The Virulence of Interracial Heterokaryons of *Phytophthora megasperma* f. sp. *glycinea*

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ABSTRACT

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In soybean, race-specific resistance to *Phytophthora megasperma* f. sp. *glycinea* is inherited as single dominant genes. However, the inheritance of virulence (cultivar-specific pathogenicity) in the fungal pathogen is unknown. Flor's gene-for-gene hypothesis predicts that avirulence will be phenotypically dominant to virulence. To determine if avirulence is dominant to virulence, *P. m. glycinea* heterokaryons containing race 1 and race 3 nuclei were tested on soybean cultivars Harasoy and Harasoy 63 for virulence phenotype. Twenty-two out of 26 heterokaryons had a race 1 phenotype, suggesting that race 1 (avirulent on Harasoy 63) is phenotypically dominant to race 3 (virulent on Harasoy 63). In addition, from two heterokaryons with race 1 phenotypes, zoospores with race 3 phenotypes were obtained. These results fit Flor's gene-for-gene hypothesis and strengthen the utility of the *P. m. glycinea*-soybean disease interaction as a model system for studying race-specific resistance in soybean.

Phytophthora root rot of soybean (*Glycine max* (L.) Merr.), caused by *Phytophthora megasperma* Drechs f. sp. *glycinea* Kuan and Erwin (10), is commonly used as a model system for studying race-specific disease resistance in plants. This system is used because both the biochemistry of phytoalexin production (2,7,9,13) and the genetics of race-specific disease resistance (1,19) in the soybean have been well studied. In soybean, disease resistance is conferred by single dominant genes (1,18) and is consistent with Flor's gene-for-gene hypothesis (5). In Flor's hypothesis avirulence is phenotypically dominant to virulence in the pathogen (6). Although it is predicted that virulence in *P. m. glycinea* will follow Flor's gene-for-gene hypothesis (5), the inheritance of virulence in *P. m. glycinea* has not been determined.

The genetics of virulence in *P. m. glycinea* is not known because crosses between races of *P. m. glycinea* are difficult. Sexual crosses cannot be readily made because *P. m. glycinea* is homothallic. However, fungal isolates can be crossed by heterokaryon formation (more than one distinct nuclear type in a common cytoplasm) (3). Heterokaryons are experimentally made by hyphal fusion (3,16) or protoplast fusion (14), or they may arise naturally from mutations or mitotic recombination in diploid heterozygous nuclei (3,4,19,20). Because heterokaryons contain more than one nucleus, they can be used to determine the dominance relationship between nuclei of different phenotypes (3,16). If the *P. m. glycinea*-soybean interaction follows Flor's gene-for-gene hypothesis, then race 1 should be phenotypically dominant to race 3 in a heterokaryon containing both race 1 and race 3 nuclei.

We have made *P. m. glycinea* heterokaryons between race 1 and race 3 drug-resistant mutants by protoplast fusion (12). Because zoospores are uninucleate, zoospore production was used to recover the parental phenotypes from the heterokaryons. With these heterokaryons we can study the relative dominance of virulence and avirulence. In this paper we show that these heterokaryons are pathogenic and that race 1 is phenotypically dominant to race 3.

MATERIALS AND METHODS

Isolates. The following *P. m. glycinea* drug-resistant mutants were obtained after mutagenesis (12): 1 Ftp (5-58 Ftp 4), race 1, fluorotryptophan resistant; 1 Mex (5-58 Mex 6), race 1, metalaxyl resistant; 3 Pfp (76-4-4 Pfp 2), race 3, fluorophenylalanine/fluorotryptophan resistant; and 3 Mex (76-4-4 Mex 2), race 3, metalaxyl resistant. Cultures were grown on V8 agar (20% clarified V8, 1.5% agar) (15) or in V8 broth (20% clarified V8) supplemented with 20 μg/ml of metalaxyl or 10 μg/ml of fluorotryptophan, as previously described (12). Heterokaryons were grown in media with 10 μg/ml of fluorotryptophan and 20 μg/ml of metalaxyl.

Chemicals. Metalaxyl was a gift of Ciba-Geigy Corp. Nantamycin was a gift of Alcon Laboratories. 6-fluorotryptophan, antibiotics, and other chemicals were from Sigma Chemical Company.

Heterokaryon formation. Protoplasts of a metalaxyl-resistant mutant and a fluorotryptophan-resistant mutant were fused to make intra- and interracial heterokaryons. The methods for protoplast production and protoplast fusion were previously described (12). Protoplasts were made by digesting rapidly growing mycelium with 10% Driselase in 1 M mannitol for 5–6 hr. Protoplasts of each parent (1×10^6–1×10^7) were mixed and pelleted by centrifugation at 1,000 *g* in a tabletop centrifuge. Pelleted protoplasts were incubated with 400 μl of fusion buffer (40% polyethylene glycol, 50 mM $CaCl_2$, 0.1 M Tris, pH 7) for 15 min at room temperature to induce protoplast fusion. The protoplasts (1×10^3–1×10^5 per petri plate) were mixed in 5 ml of 1 M mannitol and 5 ml of regeneration agar (40% V8 broth, 1 M mannitol, 1.5% agar). The cell walls were allowed to regenerate overnight, and the protoplasts were overlaid with selective agar (1.5% V8 agar, 40 μg/ml of metalaxyl, 20 μg/ml of 6-fluorotryptophan). Colonies that grew through the agar within 1–3 wk were transferred to V8 agar plates with 20 μg/ml of metalaxyl and 10 μg/ml of 6-fluorotryptophan to maintain selective pressure for resistance to both drugs. After one transfer on V8 agar, heterokaryons were transferred to potato dextrose agar (PDA) slants with 20 μg/ml of metalaxyl and 10 μg/ml of 6-fluorotryptophan. Heterokaryons were obtained at a rate of 3×10^{-4} protoplasts. This rate was 100-fold higher than the spontaneous mutation rate of the parent isolates to double drug resistance. Heterokaryons were maintained in the PDA slants, and fresh transfers were made from slants to the appropriate growth medium for each experiment.

Four *P. m. glycinea* × *P. m. glycinea* heterokaryon crosses were made: A = 1 Ftp × 3 Mex; B = 3 Pfp × 1 Mex; C = 1 Ftp × 1 Mex, D = 3 Pfp × 3 Mex. Individual heterokaryons were named by cross letter, followed by a heterokaryon number.

Resolution of heterokaryons by zoospore production. Heterokaryons were resolved into parental components by zoospore production (12). Zoospores of heterokaryon B13 and parental isolate 1Ftp4 were stained with mithramycin (12), and nuclei were counted by fluorescence microscopy. For each

heterokaryon and the parental isolates, 100–200 zoospores per plate were placed by pipette onto two or three plates each of V8 agar with no drugs, V8 agar with 10 μg/ml of 6-fluorotryptophan, V8 agar with 20 μg/ml of metalaxyl, and V8 agar with 10 μg/ml of 6-fluorotryptophan and 20 μg/ml of metalaxyl. Colonies arising from single germinated zoospores were counted 3 and 5 days later. The number of colonies was totaled from the same number (two or three) of plates of each selective medium. The numbers of fluorotryptophan and metalaxyl-resistant zoospores for each heterokaryon from crosses A (1 Ftp × 3 Mex) and B (3 Pfp × 1 Mex) were determined within 2 wk of pathogenicity testing.

The percentage of successful colony formation on each type of drug was calculated relative to the percentage of colony formation on plates with no drugs. The percentage of colony formation on plates with no drugs was set at 100%.

Inoculations. Parental isolates were grown in V8 broth in 10-cm petri plates with appropriate drugs for 7–10 days. Heterokaryons were grown in V8 broth plus metalaxyl and fluorotryptophan for 10–14 days. The mycelial mat was cut into approximately 1-mm squares and used as inoculum. Plants were inoculated by the hypocotyl method (1). Inoculum was inserted into the hypocotyls of 7-day-old seedlings of Harosoy or Harosoy 63 (7–10 seedlings per test) and Altona and Wells II (3–5 seedlings per test). The inoculation sites were covered with petrolatum jelly, or plants were incubated in a humidity chamber (100% relative humidity) to prevent desiccation at the inoculation site. Plants were scored 4 days later as resistant (no apparent infection) or susceptible (dead or large necrotic lesions). Most heterokaryons were tested twice, 2–4 wk apart, and the total number of susceptible plants and plants inoculated from both tests recorded.

Soybean cultivars Harosoy or Williams with the *P. m. glycinea*-resistance genotype rps and Harosoy 63 with Rps1[b] were used to distinguish between *P. m. glycinea* race 1 and other races (1). Two other cultivars, Altona with Rps[6] and Wells II with Rps1[c], were used to determine if the heterokaryons were races other than races 1 and 3. Altona could be used to identify races 4 and 5, and Wells II could be used to identify races 5, 7, and 9 (1). Soybean differential cultivars Harosoy, Harosoy 63, Wells II, and Altona were obtained from J. R. Wilcox. Williams was later substituted for Harosoy and was obtained from Ag Alumni Seed Foundation.

Each fungal isolate was reisolated from susceptible plants onto V8 agar plates with 20 μg/ml of chloramphenicol, 50 μg/ml of streptomycin, 50 μg/ml of benomyl, and 50 μg/ml of nantamycin and onto V8 agar plates with 10 μg/ml of fluorotryptophan and 20 μg/ml of metalaxyl plus the above antibiotics and benomyl. Infected hypocotyls were cut 1 cm above and below the lesion, and the excised hypocotyl piece was surface-sterilized in 10% chlorine bleach for 20–30 sec. A flamed razor blade was used to slice the excised hypocotyl piece into 2- to 3-mm sections, and a flamed forceps was used to transfer individual sections to the reisolation agar. The reisolation plates were incubated at 25 C. Colonies that grew onto the reisolation plates were tested for fluorotryptophan and metalaxyl resistance.

TABLE 1. Growth of parental isolates and heterokaryons on selective media

Isolate	Increase in colony diameter (mm)[a]			
	Nd	Mex	Ftp	FtpMex
1 Ftp	26 ± 1	0 ± 0	15 ± 1	0 ± 0
3 Mex	26 ± 1	25 ± 1	0 ± 0	0 ± 0
A2–A7[b]	24 ± 1	19 ± 1	7 ± 1	6 ± 1

[a] Increase in colony diameter after 5 days on each type of V8 agar plate. Nd = no drugs; Mex = 20 μg/ml of metalaxyl; Ftp = 10 μg/ml of 6-fluorotryptophan; FtpMex = 10 μg/ml of 6-fluorotryptophan, 20 μg/ml of metalaxyl.

[b] Average growth of six heterokaryons, A2–A7.

RESULTS

Eighty-three putative heterokaryons able to grow in the presence of both fluorotryptophan and metalaxyl were obtained from seven fusion experiments. The average growth of six heterokaryons from cross A was less than the parental isolates on V8 agar plates without drugs and V8 agar plates with only 10 μg/ml of fluorotryptophan or 20 μg/ml of metalaxyl (Table 1). These values are representative of all 83 putative heterokaryons. Of the 83 heterokaryons, 28 were not further characterized after the initial identification either because they were from cross C, in which sufficient heterokaryons were tested, or they were slow growing and insufficient inoculum was obtained, or they could not be maintained in PDA slants.

Virulence of heterokaryons. Fifty-five heterokaryons and the parental isolates were tested for virulence on soybean cultivars (Tables 2–6). Six of the 55 tested were nonpathogenic or infected fewer than 50% of the inoculated Harosoy plants (susceptible to both races 1 and 3). One had an unstable pathogenicity phenotype; it initially had a race 1 phenotype but had a race 3 phenotype on retesting. These seven heterokaryons are not included in this study. Some of the heterokaryons (e.g., A2, B3, C4, and D2) were less aggressive than the parental isolates (Tables 2–6). From cross A (1 Ftp × 3 Mex), 13 heterokaryons had a race 1 phenotype (virulent on Harosoy but not Harosoy 63), and one heterokaryon had a race 3 phenotype (virulent on Harosoy and Harosoy 63) (Table 3). From cross B (3 Pfp × 1 Mex), nine heterokaryons had a race 1 phenotype and two had a race 3 phenotype. None of the heterokaryons from crosses A and B had a virulence phenotype other than race 1 or race 3.

Heterokaryons from crosses C (1 Ftp × 1 Mex) and D (3 Pfp × 3 Mex) were tested for pathogenicity to see if heterokaryon formation alone would change virulence phenotype. All 13 of the heterokaryons from cross C (1 Ftp × 1 Mex) had a race 1 phenotype, and all 10 of the heterokaryons from the cross D (3 Pfp × 3 Mex) had a race 3 phenotype (Table 1). None of the heterokaryons from crosses C and D had a race phenotype other

TABLE 2. The virulence, reisolation frequency from infected plants, and colony formation from zoospores on selective media for drug-resistant mutants of *Phytophthora megasperma* f. sp. *glycinea*

Mutant	Infected plants (I/T)[a]				Reisolation[a]			Colony formation[d]				
	H	H63	A	W	Cultivar	Plants[b]	FtpMex[c]	Nd	Ftp	Mex	FtpMex	%
1 Ftp	66/69	1/69	0/15	0/11	H	6/19	0/6	1,112	1,118	0	0	55
1 Mex	63/68	1/71	0/10	0/12	H	6/17	0/6	1,220	0	1,213	0	53
3 Pfp	60/61	59/63	0/12	0/12	H	4/18	0/4	611	610	0	0	60
					H63	4/18	0/4					
3 Mex	54/54	48/53	0/15	0/14	H	3/19	0/3	795	0	841	0	41
					H63	7/19	0/7					

[a] Infected plants (I/T) = number of infected plants/total number of inoculated plants in 6–8 experiments. H = Harosoy; H63 = Harosoy 63; A = Altona; W = Wells II.

[b] Plants = number of plants from which *P. m. glycinea* could be reisolated/number of plants from which reisolation was attempted.

[c] FtpMex = number of plants containing FtpMex-resistant colonies/number of plants from which *P. m. glycinea* was reisolated.

[d] Colony formation = number of colonies formed from zoospores plated on each type of V8 agar plate. Nd = no drugs; Ftp = 10 μg/ml of 6-fluorotryptophan; Mex = 20 μg/ml of metalaxyl; FtpMex = 10 μg/ml of 6-fluorotryptophan, 20 μg/ml of metalaxyl. % = percentage of zoospores that formed colonies on plates with no drugs compared with the number of zoospores plated.

TABLE 3. The virulence and reisolation of *Phytopthora megasperma* f. sp. *glycinea* heterokaryons from the cross 1 Ftp × 3 Mex

Heterokaryon	Infected plants (I/T)[a]				Reisolation			
	H	H63	A	W	Race	Cultivar	Plants[b]	FtpMex[c]
A2	15/19	0/18	0/10	0/12	1	H	6/9	5/6
A3	15/16	0/16	0/5	0/6	1	H	6/7	5/6
A4	19/20	0/18	0/9	0/11	1	H	9/10	7/9
A5	17/17	2/16	0/8	0/8	1	H	8/9	4/8
A6	11/17	1/16	0/7	0/8	1	H	6/10	5/6
A7	9/17	0/18	0/9	0/10	1	H	5/9	5/5
A10	14/18	0/18	0/9	0/10	1	H	4/10	4/4
A11	15/17	16/18	0/7	0/9	3	H	5/10	5/5
						H63	3/8	1/3
A21	15/15	0/16	0/5	0/5	1	H	2/7	2/2
A22	12/18	0/17	0/8	0/6	1	H	2/5	2/2
A23	19/19	2/20	0/10	0/8	1	H	6/9	6/6
A24	17/18	0/18	0/10	0/7	1	H	3/8	3/3
A31	12/17	1/16	0/5	0/4	1	H	0/7	0/0
A32	15/19	3/17	0/8	0/8	1	H	0/9	0/0

[a] Infected plants (I/T) = number of infected plants/total number of inoculated plants in 2-3 experiments. H = Harosoy; H63 = Harosoy 63; A = Altona; W = Wells II.
[b] Plants = number of plants from which *P. m. glycinea* could be reisolated/number of plants from which reisolation was attempted.
[c] FtpMex = number of plants containing FtpMex-resistant colonies/number of plants from which *P. m. glycinea* was reisolated.

TABLE 4. The virulence and reisolation frequency of *Phytophthora megasperma* f. sp. *glycinea* heterokaryons from the cross 3 Pfp × 1 Mex

Heterokaryon	Infected plants (I/T)[a]				Reisolation			
	H	H63	A	W	Race	Cultivar	Plants[b]	FtpMex[c]
B2	20/21	0/20	0/10	0/7	1	H	5/20	5/5
B3	13/16	15/18	0/3	0/4	3	H	3/13	2/3
						H63	2/15	2/2
B4	18/19	12/17	0/4	0/4	3	H	1/18	1/1
						H63	3/12	2/3
B8	18/18	0/18	0/9	0/6	1	H	5/11	4/5
B11	9/9	0/8	0/5	0/3	1	H	0/9	0/0
B12	13/17	1/21	0/10	0/8	1	H	4/8	2/4
B13	18/18	0/16	0/10	0/7	1	H	5/8	5/5
B21	15/17	4/16	2/4	0/5	1	H	3/15	0/3
B22	19/19	3/18	0/10	0/9	1	H	2/19	0/2
B23	16/16	0/18	0/9	0/10	1	H	4/16	1/4
B24	17/18	0/17	0/9	0/9	1	H	3/17	3/3

[a] Infected plants (I/T) = number of infected plants/total number of inoculated plants in 2-3 experiments. H = Harosoy; H63 = Harosoy 63; A = Altona; W = Wells II.
[b] Plants = number of plants from which *P. m. glycinea* could be reisolated/number of plants from which reisolation was attempted.
[c] FtpMex = number of plants containing FtpMex-resistant colonies/number of plants from which *P. m. glycinea* was reisolated.

TABLE 5. The virulence and reisolation frequency of *Phytopthora megasperma* f. sp. *glycinea* heterokaryons from the cross 1 Ftp × 1 Mex

Heterokaryon	Infected plants (I/T)[a]			Reisolation		
	H	H63	Race	Cultivar	Plants[b]	FtpMex[c]
C2	11/17	0/17	1	H	3/7	2/3
C3	15/18	0/16	1	H	2/11	2/2
C4	14/18	0/18	1	H	0/7	0/0
C6	14/19	0/15	1	H	2/10	1/2
C7	19/20	1/19	1	H	6/9	5/6
C8	17/19	0/19	1	H	1/8	1/1
C9	9/9	0/8	1	H	2/9	2/2
C10	13/14	0/15	1	H	1/7	1/1
C11	21/21	0/16	1	H	2/11	2/2
C14	10/10	0/11	1	H	0/10	0/0
C21	14/16	0/10	1	H	3/8	3/3
C22	17/20	0/15	1	H	1/10	1/1
C30	8/8	0/8	1	H	1/8	1/1

[a] Infected plants (I/T) = number of infected plants/total number of inoculated plants in 2–3 experiments. H = Harosoy; H63 = Harosoy 63.
[b] Plants = number of plants from which *P. m. glycinea* could be reisolated/number of plants from which reisolation was attempted.
[c] FtpMex = number of plants containing FtpMex-resistant colonies/number of plants from which *P. m. glycinea* was reisolated.

than the parental isolate.

Reisolation of heterokaryons from infected plants. We attempted to reisolate the fungus from the infected plants to determine if the isolates remained fluorotryptophan and metalaxyl resistant after growth in the plants in the absence of fluorotryptophan and metalaxyl. Reisolation was difficult because of bacterial contamination. In no experiments could fungus be reisolated from all of the infected plants, and in some experiments fungus could not be reisolated from any of the plants. From plants inoculated with race 1 × race 3 heterokaryons (crosses A and B), fungus resistant to fluorotryptophan and metalaxyl was obtained for 19 of the 25 heterokaryons (Tables 3 and 4). No fluorotryptophan- and metalaxyl-resistant colonies were recovered from plants inoculated with the parental isolates (Table 2). From plants inoculated with the race 1 × race 1 or race 3 × race 3 heterokaryons (crosses C and D), fungus resistant to fluorotryptophan and metalaxyl was recovered for 20 of 23 heterokaryons (Tables 5 and 6).

Zoospore segregation of heterokaryons. Zoospores of *P. m. glycinea* are predominantly uninucleate. We tested heterokaryon B13 and a parental isolate for multinucleate zoospores. Of 177 zoospores produced by heterokaryon B13, 174 zoospores had a single nucleus and three zoospores had two nuclei. Of 162

TABLE 6. The virulence and reisolation frequency of *Phytophthora megasperma* f. sp. *glycinea* heterokaryons from the cross 3 Pfp × 3 Mex

Heterokaryon	Infected Plants (I/T)[a]				Reisolation			
	H	H63	A	W	Race	Cultivar	Plants[b]	FtpMex[c]
D1	14/17	12/20	0/10	0/7	3	H	3/9	3/3
						H63	2/9	2/2
D2	12/16	7/20	0/9	0/8	3	H	3/5	3/3
						H63	3/6	2/3
D10	14/17	16/18	0/10	0/8	3	H	5/14	5/5
						H63	4/16	4/4
D16	10/15	15/19	0/9	0/7	3	H	3/10	3/3
						H63	3/15	3/3
D17	12/18	13/19	0/7	2/8	3	H	3/12	3/3
						H63	4/13	4/4
D21	16/16	19/19	0/7	0/9	3	H	3/16	3/3
						H63	4/19	4/4
D22	18/18	19/19	2/7	0/9	3	H	1/9	1/1
						H63	2/10	0/2
D23	26/29	24/28	0/12	1/11	3	H	1/26	0/1
						H63	1/24	0/1
D24	20/20	13/17	0/10	0/10	3	H	5/20	3/5
						H63	1/13	1/1
D25	14/19	16/19	0/5	0/3	3	H	3/8	3/3
						H63	2/9	2/2

[a] Infected plants (I/T) = number of infected plants/total number of inoculated plants in 2–3 experiments. H = Harosoy; H63 = Harosoy 63; A = Altona; W = Wells II.
[b] Plants = number of plants from which *P. m. glycinea* could be reisolated/number of plants from which reisolation was attempted.
[c] FtpMex = number of plants containing FtpMex-resistant colonies/number of plants from which *P. m. glycinea* was reisolated.

TABLE 7. Colony formation from zoospores of *Phytopthora megasperma* f. sp. *glycinea* race 1 × race 3 heterokaryons, which contain bothy metalaxyl-resistant zoospores and fluorotryptophan-resistant zoospores

Heterokaryon	Race	Colony formation[a]				
		Nd	Ftp	Mex	FtpMex	%
A4	1	116	87	18	0	20
A22	1	37	38	4	1	11
A31	1	95	99	22	0	22
B13	1	922	208	662	0	58
B22	1	189	67	139	1	31
B24	1	126	25	112	3	24

[a] Colony formation = number of colonies formed from zoospores plated on each type of V8 agar plate. Nd = no drugs; Ftp = 10 μg/ml of 6-fluorotryptophan; Mex = 20 μg/ml of metalaxy; FtpMex = 10 μg/ml of 6-fluorotryoptophan, 20 μg/ml of metalaxyl. % = percentage of zoospores that formed colonies on plates with no drugs compared with the number of zoospores plated.

TABLE 8. Colony formation from zoospores of *Phytophthora megasperma* f. sp. *glycinea* race 1 × race 3 heterokaryons, which have a large proportion of zoospores resistant to both metalaxyl and fluorotryptophan or a race 3 phenotype

Heterokaryon	Zoospore germination[a]					
	Race	Nd	Ftp	Mex	FtpMex	%
A11	3	293	81	259	61	81%
B2	1	335	183	287	242	62%
B3	3	152	101	145	93	39%
B12	1	201	121	162	102	35%
B4	3	168	88	3	0	43%

[a] Nd = no drugs; Ftp = 10 μg/ml of 6-fluorotryptophan; Mex = 20 μg/ml of metalaxyl; FtpMex = 10 μg/ml of 6-fluorotryptophan, 20 μg/ml of metalaxyl. % = percentage of zoospores that formed colonies on plates with no drugs compared with the number of zoospores plated.

zoospores produced by parental isolate 1Ftp4, 159 zoospores had a single nucleus and three zoospores had two nuclei. Because the majority of zoospores are uninucleate, colonies established from single zoospores from the heterokaryons may reflect the types of nuclei present in the heterokaryotic mycelium.

Forty-four heterokaryons were tested for zoospore segregation, and 23 of these were from crosses A and B. Of these 23, four (A6, A7, B8, and B23) produced only zoospores of one parental phenotype and no zoospores that were resistant to both fluorotryptophan and metalaxyl. Two (A6 and B8) produced only zoospores with the same drug-resistance phenotype as the race 3 parent, although the heterokaryons had a race 1 phenotype (Tables 3 and 4). Nine heterokaryons produced zoospores of both parental phenotypes, with the great majority being of one parental phenotype. Six heterokaryons produced more balanced numbers of both parental phenotypes (Table 7). Four produced significant numbers of zoospores able to grow in the presence of both metalaxyl and fluorotryptophan (Table 8), thus representing a new nonparental phenotype (Table 1). Two of these heterokaryons, B2 and B12, had a race 1 phenotype, and two, A11 and B3, had a race 3 phenotype. The only other heterokaryon with a race 3 phenotype, B4, produced predominantly fluorotryptophan-resistant zoospores, the drug-resistant phenotype of the race 3 parent. However, only half of the B4 zoospores formed colonies in the presence of either fluorotryptophan or metalaxyl (Table 8). For each experiment, zoospores from the parental isolates were tested for drug resistance. The zoospores from parental isolates formed colonies only on plates with no drugs and plates supplemented with the drugs for which they were resistant (Table 2). Occasionally, mutant zoospores formed colonies at a higher frequency on media supplemented with drugs than on media without drugs.

If race 1 is phenotypically dominant to race 3, a race 3 phenotype may be recovered in the zoospores from a heterokaryon containing both race 1 and race 3 nuclei. The heterokaryons B13 and B22 from cross B (3 Pfp × 1 Mex) both contained an equal number of fluorotryptophan-resistant zoospores and metalaxyl-resistant zoospores and few or no zoospores resistant to both drugs (Table 7). Therefore, they were good candidates for the recovery of the race 3 fluorotryptophan phenotype. B13 and B22 were resolved into metalaxyl-resistant single-zoospore colonies and fluorotryptophan-resistant single-zoospore colonies by zoospore production, and the colonies were tested for virulence. In B13, 36 colonies had the race 1 parental phenotype (race 1 and metalaxyl resistance) and 31 had the race 3 parental phenotype (race 3 and fluorotryptophan resistance). Two colonies had a nonparental phenotype (race 3 and fluorotryptophan and metalaxyl resistance). In B22, 29 colonies had the race 1 parental phenotype, and 33 colonies had the race 3 parental phenotype. No colonies had a nonparental phenotype.

DISCUSSION

P. m. glycinea heterokaryons were obtained once before between a race 3 methionine requiring auxotroph and a race 3 (nonpathogenic) purine requiring auxotroph (11). Although the heterokaryons were pathogenic, they could not be used to study the relative dominance of virulence because both parents had a race 3 phenotype. The heterokaryons in this study are the first *Phytophthora* heterokaryons that could be used to determine whether virulence is dominant or recessive. Three lines of evidence suggest that race 1 was phenotypically dominant to race 3 in race 1 × race 3 heterokaryons. First, 22 of 25 heterokaryons had a race 1 phenotype. Second, 20 of 21 heterokaryons that did not produce significant numbers of zoospores resistant to both metalaxyl and fluorotryptophan had a race 1 phenotype. These heterokaryons exhibited a race 1 phenotype when they contained both race 1 and race 3 nuclei. Third, the heterokaryons B13 and B22 had a race 1 phenotype and produced an approximately equal number of fluorotryptophan-resistant zoospores and metalaxyl-resistant zoospores. When the fluorotryptophan-resistant zoospores were tested for pathogenicity, they had a race 3 phenotype (the same as the fluorotryptophan parent). Therefore, race 3 zoospores were obtained from a heterokaryon with a race 1 phenotype.

Three heterokaryons had a race 3 phenotype. Two of the three heterokaryons produced a large number of zoospores resistant to both fluorotryptophan and metalaxyl. Because zoospores are uninucleate, the presence of both drug-resistance markers in a single zoospore indicates that karyogamy occurred in these heterokaryons. When karyogamy occurs, the tetraploid nucleus often loses chromosomes during mitosis until the nucleus returns to the diploid state (3,15). In these two heterokaryons, race 1 avirulence genes may have been lost, allowing the race 3 phenotype to be expressed. Alternatively, the race 3 parent may have mutated to fluorotryptophan resistance in B3 and to metalaxyl resistance in A11, which could explain the high numbers of double drug-resistant zoospores. Mutation, however, cannot explain the absence of zoospores of one parental phenotype from heterokaryons A6, A7, B8, and B23, because they produced no double drug-resistant zoospores. We assume that both parental nuclei are present in these heterokaryons but only one parental type leads to viable zoospores. Heterokaryon B4 had a race 3 phenotype and produced zoospores predominantly of the same phenotype as the race 3 parent and no zoospores resistant to both drugs. B4 may also have undergone karyogamy because only 50% or less of the zoospores formed colonies on plates with fluorotryptophan or metalaxyl when compared with colony formation on no drug plates. In this case, the new nonparental phenotype of the zoospores could be the lack of drug resistance rather than double drug resistance. The race 1 avirulence gene may also have been lost by recombination, leading to the race 3 phenotype of the heterokaryon.

In this study, races other than race 1 and race 3 were not observed in 25 race 1 × race 3 heterokaryons when they were tested for virulence on two other differential cultivars. Other races would not be expected in these heterokaryons because race 1 and race 3 may differ by only one avirulence gene, and avirulence is dominant to virulence. However, if complex races carrying several different virulence genes were fused, the resulting heterokaryons might have a different phenotype than either parent. The pathogenicity phenotype of the heterokaryons would result from the sum of the dominant avirulence genes from each of the parental isolates. Also, different races could be found in the sexual progeny from heterokaryons heterozygous at avirulence loci.

In *Phytophthora*, heterozygosity at the the virulence locus could occur in two ways. First, the mycelium could be heterokaryotic and carry different nuclei. Therefore, the zoospores produced by the heterokaryon would have different phenotypes. Changes in race phenotype of zoospores from that of the initial isolate have been reported several times (8,17). These results could be explained if these isolates were heterokaryotic. Second, as a diploid, *Phytophthora* can be heterozygous at a single locus within a single nucleus. After selfing in the sexual cycle, the recessive phenotype would be found in the progeny.

Heterokaryosis might be a means for *P. m. glycinea* to generate genetic variability in the sexual and asexual progeny and might explain how new races arise. New races might arise from the sexual progeny of the heterokaryons if the parental isolates were heterozygous at the virulence loci. New virulence phenotypes would be found in the sexual progeny after selfing. The mutants used in this study were homokaryotic for virulence because they were started from single zoospores and new virulence phenotypes were never found in the zoospore progeny. To determine if they were homozygous at the virulence loci, single oospore progeny should be tested for new race phenotypes. This is currently under investigation in our laboratory. New virulence phenotypes could also be found in the asexual progeny of the heterokaryons if karyogamy or a mutation in an avirulence gene occurred. Because karyogamy may have occurred in several of the heterokaryons, single zoospore progeny from these heterokaryons could be tested for new race phenotypes.

In soybean, resistance to *P. m. glycinea* is inherited as single dominant alleles as predicted by Flor's gene-for-gene hypothesis for resistance in the host. For the pathogen, Flor's gene-for-gene hypothesis predicts that avirulence is dominant to virulence. Therefore, race 1 (avirulent on Harosoy 63) is expected to be dominant to race 3 (virulent on Harosoy 63). In this study, *P. m. glycinea* race 1 was phenotypically dominant to race 3 when a mixture of nuclei were present in the same mycelium. These data support Flor's gene-for-gene hypothesis. Additional genetic studies are needed to show that virulence is inherited as single recessive genes. We are currently examining oospores from one of the heterokaryons to look for sexual recombination. If segregation of avirulence occurs in the sexual progeny, we can determine the number of genes involved in virulence in *P. m. glycinea* race 1 and race 3.

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