates 10 of these genes. New markers and avirulence genes can be continually integrated into this map to increase its coverage of the *P. sojae* genome. Any molecular marker closely linked to an avirulence gene may be used as a starting point for chromosome walking and map-based cloning of avirulence genes. Cloning and characterisation of these genes is a prerequisite for deducing the molecular mechanism controlling virulence/avirulence during host–pathogen interactions.

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