Genetic Analysis of Virulence in the Rice Blast Fungus *Magnaporthe grisea*

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ABSTRACT

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Crosses between rice isolates of *Magnaporthe grisea* were made to determine the genetic control of virulence to rice. A cross between a hermaphroditic rice isolate, Guy11, and a laboratory strain 2539, yielded more than 50% viable ascospores. Random spore and tetrad analysis showed that two loci, Pos1 (pathogenicity on *Oryza sativa*) and Pos2, were involved in conditioning virulence to rice lines 51583 and Sha-tiao-tsao. A buff mutation was epistatic to virulence. Fertility and ascospore viability were low in crosses between field rice isolates. In a cross between two rice isolates, Guy11 and CH104-3, only 10% of the ascospores was viable, but all progeny were virulent to 51583 and Sha-tiao-tsao. From this cross, we identified two different loci, Pos3 and Pos4, controlling virulence on rice lines K59 and Kinandang Patong, respectively. Fertility and virulence were maintained in progeny recovered from F_2 and F_3 generations. Joint segregation analysis showed an excess of parental types with respect to virulence. This might reflect genetic linkage of virulence genes or segregation of other genetic factors epistatic to virulence.

Additional keywords: host-pathogen interaction, *Pyricularia oryzae*.

The blast fungus *Magnaporthe grisea* (Hebert) Barr comb nov. (anamorph *Pyricularia oryzae* Cavara) (2) shows high variability with respect to host range and cultivar specificity. Asuyama (1) and Ou (20) reported more than 50 gramineous hosts and some dicotyledonous species that are parasitized by the blast fungus. Many pathogenic races have been found on cultivated rice (*Oryza sativa* L.) in different rice-growing regions of the world. Ou and others (8,21,22) have reported an unusually high degree of pathogenic instability within a single race, whereas other workers (4,14,15) consider each race relatively stable. Although the level of pathogenic stability within a single race is still unresolved, it is generally agreed that the blast fungus populations evolve rapidly on resistant cultivars. Consequently, the average life span of most resistant cultivars is only 2–3 years in blast-prone environments. An understanding of the extent and mechanisms of pathogenic variation would help us design rice genotypes with more durable resistance. As a prerequisite to understanding the basis of genetic variation, we need to identify genes controlling virulence to rice cultivars.

Since the discovery of the perfect state of the blast fungus (9,11,31), research has focused on obtaining fertile matings among isolates from rice and other gramineous hosts to conduct genetic analysis. Crosses among isolates from finger millet (*Eleucine* coracana (L.) Gaertn.), weeping lovegrass (*Eragrostis curvula* (Schrad.) Ness), and goosegrass (*Eleucine indica* L.) have been used for genetic analysis of auxotrophy (6,18), drug resistance (23,26), and isozymes (16). In studying the genetics of host range, Yaegashi (29) identified two loosely linked genes conditioning specificity to finger millet and weeping lovegrass. Valent et al (27) demonstrated two independent genes, Pgg and Pwl, controlling pathogenicity to goosegrass and weeping lovegrass, respectively.

Despite the progress made on the genetic analysis of nonrice isolates, little is known about the genetics of specificity on rice cultivars. Kato and Yamaguchi (11) first reported mating between two rice isolates, but others were unable to repeat the cross apparently because of a loss of fertility after prolonged vegetative propagation (27). Yaegashi and Asaga (30) obtained 25 progeny virulent to rice from a cross between Ken60-19 and a finger millet isolate WGG-FA40, but all F_1 rice strains were infertile. Valent et al (27) obtained rice-infecting progeny from the same cross Ken60-19 × WGG-FA40 and repeatedly backcrossed its progeny to the hermaphroditic parent WGG-FA40; however, none of the 27 rice-infecting strains obtained from these backcrosses showed improved fertility over Ken60-19. Kolmer and Ellingboe (13) obtained fertile isolates that produced intermediate infection types, but they were unable to recover female fertile strains that produced fully compatible lesions on rice. One possible explanation is that many genes are needed for successful pathogenesis on rice and such a favorable combination is broken up by sexual recombination. Because only a small number of ascospores are recovered from crosses with rice isolates, it is not surprising that the desired recombinants have not been detected.

In 1979, Notteghem collected a field rice isolate, Guy11, from French Guyana that was later found to be hermaphroditic. When this naturally occurring hermaphroditic isolate was crossed to laboratory strains of improved fertility, we were able to recover progeny that were fertile and pathogenic to certain rice lines. With these crosses, we have determined the genetic control of specificity on selected rice lines. In this paper, we report the inheritance of virulence factors and the joint segregation analysis of virulence and isozymes. The use of a genetically defined locus to study pathogenic variation is discussed.

MATERIALS AND METHODS

Fungal strains and crossing scheme. During 1982-1984, a mating population was developed at the Department of Plant Pathology at the University of Wisconsin, Madison by random intercrossing fertile isolates from goosegrass, weeping lovegrass, finger millet, and two Japanese rice isolates, Ken73-01 and Ina168 (16). From this mating population, fertile ascospore cultures with isozyme markers were selected and used in the present study conducted at the International Rice Research Institute, Los Baños, Philippines during 1986-1987. Table 1 shows the characteristics and origins of the parental rice isolates used for virulence analysis. Isolates CH104-3 and CH40-1 were the same as 104-3 and 40-1 described by Kolmer and Ellingboe (13). However, after many transfers, CH40-1 sporulated very poorly and hence the pathogenicity of CH40-1 could not be determined. Strains 4134-11-2 and 4091-5-8, laboratory strains developed by Valent, were nonpathogenic to rice. Figure 1 shows the mating scheme by which fertile laboratory strains are developed. Progeny derived from 943 × CH40-1 were intercrossed or backcrossed to the parents to determine their

mating types and fertility. Fertile cultures (in terms of perithecia formation) were designated as mating type testers for the determination of mating types of progeny derived from subsequent crosses. Strains 2539 and 2540 were single conidial cultures recovered from a single lesion on rice plant inoculated with an ascus culture from cross CH40-1 × 4162.

Crosses and ascospore isolation were made as previously described (16). Most crosses with rice isolates had low ascospore viability. Therefore, individual germinated asci were isolated and designated as ascus cultures to speed up the selection for fertility. In later crosses, when a higher percentage of viable ascospore was obtained, individual ascospores were isolated and designated as ascospore cultures. Single hyphal tips from individual germinated asci were also isolated, and such hyphal tip cultures were considered equivalent to single ascospore cultures. This was justified based on the cytological observation that the nuclei in the hyphal branches were derived from successive nuclear division of one mother nucleus and no nuclear migration or anastomosis had been observed in living hyphal branches by using light microscopy (H. Leung, unpublished data). In advancing early generations of fertile and pathogenic strains, both ascus and single ascospore cultures were used to inoculate rice plants. Conidial cultures recovered from lesions were used for the next cycle of mating. Reisolated conidial cultures were either crossed to mating type testers, as shown in Figure 1, or backcrossed to the parental strains to determine the mating type.

Culture maintenance and media. Oatmeal agar was used for all matings, and prune agar (20 g of prunes, 5 g of lactose, 1 g of yeast extract, and 17 g of agar per liter of water) was used for vegetative propagation. To maintain stability of the cultures, all ascospore progenies were stored on filter paper discs after isolation. Sterile paper discs (0.5 cm^2) were placed on actively growing cultures until fully colonized and then dried in a desiccator under vacuum for 4 days at room temperature and stored in coin envelopes in a sealed plastic bag at −20 C.

Plant materials and virulence assay. Because the ability to detect pathogen virulence depended on the susceptibility of the rice plant, we first screened different sources of germplasm to identify suitable rice lines for pathogenicity assays. More than 30 rice lines from the International Rice Germplasm Center at the International Rice Research Institute were tested for susceptibility to the sexual isolates CH104-3, Guy11, and Ken60-19. Eleven lines were selected for routine testing. Seeds were pregerminated in water and then transplanted to field soil in 23 × 11 × 11 cm plastic boxes. Six plants per line were planted in a row, with seven rows per box. All plants were grown in a greenhouse until inoculation.

Spore inocula were produced by using the corn leaf method of Latterell and Rossi (15). Actively growing mycelial pieces were evenly spread on sterilized corn leaves (5 × 6 cm) in a petri dish and incubated under fluorescent light at 26–28 C for 5–6 days. The resultant conidia were washed off from the corn leaves with 0.1% Tween 20 aqueous solution and the inoculum concentration adjusted to 10^5 conidia per milliliter. In testing segregation of virulence, parental isolates were included as checks. Seventeen-day-old seedlings in each plastic box were sprayed with 20 ml of inoculum and then incubated in a dew chamber at 24 C for 24 hr, followed by incubation in an air-conditioned mist room (24–28 C) under natural light for 3 days. Plants were returned to the greenhouse at ambient temperature for 1 day and then scored for disease reaction with a six-point scale: 0 = no infection; 1 = a few to many brown specks, no sporulation can be induced; 3 = brown margined, irregularly shaped, 1- to 2-mm long lesions, sometimes coalescing on leaf edges, lesions sporulate sparingly; 5 = several spindle-shaped, 3–7 mm long lesions, with greyish centers; 7 = five to many spindle-shaped lesions per inoculated leaf, lesions often coalescing; 9 = many coalesced lesions, the leaf wilts and dies (Fig. 2). Although only six interaction phenotypes were used in this study, the 0–9 scale would allow for finer quantification of interaction phenotypes if needed.

Each plant from a rice line was rated as a single interaction phenotype (IP), and the modal IP was designated as the overall disease reaction for the rice line. When both incompatible (IP ≤ 3) and compatible phenotypes (IP ≥ 5) were equally frequent (about 5% of the time), the pathogenicity test was repeated. If mixed phenotypes were observed again, the interaction phenotype was considered heterogeneous, and the data were not included in the genetic analysis. Progenies from the initial crosses 2539 × Guy11 and Guy11 × CH104-3 were evaluated at least twice. Progenies from other crosses were tested once because of seed shortage.

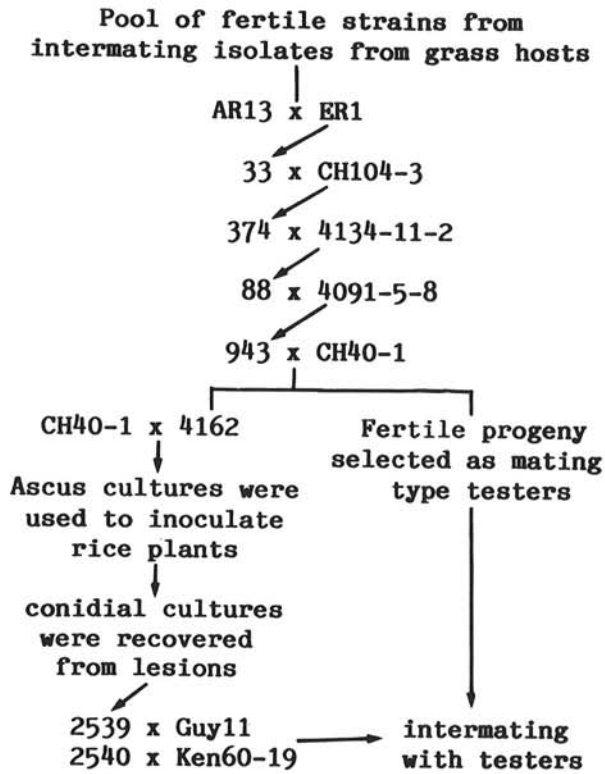

Fig. 1. Mating scheme by which fertile laboratory strains are developed. Strains 4134-11-2 and 4091-5-8 are laboratory strains nonpathogenic to rice.

TABLE 1. Origins and characteristics of sexual isolates of *Magnaporthe grisea*

Isolate	Origin	Characteristics				Source or reference
		Sporulation	Pathogenicity on rice	Sex	Mating type	
CH104-3	China	Poor	Yes	Male	MAT-1	13
CH40-1	China	Very poor	ND[a]	Male	MAT-1	13
Ken60-19	Japan	Normal	Yes	Male	MAT-2	27,30
Guy11	French Guyana	Normal	Yes	Hermaphrodite	MAT-2	This study
2539	Laboratory strain	Normal	No	Hermaphrodite	MAT-1	This study
4134-11-2	Laboratory strain	Normal	No	ND	MAT-2	B. Valent
4091-5-8	Laboratory strain	Normal	No	ND	MAT-2	B. Valent

[a] ND = not determined.

Assays of pigmentation, mating type, and lactate dehydrogenase. Progenies from crosses that segregated at the lactate dehydrogenase (E.C.1.1.1.27) loci were analyzed by starch gel electrophoresis (17). All electrophoretic phenotypes were analyzed twice. The mating type of the progeny was determined by crossing to mating testers, as shown in Figure 1. Pigmentation was classified as wild type (greyish black) or buff on prune agar 8–10 days after isolation. Single-gene segregation and independent assortments of genetic markers and virulence factors were analyzed by using X^2 goodness-of-fit tests (25).

Terminology. In this paper, the term *pathogenicity* is used in a broad sense as the ability of the fungus to infect particular species (e.g., rice or a nonrice host). Thus, it describes the genes conditioning host range and the basic ability to cause infection. The terms *avirulence* and *virulence* are used to describe genes conditioning incompatible and compatible interaction with cultivars within a host species. Gene symbols are assigned according to the suggestions of Yoder et al (32).

RESULTS

Sexual crosses between rice isolates. Recurrent selection for fertility in laboratory strains (Fig. 1), together with the use of fertile field isolates of *M. grisea*, was effective in producing fertile rice strains. Fertility, in terms of perithecia formation and ascospore germination, was improved by early generation selection. Most progenies obtained from these early generations, however, did not infect rice. Of more than 1,000 ascus cultures tested, only 3% produced small brown lesions that sporulated sparingly. Most conidial cultures recovered from these lesions were fertile when backcrossed to mating testers from cross 943 × CH40-1, but they were always the same mating type (MAT-1) as the rice isolates CH104-2 and CH40-1. Only after five more generations of intermatings between recovered conidial cultures and mating type testers were a few rare recombinants of MAT-2 and partial virulence recovered.

When progeny from 943 × CH40-1, 2539 and 2540, were crossed to rice isolates, Guy11 and Ken60-19, a range of fertility was observed. For example, the cross between Guy11 and 2539 gave 60–80% ascospore viability. Crosses between Guy11 and field rice isolates, CH104-3 and CH40-1, however, were less fertile, with only 10% ascospore viability. The difference in fertility between rice isolate × rice isolate and rice isolate × laboratory testers crosses also was evident in F_1 progenies. When 68 F_1 progeny from cross 2539 × Guy11 were crossed to mating testers, 56 (82%) were fertile, whereas only 25 out of 65 (38%) F_1 progeny of CH104-3 × Guy11 were fertile. Ken60-19 was less fertile than Guy11 and could only function as a male. Ken60-19 yielded about 20% ascospore viability when crossed to 2540 but did not cross with other field isolates.

Host reaction. More than 30 rice lines were tested with CH40-1, Guy11, and Ken60-19. This collection represented a diverse group of germplasm that included indica and japonica rices. The interaction phenotypes of three field rice isolates on 11 rice lines are shown in Table 2. Strain Guy11 showed a wider spectrum of virulence than did CH104-3 and Ken60-19. Two lines, Taichung tcwc and 60021, showed intermediate interaction with small irregularly-shaped lesions. Based on this survey of host reaction, appropriate rice lines were selected to test the segregation of virulence.

TABLE 2. Disease reaction of 11 rice lines to three fertile rice isolates of *Magnaporthe grisea*

Rice line[a]	Origin	Type	Interaction phenotype[b]		
			CH104-3	Guy11	Ken60-19
51583	USSR	Indica	7	7	5
Sha-tiao-tsao	China	Japonica	7	7	7
Taichung tcwc	China	Indica	3	3	3
28558	India	Indica	7	7	3
59640	China	Indica	5	7	3
Maratelli	Italy	Japonica	9	9	9
Kinandang Patong	Philippines	Japonica	1	7	0
K59	Japan	Japonica	1	7	1
19561	India	Japonica	0	3	5
25930	Brazil	Japonica	0	5	7
60021	China	Indica	1	3	1

[a] Numbers are accessions maintained in the International Rice Germplasm Center at the International Rice Research Institute.
[b] 0–3 considered incompatible; 5–9 considered compatible.

Inheritance of virulence. Table 3 summarizes the segregation of virulence among progenies from three crosses on selected rice lines. The main effect of virulence was analyzed by classifying progeny exhibiting IP $\geq$ 5 as virulent and those exhibiting IP $\leq$ 3 as avirulent (Fig. 3). This simplification was justified because IP5 was distinguished from IP3 in having compatible lesions that could sporulate profusely when incubated in a moist chamber. However, the different IP profiles shown by the same segregating progeny on different rice lines suggested a type of quantitative interaction different from the distinct phenotypes of compatibility and incompatibility.

In cross 2539 × Guy11, 68 random spores and 10 complete or partial tetrads were isolated. Of the 68 random spores, 51 had wild-type pigmentation and 17 were buff. Segregation analysis of the pigmented ascospore cultures suggested single-gene control of virulence on Sha-tiao-tsao and 51583, although there was a slight excess of virulent progeny on Sha-tiao-tsao (Table 3). The single-gene segregation pattern was also evident for tetrads (Table 4). The determinants of virulence on 51583 and Sha-tiao-tsao appeared to be associated because most progeny virulent to 51583 were also virulent to Sha-tiao-tsao; however, there were 10 progeny virulent to Sha-tiao-tsao but avirulent to 51583, suggesting that different genes were involved.

All buff mutants from the cross 2539 × Guy11 were nonpathogenic to rice, as illustrated in ascus no. 105-8 in Table 4. Previous studies showed that a deficiency in melanin synthesis was responsible for the nonpathogenicity of buff mutants (3,28). To demonstrate that buff was epistatic to the genes controlling virulence, a buff strain 6025 was backcrossed to Guy11. Strain 6025 was expected to have a "hidden" virulence allele because it came from ascus no. 105-8 from which all four pigmented segregants were nonpathogenic. Of 37 random ascospores recovered from 6025 × Guy11, 18 were pigmented and 19 were buff. All pigmented cultures were virulent to 51583, confirming that the buff mutation was epistatic to virulence.

Cross 2540 × Ken60-19 was equivalent to a "half-sib" family of 2539 × Guy11 because 2539 and 2540 were considered identical, as they were conidia reisolated from a single lesion on rice line 51583. Both 2539 and 2540 were MAT-1 and had the same lactate dehydrogenase isozyme genotypes. In this cross, single-gene

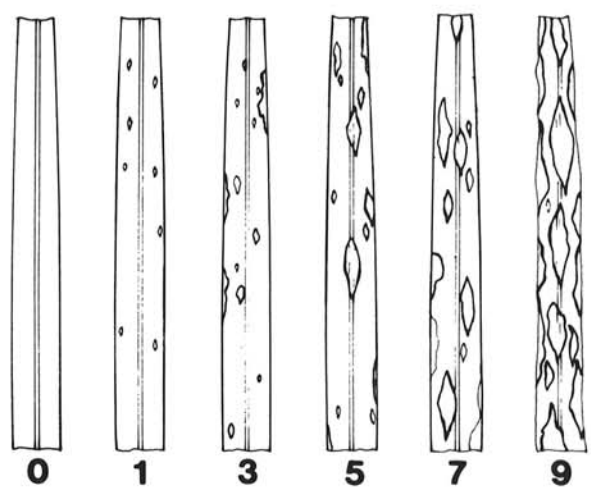

Fig. 2. Interaction phenotype (IP) scale of disease reaction. Ascospore cultures giving IP $\leq$ 3 and IP $\geq$ 5 are considered avirulent and virulent, respectively.

segregation was observed on 51583 but not on Sha-tiao-tsao (Table 3). The highly significant deviation from a 1 avirulent:1 virulent pattern on Sha-tiao-tsao indicated that there were epistatic interactions between the genes from Ken60-19 and 2540, resulting in a suppression of virulence on Sha-tiao-tsao (Table 3). Such epistatic interactions, however, did not affect the expression of virulence on 51583.

Cross Guy11 × CH104-3 represented the first reported case of two field isolates from rice being successfully crossed in the laboratory (Table 3). Although the ascospore viability was low (about 10%), all ascospore cultures were virulent to 51583 and Sha-tiao-tsao except one ascospore culture, which was avirulent to 51583. This culture might be a nonpathogenic mutant. Segregation on K59 and Kinandang Patong fitted a 1 avirulent:1 virulent ratio, suggesting single-gene control of virulence on these rice lines. The 1:1 segregation on 28558 was unexpected because both parental isolates were virulent to 28558.

To have a system for genetic analysis, it was important to demonstrate that fertility and virulence could be transmitted in subsequent generations. Secondary crosses were made by crossing progenies derived from the initial crosses (Table 5). Strain 6554, a progeny from Guy11 × CH104-3, was crossed to a nonsporulating culture of CH40-1. This cross showed that a large proportion of the progenies were virulent to 51583, Sha-tiao-tsao, and Maratelli. In crossing 6039 and 6058, two ascospore progeny from 2539 × Guy11, 1 avirulent:1 virulent segregation were observed on 51583, confirming monogenic control of virulence on 51583. However, with respect to virulence on Sha-tiao-tsao, an unexpected segregation pattern was observed. Instead of recovering all progeny virulent on Sha-tiao-tsao, about one-third of the progeny was avirulent. This 1 avirulent:3 virulent segregation pattern suggested that strains 6039 and 6058 might differ by two genes that acted complementarily to give an incompatible interaction on Sha-tiao-tsao. Similarly, a digenic ratio was observed on Maratelli. Cross (6063 × 6068) (×) was considered an F_3 generation because the progeny were obtained by allowing a single ascus culture derived from an F_2 cross 6063 × 6068 to intermate. Almost all ascospore cultures from this cross were virulent on Maratelli, Sha-tiao-tsao, and 51583.

Based on the results of the segregation analysis, four genes, Pos1, Pos2, Pos3, and Pos4, were assigned to the loci conditioning virulence to four rice lines (Table 6). No gene symbol was assigned to the segregating factor on 28558 because the data was inadequate to explain why there was 1 avirulent:1 virulent segregation when both parental strains were virulent. The Pos locus (pathogenicity on *Oryza sativa*) denoted the locus carrying alleles that enabled the fungus to cause either a compatible or incompatible phenotype on rice. It did not imply any functional relationship with resistance genes in the rice plant. Thus, the locus could carry alleles that code for basic pathogenic ability on rice or for specificity on a cultivar

TABLE 3. Segregation of virulence on rice in random ascospore progenies from crosses of *Magnaporthe grisea*

Cross A × B	Rice line	Interaction phenotype of parental isolates A	B	Frequency distribution of interaction phenotypes among F_1 ascospore progenies 0	1	3	5	7	9	Avir:Vir[a]	Genetic ratio	χ^{2b}
2539 × Guy11	51583	0	9	3	6	17	7	11	4	26:22	1:1	0.33
	Sha-tiao-tsao	0	9	4	5	8	18	11	1	17:30	1:1	3.59
	Taichung tcwc	0	9	29	9	5	0	0	0	43:0	1:0	···
	60021	0	9	19	21	3	0	0	0	43:0	1:0	···
2540 × Ken60-19	51583	0	5	14	2	6	8	3	4	22:15	1:1	1.32
	Sha-tiao-tsao	0	7	28	0	1	2	0	3	29:5	1:1	16.94**
	25930	0	7	26	5	1	4	1	0	32:5	1:1	19.70**
	K59	0	1	40	1	0	0	0	0	41:0	1:0	···
Guy11 × CH104-3	51583	9	7	0	0	1	5	13	40	1:58	0:1	···
	Sha-tiao-tsao	9	7	0	0	0	6	11	26	0:43	0:1	···
	K59	7	1	11	4	9	8	7	9	24:24	1:1	0:0
	Kinandang Patong	5	1	5	3	12	18	11	3	20:32	1:1	2.76
	28558	5	7	2	5	14	6	11	8	21:25	1:1	0.34

[a] Ascospore cultures are considered avirulent (Avir) if IP ≤ 3, virulent (Vir) if IP ≥ 5.
[b] ** significant at $P = 0.01$.

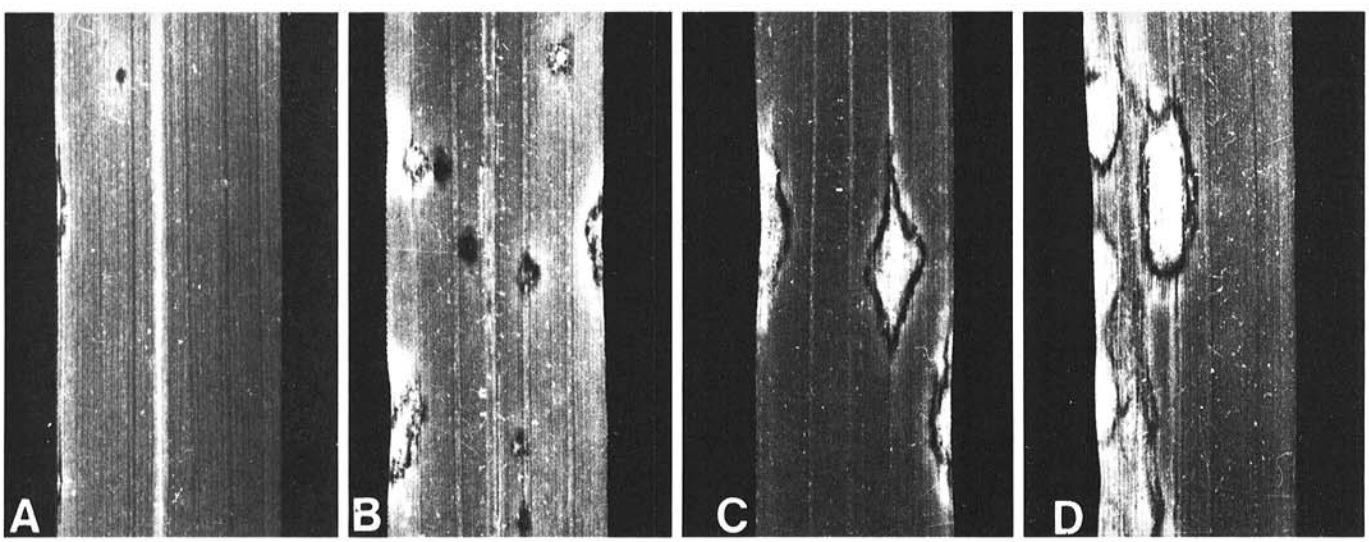

Fig. 3. Interaction phenotypes (IP) of segregating progeny on rice line 51583. **A,** IP = 0, no infection except a few brown speckes; **B,** IP = 3, small brown lesions, some of which coalesce on the leaf edges; **C**, IP = 5, spindle-shaped compatible lesions; **D**, IP = 7, many compatible lesions coalesce.

(i.e., virulence). The present analysis indicated that at least two alleles were present at each locus. The virulence alleles at Pos3 and Pos4 were responsible for cultivar specificity because all segregants from Guy11 × CH104-3 were shown to be competent rice pathogens on 51583 and Sha-tiao-tsao. On the other hand, the specificities of the alleles at Pos1 and Pos2 were not known because no rice line susceptible to all the segregants from cross 2539 × Guy11 was identified. For simplicity, we tentatively considered all four alleles virulence alleles.

Segregation of other genetic markers and linkage tests. Because the viability of ascospores was low (10–60%) in most crosses, it was important to check how lethality affected segregations of other markers. The segregations of the two Ldh loci and the mating type provided a measure of segregation distortion due to lethality (Table 7). In all three crosses tested, Ldh1 conformed to a 1:1 segregation ratio. For Ldh3, there was a slight excess of $Ldh3^{83}$ allele in cross Guy11 × 2539, but the deviation was not highly significant ($0.01 < P < 0.05$). Segregation at the mating type loci

TABLE 4. Segregation of virulence, lactate dehydrogenase, and buff mutation in tetrads of cross 2539 × Guy11 of *Magnaporthe grisea*

Ascus number	Ascospore number	MAT[a]	Pigment[b]	Ldh1[c]	Ldh3[c]	Interaction phenotype[d]	
						51583	Sha-tiao-tsao
105-10	6039	2	+	n	100	7	7
	6043	2	+	n	100	7	7
	6041	1	+	n	83	0	0
	6045	1	+	n	83	0	0
	6042	1	+	100	83	0	0
	6044	1	+	100	83	1	1
	6040	2	+	100	100	9	7
	6046	2	+	100	100	7	5
105-6	6008	1	+	100	83	9	5
	6014	1	+	100	83	7	5
	6009	2	+	n	83	1	3
	6010	2	+	n	83	0	...
	6011	1	+	n	100	5	...
	6012	1	+	n	100	5	...
	6013	2	+	100	100	3	1
105-2	5982	2	+	100	100	9	9
	5983	2	+	100	100	9	9
	5986	2	+	100	83	5	5
	5987	2	+	100	83	9	9
	5985	1	+	n	100	0	0
	5988	1	+	n	100	3	3
105-8	6022	1	Buff	100	83	0	0
	6025	1	Buff	100	83	0	0
	6024	2	Buff	n	100	0	0
	6027	2	Buff	n	100	0	0
	6023	2	+	n	83	0	0
	6028	2	+	n	83	0	0
	6026	1	+	100	100	3	3
	6029	1	+	100	100	0	1

[a] MAT = mating type locus with two alleles, MAT-1 and MAT-2.
[b] + indicates greyish black pigmentation of the wild type.
[c] Numbers indicate the relative mobilities of the electrophoretic alleles of the lactate dehydrogenase (Ldh) loci; n = null.
[d] 0–3 = incompatible; 5–9 = compatible; ··· = not determined.

TABLE 5. Transmission of virulence in F_2 and F_3 progenies derived from the primary crosses of 2539 × Guy11 and Guy11 × CH104-3 in *Magnaporthe grisea*

Cross[a] A × B	Generation	Rice line	Interaction phenotype of parental isolates		Distribution of phenotypic classes among ascospore progenies[b]						Avir:Vir	Genetic ratio	χ^2
			A	B	0	1	3	5	7	9			
6554 × CH40-1[c]	···	51583	9	ND[d]	11	3	11	15	6	9	25:30	...[e]	...
		Sha-tiao-tsao	9	ND	15	0	3	13	7	13	18:33	...	...
		Maratelli	7	ND	10	0	5	16	5	10	15:31	...	...
		taichung tcwc	3	ND	16	9	18	9	0	0	43:9	...	...
		28558	7	ND	12	5	26	10	0	0	43:10	...	...
6039 × 6058	F_2	51583	7	1	2	15	12	5	6	18	29:29	1:1	0.00
		Sha-tiao-tsao	7	5	3	1	6	12	13	21	10:46	1:3	1.52
		Maratelli	7	5	3	2	4	10	14	18	9:42	1:3	1.46
(6063 × 6069) (x)[f]	F_3	Maratelli	ND	ND	0	0	0	7	13	38	0:58	...	...
		Sha-tiao-tsao	ND	ND	0	2	4	7	12	32	6:51	...	...
		51583	ND	ND	0	1	1	6	12	37	2:55	...	...
		19561	ND	ND	22	31	5	0	0	0	58:0	...	...
		25930	ND	ND	2	9	7	12	13	7	18:32	...	...

[a] 6554 is a progeny from Guy11 × CH104-3; 6039, 6058, 6063, and 6068 are progeny from 2539 × Guy11.
[b] 0–3 = avirulent (Avir), and 5–9 = virulent (Vir).
[c] Virulence of CH40-1 is not determined because the culture sporulates very poorly.
[d] ND = not determined.
[e] ··· = no genetic interpretation because of unknown parental phenotypes.
[f] Selfing of an ascus derived from a cross between 6063 and 6068.

deviated significantly from the expected 1:1 ratio in cross Guy11 × 2539 ($P < 0.01$) but not in cross Guy11 × CH104-3. Because a considerable number of the progeny were not fertile, mating type segregation was a less useful measure for segregation distortion.

Independent assortment was tested between loci that conformed to single-gene segregations (Table 8). There was an apparent association of virulence on the rice lines tested, but all possible recombinants were recovered for each gene pair, indicating that independent factors for virulence were involved. Highly significant deviations ($P<0.01$) from independent assortment were observed for three gene pairs, Pos1/Pos2, Pos1/Ldh1, and Pos3/Pos4.

DISCUSSION

We have demonstrated that by selecting for fertility in the progeny of mating strains and by using a hermaphroditic rice isolate, fertile and virulent rice strains can be recovered from sexual recombination. By screening a diverse source of rice germplasm, we have identified rice lines that are susceptible to progenies from crosses between rice isolates. These rice lines are useful for detecting virulence, and they serve as susceptible checks in virulence tests of segregating progeny. We also have shown that fertility and virulence can be transmitted to the next generation either by backcrossing or by sib-mating, an important criterion for genetic analysis of the fungus.

Our data support the idea that epistatic factors are present in the nonrice isolates that suppress the expression of virulence genes on rice. These epistatic factors appear to be closely associated with fertility factors, such that when fertility is selected, the epistatic genes are also selected. Consequently, even in progeny with about 87% of the rice isolate genome (the fourth backcross), virulence on rice is not restored. The epistatic effect also appears to be genotype specific. There is a marked difference in segregation patterns between the sib-crosses 2539 × Guy11 and 2540 × Ken60-19. A much higher percentage of virulent progeny on Sha-tiao-tsao was recovered from 2539 × Guy11 than from 2540 × Ken60-19. Because 2539 and 2540 are identical, the difference in segregation patterns reflects different specificity of epistatic genes in the genotypes of Guy11 and Ken60-19.

When interaction phenotypes are classified as either compatible (IP ≥ 5) or incompatible (IP ≤ 3), simple Mendelian segregations of virulence were observed on several rice lines. In the cross 2539 × Guy11, both random spore and tetrad data showed that single loci were involved in conditioning virulence to Sha-tiao-tsao and 51583. In the cross Guy11 × CH104-3, two different genes were involved in conditioning virulence to Kinandang Patong and K59. The single-gene segregation on 28558 was unexpected because both parental isolates Guy11 and CH104-3 are virulent. This might be caused by premeiotic mutational events that affect the virulence locus, resulting in a loss of virulence to 28558 in one of the parental isolates. Another unexpected segregation pattern was found in the F_2 cross 6039 × 6058, where a digenic pattern was observed on Sha-tiao-tsao (Table 5). It appears that additional genes affecting virulence on Sha-tiao-tsao are expressed in a new genotypic background. The data obtained so far are not sufficient for a detailed analysis of complementary or epistatic gene action.

TABLE 6. Single virulence genes identified in two crosses of *Magnaporthe grisea*

Gene symbol	Cross origin	Virulence on
Pos1	2539 × Guy11	51583
Pos2	2539 × Guy11	Sha-tiao-tsao
Pos3	Gyy11 × CH104-3	K59
Pos4	Guy11 × CH104-3	Kinandang Patong

TABLE 7. Segregation of lactate dehydrogenase-1, lactate dehydrogenase-3, and mating type in crosses of *Magnaporthe grisea*

Locus	Cross	Parental genotype[a] a:b	Acospore segregant a:b	Expected ratio	χ^{2b}
Ldh1	Guy11 × 2539	100:null	54:57	1:1	0.08
	6063 × 6061		24:22	1:1	0.08
	6058 × 6039		43:40	1:1	0.10
			121:119	1:1	0.01
	Heterogeneity (df = 2)				0.25
Ldh3	Guy11 × 2539	100:83	66:45	1:1	3.97*
	6063 × 6061		20:26	1:1	0.78
			86:71	1:1	1.43
	Heterogeneity (df = 1)				3.32
MAT	2539 × Guy11	MAT 1:MAT 2	16:37	1:1	8.32**
	CH104-2 × Guy11		16:9	1:1	1.96
			32:46	1:1	2.50
	Heterogeneity (df = 1)				7.78**

[a] Numbers indicate the relative mobilities of the electrophoretic alleles.
[b] * significant at $P = 0.05$; ** significant at $P = 0.01$.

TABLE 8. Joint segregation of virulence and isozyme loci in crosses of *Magnaporthe grisea*

Gene pair[a]	Cross	Genotype[b]	No. of acospores	PT:NPT[c]	χ^{2d}
Pos1/Pos2	2539 × Guy11	+/+	19	34:13	9.38**
		−/−	15		
		+/−	2		
		−/+	11		
Pos1/Ldh1	2539 × Guy11	+/100	18	31:17	4.08*
		−/n	13		
		+/n	4		
		−/100	13		
	6039 × 6058	+/n	15	32:25	0.85
		−/100	17		
		+/100	13		
		−/n	12		
Pos1/Ldh3	2539 × Guy11	+/100	14	24:24	0.00
		−/83	10		
		+/83	8		
		−/100	16		
Pos2/Ldh1	2539 × Guy11	+/100	24	34:13	9.38**
		−/n	10		
		+/n	6		
		−/100	7		
Pos2/Ldh3	2539 × Guy11	+/100	19	25:22	0.19
		−/83	6		
		+/83	11		
		−/100	11		
Ldh1/Ldh3	2539 × Guy11	100/100	35	61:50	1.09
		n/83	26		
		100/83	19		
		n/100	31		
Pos3/Pos4	CH104 × Guy11	+/+	19	32:14	7.04**
		−/−	13		
		+/−	4		
		−/+	10		
Pos3/Ldh1	6039 × 6058	+/n	15	33:19	2.66
		−/100	18		
		+/100	7		
		−/n	12		

[a] Pos1, Pos2, Pos3, and Pos4 represent four loci-carrying alleles that condition virulence on rice lines 51583, Sha-tiao-tsao, K59, and Kinandang Patong, respectively. Ldh1 and Ldh3 represent the lactate dehydrogenase-1 and lactate dehydrogenase-3 loci.
[b] + and − designate compatible IIP ≥ 5) and incompatible (IP ≤ 3) interaction phenotype; 100 and 83 indicate the relative mobilities of the electrophoretic alleles at the Ldh1 and Ldh3 loci; n = null allele. The first two genotypes in each gene pair are the parental genotypes.
[c] PT = parental type; NPT = nonparental type.
[d] Chi-square values calculated based on a 1:1 expected ratio; * significant at $P = 0.05$; ** significant at $P = 0.01$.

Nonetheless, these observations suggest that genetic systems other than the single-gene model can play a role in the expression of virulence. Further analysis is needed to clarify the genetic basis for these observations.

The excess of parental types with respect to virulence and avirulence suggests that the virulence factors identified in *M. grisea* might be genetically linked. Clustering of disease resistance genes has been reported in a number of plant species, e.g., bacterial blight resistance in rice (33), downy mildew resistance in lettuce (10), and rust resistance in flax (24), but linkages among virulence genes in pathogens have been rarely observed (7). In the apple scab pathogen *Venturia inaequalis*, 19 pathogenicity genes have been identified, three of which are linked (5). In *M. grisea*, genetic linkages among auxotrophic markers (6,18,19) and isozymes (16) have been reported. Yaegashi (29) also suggested a loose linkage between the genes conditioning host range on finger millet and weeping lovegrass. Because of the small number of families examined in this study, no attempt is made to estimate the recombination fractions between virulence genes. Detailed mapping by restriction fragment length polymorphism analysis is now in progress.

Segregation of other genetic factors that affect the pathogen's ability to infect rice can also give the impression of genetic linkage. An example is the buff mutation, which appears to cosegregate with avirulence, but in fact is epistatic to virulence due to a genetic block in melanin synthesis (3,28). Similarly, other genetic factors that affect the vigor of segregants may also cause a differential ability to infect rice. Such confounding effects can be removed by incorporating virulance factors into a common tester strain so that the characters can be evaluated in a common genetic background.

The ability to define a virulence locus by genetic crosses will allow a more rigorous analysis of pathogenic variation exhibited by the fungus. Ou and others (8,21) reported great variation in virulence in certain rice isolates, whereas Kiyosawa (12) reported different mutation rates for different "virulence" genes based on phenotypic analysis. Without genetic proof, the observed phenotypic variability could be due to a variety of factors unrelated to changes at the virulence locus. With a genetic system, we could monitor the changes occurring at a specific locus and confirm any mutational change by genetic crosses. Such an approach would help resolve the debate on pathogenic stability in the blast fungus. Furthermore, the recent development of a transformation system in *M. grisea* (23) offers an even more direct approach to understanding the genetic fine structures of virulence genes through molecular cloning and characterization. We have demonstrated Mendelian inheritance of several virulence genes, yet we have no knowledge of the biochemical function or epistatic relationships of these genes. Such information will help the design of strategies in cloning virulence genes.

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