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Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulence genes

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Abstract Two genetically different isolates of the homothallic Oomycete, *Phytophthora sojae*, were demonstrated to outcross and form hybrid oospores after co-culturing in vitro. Random amplified polymorphic DNA (RAPD) markers revealed ten hybrids among 354 oospores analysed. One F_1 hybrid was allowed to self fertilise and produce an F_2 population of 247 individuals. Among 53 F_2 individuals, selected at random, 18 polymorphic RAPD markers were observed to segregate at near 3:1 Mendelian ratios, consistent with segregation for dominant alleles at single loci. Segregation of virulence against soybean resistance genes *Rps1a*, *3a*, and *5* revealed that the avirulence genes *Avr1a*, *3a* and *5* were dominant to virulence. Avirulence against these three resistance genes appeared to be conditioned by one locus for *Avr1a* and two independent, complementary dominant loci for both *Avr3a* and *Avr5*. Segregation of virulence against *Rps6* was in the ratio of 1:2:1 (avirulent:mixed reaction:virulent), suggesting a semi-dominant allele at a single locus. Two avirulence genes and one RAPD marker formed one linkage group, in the order *Avr3a*, OPH4-1, *Avr5*, each separated by approximately 5 cM. Our results confirm that outcrossing occurred between the parental isolates, and that sexual recombination under field conditions may play an important role in generating and maintaining genetic diversity in populations of *P. sojae*.

Key words *Phytophthora megasperma* f. sp. *glycinea* · Avirulence · Gene-for-gene · RAPD

Introduction

The genus *Phytophthora* (class Oomycetes) includes many very destructive plant pathogens. Some *Phytophthora* species have a very broad host range, as exemplified by *P. cinnamomi* which infects almost a 1000 different species of dicotyledonous plants (Zentmeyer 1980), whereas the host range of other *Phytophthora* species is restricted to one or few species of plants. *P. sojae* (syn. *Phytophthora megasperma* f. sp. *glycinea*) is one such host-specific species and causes root and stem rot of soybean (*Glycine max*). *P. sojae* is homothallic (self fertile), producing sexually derived oospores in single culture with the oogonia predominantly fertilised by paragynous antheridia (Hansen and Maxwell 1991). As in other Oomycetes the vegetative growth stage is diploid (Shaw 1983).

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. However, this approach to disease management 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 25 races of *P. sojae* are known to exist worldwide (Ward 1990). In Australia, five races, 1, 4, 13, 15 and an undescribed race, are known to occur (Ryley et al. 1991; M. J. Ryley, personal communication). The genetics of resistance to *P. sojae* has been extensively studied in soybean (Ward 1990). Twelve dominant *Rps* genes have been identified at seven loci. Five alleles have been identified at the *Rps1* locus, three at the *Rps3* locus, whereas only one allele has been identified at each of the *Rps2*, *Rps4*, *Rps5*, *Rps6*, and *Rps7* loci, respectively.

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*. Layton and Kuhn (1988) obtained forced heterokaryons between drug and fungicide resistant mutants but were unable to establish the occurrence of outcrossing. Recently, Bhat and Schmitthenner (1993) isolated sexually derived hybrids by complementation of drug and fungicide resistant mutants of *P. sojae*. Both groups of researchers noted loss of specific virulence as a problem when inducing mutations towards drug and fungicide resistance for use as markers to select for outcrossing. Hence, a paucity

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of easily scorable markers, able to confirm the genetic identity of putative hybrids in the event of outcrossing, has hindered genetic studies of virulence/avirulence in *P. sojae*.

Single dominant alleles at independent loci, characteristic of each parental isolate, are preferable as genetic markers for the identification of hybrids among selfed progeny of parental isolates. A class of such markers, random amplified polymorphic DNA (RAPD), were used recently by Francis and St. Clair (1993) to detect hybrids amongst selfed progeny of different isolates of *Pythium ultimum*. These workers demonstrated unambiguously the occurrence of outcrossing in this homothallic Oomycete and subsequently were able to produce an F_2 generation in which different molecular markers were shown to segregate in a Mendelian fashion. Because *P. sojae* resembles *P. ultimum* in being a homothallic Oomycete, it was considered possible that *P. sojae* may also have the potential to outcross. This would enable the formation of an F_2 generation and allow studies of genetics of virulence/avirulence in *P. sojae* against resistance genes in soybean. Furthermore, molecular markers could be used to map regions of the *P. sojae* genome conditioning virulence/avirulence.

The aims of the current investigation were threefold: (1) to demonstrate the occurrence of outcrossing among different isolates of *P. sojae*; (2) to obtain an F_2 population originating from a cross between different isolates and (3) to study the inheritance of both virulence/avirulence and molecular markers in the F_2 population. Such information would provide an invaluable basis for further molecular characterisation of the soybean/*P. sojae* interaction. It forms a starting point for further genetic studies into the processes involved in generating and maintaining genetic diversity in *P. sojae* and other homothallic *Phytophthora* species.

Materials and methods

P. sojae isolates. The two parental isolates of *P. sojae* used in this investigation were UQ244 and US7 from the culture collection of the Cooperative Research Centre for Tropical Plant Pathology, the University of Queensland, Australia. These two isolates are readily distinguished by DNA markers (Whisson et al. 1992; S. C. Whisson, unpublished data), and exhibit different virulence patterns on soybean differentials. UQ244 is an Australian isolate belonging to race 1 of *P. sojae* and is only able to overcome the resistance gene *Rps7*. Isolate US7, belonging to race 7, was isolated from soybean in the United States and is able to overcome resistance genes *Rps1a*, *2*, *3a*, *3c*, *4*, *5*, *6*, and *7*.

Crosses, isolation and analysis of F_1 progeny. Mycelial plugs from both parental isolates were placed on opposite sides of ten plates of clarified V8 agar and the resultant colonies allowed to grow together. Oospores were harvested 40 days after inoculation and were isolated by maceration of agar strips in a domestic blender at full speed. The oospore suspension was sieved through nylon mesh (75 µm), plated on 1.5% water agar plates (diameter 90 mm) and allowed to germinate in the dark at 25 °C. Germinating oospores observed through a binocular microscope (25× magnification) were picked individually, transferred to V8 agar plates, and incubated in the dark at 25 °C. Mycelia scraped from the surface of agar plate cultures were used for small scale DNA extraction by the method of Edwards et al. (1991). Individuals were identified with RAPD markers as described by Whisson et al. (1993) using the decanucleotide primer OPH13 (Operon Technologies, Alameda, Calif., USA) which generates one polymorphic RAPD marker from UQ244 and two from US7. Putative hybrids were further screened using five additional RAPD primers (OPH3, 4, 9, 12, and OPR2). Each hybrid culture was then initiated from a single hyphal tip or single zoospore (generated by the method of Eye et al. 1978) and verified again using the same set of RAPD primers to confirm their hybrid identity.

Generation of an F_2 population. One selected F_1 hybrid was allowed to self fertilise and form oospores. Oospores were harvested from a 40-day-old culture, allowed to germinate as described above, and germinating oospores were transferred to clarified V8 agar plates. Mycelia from these cultures were used to inoculate liquid-clarified V8 media to grow mycelia for large-scale DNA extraction.

DNA extraction and RAPD analysis of F_2 individuals. DNA was extracted from mycelia of the parents, F_1, and F_2 individuals using the method of Panabiéres et al. (1989) with the modifications described by Whisson et al. (1992). RAPD analysis was conducted as described by Whisson et al. (1993) using 14 different decanucleotide primers (see Table 1).

Virulence/avirulence testing. Soybean cultivars Harosoy 63 (*Rps1a*), PI86972-1 (*Rps3a*), Harosoy 5272 (*Rps5*), Altona (*Rps6*), and Harosoy (*Rps7*) were used as differentials to evaluate the virulence spectrum of parental, F_1, and F_2 individuals of *P. sojae*. Virulence was tested using the hypocotyl inoculation technique described by Ryley et al. (1991) in which, for each individual tested, at least ten plants of each soybean cultivar were inoculated. The seedlings were assessed 4 days post-inoculation, and were rated as dead (hypocotyl collapsed), resistant (no lesion) or intermediate (restricted non-killing lesion), Cultures were classified as avirulent if at least nine out of ten plants were resistant. Cultures were classified as virulent if at least nine out of ten plants were dead. Cultures which killed between 10 and 90% of the plants were classed as a mixed response.

Linkage analysis. RAPD and virulence data were subjected to linkage analysis using the computer program 'Mapmaker' version 3.0 (Whitehead Institute, Cambridge, Mass., USA) to provide map distances in centi Morgan (cM) and LOD scores for linked markers. LOD scores are defined as the ^{10}log of the ratio of the probability of linkage between loci at a given recombination fraction divided by the probability of independent segregation among these loci (Chang et al. 1988).

Results

Identification of F_1 hybrids

From the plates containing both the parental isolates US7 and UQ244, cultures derived from 354 oospores were screened with primer OPH13. RAPD patterns identical to parental type UQ244 were detected in 332 cultures and RAPD patterns identical to parental type US7 in 12 cultures. Ten cultures gave RAPD fragments characteristic of both parents, and their putative hybrid identity was verified using five additional RAPD primers (OPH3, 4, 9, 12, and OPR2). Examples of amplified DNA fragments obtained from one of these putative hybrids are shown in Fig. 1. For all primers used, polymorphic fragments characteristic of each of the parental isolates were all present in the putative hybrids. To verify that these putative hybrids were not heterokaryons or mixed cultures, hyphal tip or zoo-

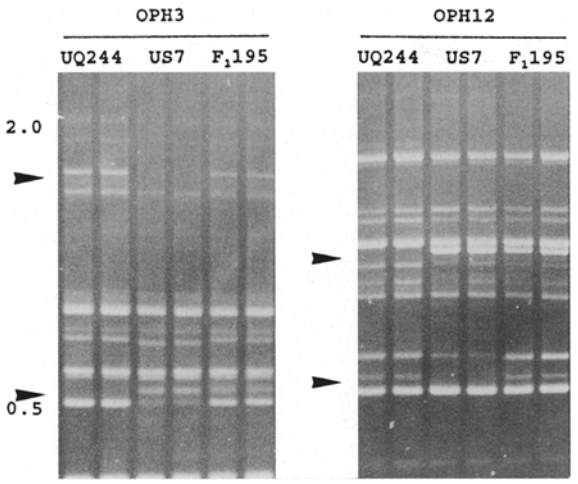

Fig. 1 Verification of a putative hybrid ($F_1$195) using RAPD markers, after co-culturing *P. sojae* isolates UQ244 and US7. DNA from each isolate was amplified in duplicate using primers OPH3 and OPH12. Polymorphic fragments present in either of the parents and in the putative hybrid are indicated by *arrows*. DNA fragment size calibrations from λ phage/*Hin*dIII are indicated as base pairs × 10^3

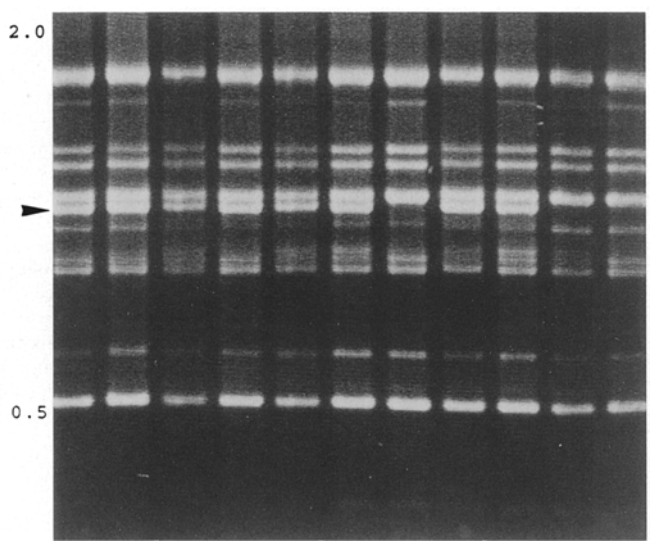

Fig. 2 Segregation of RAPD marker OPH12-1 (indicated by *arrow* at left) among a subset of F_2 individuals of *P. sojae*. RAPD patterns for both parental isolates and the F_1 hybrid used to produce this F2 population are shown in Fig. 1. DNA fragment size calibrations from λ phage/*Hin*dIII are indicated as base pairs × 10^3

spore derived cultures were prepared and retested using the same set of RAPD markers. In each case the initially obtained hybrid RAPD patterns could be confirmed.

Virulence/avirulence tests of F_1 hybrids

All ten F_1 hybrids exhibited avirulence towards resistance genes *Rsp1a*, *3a*, and *5*. A mixed reaction (disease symptoms in a pot of at least ten seedlings included 30–90% fully resistant plants, between 10–60% of the plants exhibiting large restricted non-killing lesions, and 0–30% fully susceptible plants) was observed on resistance gene *Rsp6* for all ten F_1 hybrids. All F_1 hybrids were virulent towards resistance gene *Rps7* and their aggressiveness was intermediate between the slightly more aggressive parental isolate UQ244 compared to parental isolate US7.

Generation of an F_2 population and segregation of RAPD markers

One hybrid culture ($F_1$195), transferred from a single zoospore, was self-fertilised to yield an F_2 population of 247 individuals. Fifty-three of these were selected at random and used for further analysis of molecular markers and virulence characters. Fourteen of the eighteen RAPD markers generated from 14 primers, segregated at or near the expected Mendelian F_2 ratio of 3:1 for a dominant allele at a single locus (Table 1). An example of a segregating RAPD fragment is shown in Fig. 2.

Virulence tests of F_2 individuals

Avirulence towards resitance gene *Rps1a* segregated as a dominant allele at a single locus with an avirulence/virulence ratio of 38:15 (Table 2). Both the *Avr3a* and *Avr5* genes exhibited an identical avirulence/virulence segregation ratio of 30:23, giving a poor fit for segregation of a dominant allele at a single locus (Table 2). However, for both *Avr3a* and *Avr5*, this ratio gave a near perfect fit to the 9:7 segregation ratio expected for two complementary dominant alleles at two independent loci. Segregation of avirulence/virulence towards *Rps6* was not as well defined, with some F_2 individuals exhibiting complete avirulence, others exhibiting complete virulence, and the remainder exhibiting a mixed reaction. The ratio of avirulence:mixed reaction:virulence was 12:23:18, which is consistent with the 1:2:1 segregation ratio of a semi-dominant allele at a single locus, with the heterozygote giving a mixed reaction. All F_2 individuals exhibited virulence towards resistance gene *Rps7*. None of the 53 randomly selected F_2 individuals were any less, or any more, aggressive than either of the two parental strains.

Linkage analysis

Analysis of all 22 segregating genetic characters in the F_2 population showed linkage between avirulence genes *Avr3a* and *Avr5* (10.27 cM, LOD 9.25) *Avr3a* and RAPD marker OPH4-1 (5.03 cM, LOD 3.25), and *Avr5* and OPH4-1 (5.03 cM, LOD 3.25). Hence, the most likely order is *Avr3a* – OPH4-1 – *Avr5*. The other 17 RAPD markers and two virulence characters segregated independently from each other under the minimum LOD and distance parameters of 3.00 and 50 cM, respectively.

Table 1 Segregation data for RAPD markers in the F_2 progeny of *P. sojae* obtained after outcrossing isolates US7 (Race 7) and UQ244 (Race 1)

Primer	Sequence	Marker	US7	UQ244	F_1	F_2 Ratio[a] (+:−)	χ^2	Probability[b]
A5	AGGGGTCTTG	OPA5-1	−	+	+	38:15*	0.31	0.70>*P*>0.50
		OPA5-2	−	+	+	36:17*	1.42	0.25>*P*>0.20
A15	TTCCGAACCC	OPA15-1	+	−	+	34:17*	1.89	0.20>*P*>0.10
		OPA15-2	−	+	+	46:5	6.28	0.02>*P*>0.01
H3	AGACGTCCAC	OPH3-1	−	+	+	36:17*	1.42	0.25>*P*>0.20
H4	GGAAGTCGCC	OPH4-1	+	−	+	42:11*	0.51	0.50>*P*>0.30
H9	TGTAGCTGGG	OPH9-1	−	+	+	38:15*	1.02	0.50>*P*>0.30
H12	ACGCGCATGT	OPH12-1	+	−	+	37:16*	0.76	0.50>*P*>0.30
H13	GACGCCACAC	OPH13-1	+	−	+	45:8*	2.77	0.10>*P*>0.05
		OPH13-2	+	−	+	37:16*	0.76	0.50>*P*>0.30
H14	ACCAGGTTGG	OPH14-1	−	+	+	40:13*	0.01	0.95>*P*>0.90
I12	AGAGGGCACA	OPI12-1	+	−	+	38:15*	0.31	0.70>*P*>0.50
M6	CTGGGCAACT	OPM6-1	−	+	+	46:7	3.93	0.05>*P*>0.025
Q9	GGCTAACCGA	OPQ9-1	−	+	+	48:5	6.85	0.01>*P*>0.005
R2	CACAGCTGCC	OPR2-1	+	−	+	44:9*	1.82	0.20>*P*>0.10
R8	CCCGTTGCCT	OPR8-1	+	−	+	28:23	10.99	0.001>*P*>0.0005
W9	GTGACCGAGT	OPW9-1	−	+	+	39:14*	0.06	0.90>*P*>0.80
		OPW9-3	+	−	+	37:16*	0,76	0.50>*P*>0.30

* Asterisk indicates significant 3:1 segregation ratio (P>0.05)
[a] Based on complete dominance of RAPD markers and expected segregation ratio of 3:1
[b] Degrees of freedom = 1

Table 2 Data obtained for segregating avirulence genes from the F_2 progeny of *P. sojae* (Race 1 × Race 7)

Resistance gene	US7	UQ244	F_1	F_2 ratio (A:V)	Expected ratio	χ^2	Probability
Rps1a	V	A	A	38:15	3:1	0.31*	0.70>*P*>0.50
Rps3a	V	A	A	30:23	3:1	9.57	0.005>*P*>0.001
					9:7	0.003*	0.975>*P*>0.95
Rps5	V	A	A	30:23	3:1	9.57	0.005>*P*>0.001
					9:7	0.003*	0.975>*P*>0.95
Rps6	V	A	M	12:23M[a]:18	1:2:1	2.28*	0.50>*P*>0.30
Rps7	V	V	V	0:53	0:1	0.00*	1.00

* Asterisk indicates significant segregation ratio (P>0.05)
[a] M denotes a mixed reaction

Discussion

In this investigation we have used molecular markers to provide unambiguous evidence for outcrossing in *P. sojae*. We have demonstrated that drug and fungicide resistant mutants are not necessary to force outcrossing when molecular markers, such as RAPDs, are used to detect hybrids. A similar approach was used to detect hybrids between different isolates of *P. ultimum* (Francis and St. Clair 1993), another homothallic Oomycete.

Confirmation of the genetic identity of the F_1 hybrids was obtained by allowing one F_1 individual to self fertilise and produce an F_2 population, which was analysed using both RAPD markers and virulence characters. Segregation ratios of most of the RAPD markers gave a good fit to the Mendelian ratio expected for a dominant allele at a single locus. Nineteen out of the total of twenty-two genetic markers segregated independently from each other, indicating that the cross between UQ244 and US7 had produced a true hybrid and not a heterokaryon or a mixed culture.

Segregation ratios of four RAPD markers (OPA15-2, OPQ9-1, OPM6-1, and OPR8-1; Table 1) gave a poor fit to a Mendelian ratio of 3:1 in the F_2 population. This may be due to genetic abnormalities, or due to the RAPD primer amplifying equal size DNA fragments from two independent polymorphic loci leading to a 15:1 segregation ratio, which fits the first two RAPD markers noted above with χ^2 values of 1.10 and 0.92, respectively.

Virulence tests of F_1 hybrids and F_2 individuals showed that, against three resistance genes (*Rps1a*, *3a*, and *5*) in the soybean differentials, avirulence in the pathogen was dominant to virulence. This supports the conclusions of

Layton and Kuhn (1988) working with interracial heterokaryons, and is in accordance with the gene-for-gene theory of Flor (1956). However, segregation ratios for avirulence/virulence toward resistance genes *Rps3a* and *Rps5* gave a poor fit to the 3:1 Mendelian ratio expected for a single dominant gene but were consistent with the expected 9:7 ratio for two independently segregating, complementary dominant genes. The observed segregation and linkage data suggest that both *Avr3a* and *Avr5* may require the same, independently segregating, dominant effector gene to obtain avirulence. Control of virulence/avirulence by two genes has been found in a number of different pathogens (Michelmore et al. 1984; Barrett 1985). Conclusive demonstration of complementation may be provided by test crosses between virulent isolates to produce progeny which are avirulent. Ilott et al. (1989) hypothesised that a system in which two complementary dominant genes conferred avirulence could operate if the pathogen component determining avirulence was the end product of a multi-step biosynthetic pathway. Mutations in any of several genes may result in virulence towards the same resistance gene and avirulence would then map to different loci in different pathogen isolates.

Deviation from simple Mendelian ratios may also be caused by modifying genes, epistatic effects, or chromosomal mismatching events. However, such events are unlikely in this region as the linked RAPD marker OPH4–1 segregated in the ratio of 42:11 (3:1), providing further support for a system of two complementary dominant avirulence genes, both of which are necessary to obtain avirulence towards resistance genes *Rps3a* or *Rps5*.

The results observed with *Avr6* appear to be consistent with a semi-dominant avirulence allele at a single locus in which the presence of both dominant alleles leads to a successful defence reaction in the soybean plants carrying the *Rps6* gene whereas in the presence of only one dominant allele a mixed reaction is observed on these plants. Further clarification is needed by generating F_3 families from F_2 individuals that exhibited a mixed reaction on *Rps6*. An alternative is that *Avr6* may be under polygenic control and should be analysed as a quantitative trait. The mixed reaciton observed for the F_1 hybrid and some F_2 individuals towards *Rps6* is a characteristic of a large proportion of Australian isolates of *P. sojae* following hypocotyl inoculation (Rose et al. 1982; Heritage et al. 1993). To assess polygenic control of avirulence, a quantitative root inoculation method such as described by Irwin and Langdon (1982) is required for further clarification.

The inheritance of avirulence loci other than those described in this paper will be studied when cultivars with additional resistance genes become available in Australia. Race 1 and Race 7 isolates of *P. sojae* also differ in virulence patterns towards the resistance genes *Rps2*, *Rps3c*, and *Rps4*. The *Rps2* gene in cultivar Davis will also be investigated using the quantitative root-inoculation method of Irwin and Langdon (1982).

Only one linkage group was observed among the 22 markers analysed. This linkage group contained two avirulence characters and one RAPD marker. The remaining unlinked markers are presumably scattered throughout the 10–13 chromosomes (Sansome and Brasier 1974) that constitute the *P. sojae* genome.

Limited information is available regarding the impact of sexual reproduction and occasional outcrossing on establishing and maintaining genetic diversity in populations of *Phytophthora* species. In the case of the heterothallic Oomycete, *P. infestans*, two mating types are required for sexual reproduction. Where populations of only one mating type exist, and sexual reproduction is not possible, the number of different races tends to remain low. However, in the presence of both mating types and the occurrence of sexual reproduction, the number and complexity of races tends to increase dramatically (Tooley et al. 1986; Drenth et al. 1994).

In Australia, new races of *P. sojae* appear to have evolved from a near-monomorphic genetic background (Whisson et al. 1992; S. C. Whisson, unpublished data). New races, such as race 13 (virulent on *Rps6* and *7*) and race 15 (virulent on *Rps3a* and *7*), are assumed to have arisen by mutation from race 1 (virulent on *Rps7*). Our results suggest the possibility that new races may arise by outcrosses among existing races in field situations. One such race was isolated recently in southern New South Wales, Australia (M. J. Ryley, personal communication). This race, virulent on *Rps1a*, *1c*, *3a*, and *7*, was obtained from a diseased soybean plant in an area where race 4 (virulent on *Rps1a*, *1c* and *7*) and race 15 (*Rps3a* and *7*) had been reported previously. Our results show that, under optimal conditions in vitro with both isolates present in close proximity, the outcrossing rate is less than 5%. The frequency at which outcrossing occurs in the field is unknown and is presumably far less than that observed in vitro.

Our results substantiate the genetic basis of virulence/avirulence in *P. sojae* and provide genetic evidence on the pathogen side towards a gene-for-gene interaction between soybean and *P. sojae*. However, the results also suggest that, in some instances, more than one gene may be responsible for avirulence towards one resistance gene. The identification of linkage between RAPD markers and avirulence genes will facilitate targeting and cloning of those genes by bulked segregant analysis (Michelmore et al. 1991). Cloned and characterised avirulence genes will aid in the understanding of plant/pathogen interactions and can be used as tools (De Wit 1992) to confer more durable resistance against *P. sojae* in soybean.

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