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Magnaporthe grisea Genes for Pathogenicity and Virulence Identified Through a Series of Backcrosses

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ABSTRACT

We have identified genes for pathogenicity toward rice (*Oryza sativa*) and genes for virulence toward specific rice cultivars in the plant pathogenic fungus *Magnaporthe grisea*. A genetic cross was conducted between the weeping lovegrass (*Eragrostis curvula*) pathogen 4091-5-8, a highly fertile, hermaphroditic laboratory strain, and the rice pathogen O-135, a poorly fertile, female-sterile field isolate that infects weeping lovegrass as well as rice. A six-generation backcrossing scheme was then undertaken with the rice pathogen as the recurrent parent. One goal of these crosses was to generate rice pathogenic progeny with the high fertility characteristic of strain 4091-5-8, which would permit rigorous genetic analysis of rice pathogens. Therefore, progeny strains to be used as parents for backcross generations were chosen only on the basis of fertility. The ratios of pathogenic to nonpathogenic (and virulent to avirulent) progeny through the backcross generations suggested that the starting parent strains differ in two types of genes that control the ability to infect rice. First, they differ by polygenic factors that determine the extent of lesion development achieved by those progeny that infect rice. These genes do not appear to play a role in infection of weeping lovegrass because both parents and all progeny infect weeping lovegrass. Second, the parents differ by simple Mendelian determinants, "avirulence genes," that govern virulence toward specific rice cultivars in all-or-none fashion. Several crosses confirm the segregation of three unlinked avirulence genes, *Avr1-CO39*, *Avr1-M201* and *Avr1-YAMO*, alleles of which determine avirulence on rice cultivars CO39, M201, and Yashiro-mochi, respectively. Interestingly, avirulence alleles of *Avr1-CO39*, *Avr1-M201* and *Avr1-YAMO* were inherited from the parent strain 4091-5-8, which is a nonpathogen of rice. Middle repetitive DNA sequences ("MGR sequences"), present in approximately 40–50 copies in the genome of the rice pathogen parent, and in very low copy number in the genome of the nonpathogen of rice, were used as physical markers to monitor restoration of the rice pathogen genetic background during introgression of fertility. The introgression of highest levels of fertility into the most successful rice pathogen progeny was incomplete by the sixth generation, perhaps a consequence of genetic linkage between genes for fertility and genes for rice pathogenicity. One chromosomal DNA segment with MGR sequence homology appeared to be linked to the gene *Avr1-CO39*. Finally, many of the crosses described in this paper exhibited a characteristic common to many crosses involving *M. grisea* rice pathogen field isolates. That is, pigment-defective mutants frequently appeared among the progeny.

THE haploid heterothallic ascomycete *Magnaporthe grisea* (Hebert) Barr [anamorph, *Pyricularia grisea* Sacc., formerly *Pyricularia oryzae* Cavara (ROSSMAN, HOWARD and VALENT 1990)] includes pathogens of many grasses, although individual field isolates are limited to one or a few host species (KATO 1978; OU 1985; MACKILL and BONMAN 1986). Strains of *M. grisea* cause the serious disease of rice called blast (OU 1985). Among isolates pathogenic to rice, hundreds of "races" have been identified as defined by their virulence or avirulence toward particular rice cultivars. Cultivars of rice that differ from one another by the presence or absence of dominant blast resistance genes have been developed (YAMADA *et al.* 1976), but the resistance of any one of these cultivars is effective only against certain races of the pathogen. The fungus shows a high degree of variability in the field; new races frequently appear with the ability to infect previously resistant rice cultivars.

Individual *M. grisea* strains produce distinctive and reproducible symptoms on particular host plants under a given environmental regime (LATTERELL 1975; OU 1985). Pathogenicity is a complex phenotype, involving such distinct components as infection efficiency (determines lesion number), rate of lesion development, extent of colonization (determines lesion size), and efficiency of sporulation. Genetic analysis of host-pathogen interactions, accomplished mainly in systems with obligate fungal pathogens such as the rusts and the mildews, confirms the complexity of the interactions (DAY 1974; CRUTE 1986). Quantitative pathogenicity factors determine at least some aspects of the interaction between the pathogen and its host (CATEN *et al.* 1984; CRUTE 1986). "Major genes,"

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single genes with large effects on a host-pathogen interaction, are also common. Except for preliminary reports of genetic analysis of host specificity (YAEGASHI and ASAGA 1981; LEUNG *et al.* 1988; ELLINGBOE, WU and ROBERTSON 1990), little is known of the genetic basis of *M. grisea* pathogenicity and host specificity.

The ability of *M. grisea* strains to undergo genetic crosses is also a complex phenotype. As with other heterothallic ascomycetes, compatibility for mating is governed by alleles of the mating type locus, *Mat1*. At least one parent must be female fertile, with the capacity to produce perithecia, and the two parents together must be able to produce asci with viable ascospores. The fertility of *M. grisea* field isolates ranges from total sterility (inability to mate with any other strain), through female sterility (ability to mate only as a male), to full fertility (ability to mate as a male or as a female; VALENT *et al.* 1986). Even among hermaphroditic strains there appears to be a fertility continuum. Hermaphroditic strains of relatively low fertility cross with only a few other strains and produce few perithecia, and strains of the highest fertility cross with many other strains and produce large numbers of perithecia. Viability of ascospores for crosses between field isolates ranges from less than 1% up to 10% in exceptional cases. The level of fertility of field isolates generally correlates with host specificity. For example, field isolates that infect weeping lovegrass, goosegrass (*Eleusine indica*) or finger millet (*Eleusine coracana*) typically are hermaphrodites that can mate to yield numerous viable ascospores. Hundreds of field isolates that infect rice have been tested and found to be female sterile (VALENT *et al.* 1986; B. VALENT, unpublished results), and those rice pathogens that do mate with hermaphroditic strains infecting grasses other than rice usually produce ascospores with poor viability. A recent unique exception to the rule of female sterility among rice pathogen field isolates is a strain, Guy-11, isolated by J. L. NOTTEGHEM in French Guyana (LEUNG *et al.* 1988). Rice pathogens of both mating types occur in the field (KATO and YAMAGUCHI 1982; YAEGASHI and YAMADA 1986).

In addition to host specificity and level of fertility, *M. grisea* field isolates differ in middle repetitive DNA sequences. That is, rice pathogens from around the world contain a family of repeated DNA sequences, "MGR sequences," that appear to be absent from or present in low copy number in field isolates that infect grasses other than rice (HAMER *et al.* 1989). MGR sequences are interspersed with single copy DNA on all chromosomes of rice pathogens. The presence of MGR sequences only in rice pathogens suggests that rice pathogens in nature are genetically isolated from nonpathogens of rice in the same geographic area.

Breeding for improved fertility with *M. grisea* weeping lovegrass and goosegrass pathogens has produced strains with excellent properties for genetic analysis (VALENT *et al.* 1986; VALENT and CHUMLEY 1987). The series of crosses reported here originated with the dual purposes of determining the genetic differences between a pathogen and a nonpathogen of rice and of introgressing high fertility into laboratory strains that infect rice. The development of highly fertile laboratory strains that infect rice as well as other grass species should permit rigorous genetic analysis of host species specificity, host cultivar specificity and mechanisms of pathogenesis in *M. grisea*. The segregation of pathogenicity, virulence, fertility and MGR markers was investigated through six generations of backcrosses. Segregation patterns suggested simple Mendelian inheritance of genes that control specificity (*i.e.*, virulence) toward rice cultivars as well as polygenic inheritance of factors that determine pathogenicity toward rice. Hermaphroditic fertile rice pathogens were generated, although no highly successful pathogens showed the very high fertility characteristic of laboratory strains that infect grasses other than rice. Most crosses reported here exhibited instability at one particular genetic locus, *BUF1* (CHUMLEY and VALENT 1990). All other markers observed, including mating type, segregated normally. Detection of genetic linkage between a particular MGR sequence and the avirulence gene *Avr1-CO39* suggested that MGR-associated restriction fragment length polymorphisms (RFLPs) might serve as physical markers for cloning genes of interest by chromosome walking.

MATERIALS AND METHODS

Strains: Most strains described in this paper (see Table 1) were derived from three field isolates. The fertile laboratory strain 4091-5-8 was a progeny strain from a cross between a Japanese field isolate that infects weeping lovegrass, K76-79 (*Mat1-2*), and a Japanese field isolate that infects finger millet and goosegrass, WGG-FA40 (*Mat1-1*) (VALENT *et al.* 1986). Both field isolates were generously provided by HIROSHI YAEGASHI of the Tohoku National Agricultural Experiment Station in Akita, Japan. Strain 4091-5-8 is a pathogen of weeping lovegrass and goosegrass, but it does not produce visible symptoms on any of the fifteen cultivars of rice tested. The field isolate O-135, which infects rice and weeping lovegrass, was collected by BV in 1985 at the China National Rice Research Institute in Hangzhou, Zhejiang, China. A rice-pathogen laboratory strain, 6043 (LEUNG *et al.* 1988), was generously provided by HEI LEUNG, Washington State University, Pullman. The laboratory strains 4360-19-1 and 4360-15-1, both pathogens of rice, were progeny from other crosses in our laboratory and will be described elsewhere. The strains commonly used in mating type tests were 4091-5-8 (*Mat1-2*, ⚥) and 4136-4-3 (*Mat1-1*, ⚥) (VALENT and CHUMLEY 1987).

Media and permanent storage of the pathogen: Isolates were grown and crossed on oatmeal agar plates, prepared as follows. Fifty grams of rolled oats were heated in 500 ml

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TABLE 1

Strains

Strain	Mating Type	Fertility[a]	Comments[b]
4091-5-8	2	⚥, H	Weeping lovegrass pathogen, laboratory strain
0-135	1	♂	Rice and weeping lovegrass pathogen, Chinese field isolate
4274-R-84	2	⚥, H	1st generation strain, parent of 2nd generation (cross 4284)
4284-R-163	2	⚥, H	2nd gen. strain, parent of 3rd gen. (cross 4306)
4306-R-127	2	⚥, H	3rd gen. strain, parent of 4th gen. (cross 4314)
4314-R-24	2	⚥, M	4th gen. strain, parent of 5th gen. "A" branch (cross 4321)
4314-R-39	2	⚥, H	4th gen. strain, type 2 on C039
4314-R-98	2	⚥, H	4th gen. strain, parent of 5th gen. "B" branch (cross 4338)
4338-R-68	2	⚥, H	5th gen. "B" branch strain, parent for 6th gen. (cross 4349)
4321-R-11	1	♂	5th gen. "A" branch strain, type 5 on C039
4321-R-13	2	⚥, H	5th gen. "A" branch strain, type 0 on C039
4321-R-14	2	♂	5th gen. "A" branch strain, type 0 on C039
4321-R-20	1	⚥, L	5th gen. "A" branch strain, type 3 on C039
4321-R-25	2	♂	5th gen. "A" branch strain, type 5 on C039
4321-R-28	1	⚥, M	5th gen. "A" branch strain, type 3 on C039
4321-R-37	1	♂	5th gen. "A" branch strain, type 0 on C039
4321-R-39	2	♂	5th gen. "A" branch strain, type 5 on C039
4321-R-63	2	⚥, L	5th gen. "A" branch strain, type 5 on C039
4321-R-317	2	⚥, M	5th gen. "A" branch strain, type 4 on C039
4349-R-1	2	⚥, H	6th gen. "B" branch strain, type 3 on C039
4349-R-6	1	♂	6th gen. "B" branch strain, type 5 on C039
4349-R-8	2	⚥, L	6th gen. "B" branch strain, type 5 on C039
4349-R-19	2	⚥, H	6th gen. "B" branch strain, type 2 on C039
4349-R-54	2	♂	6th gen. "B" branch strain, type 5 on C039
4349-R-69	1	♂	6th gen. "B" branch strain, type 5 on C039
4349-R-81	2	⚥, H	6th gen. "B" branch strain, type 1 on C039
4349-R-128	2	⚥, H	6th gen. "B" branch strain, type 1 on C039
6043	2	⚥, M	From Hei Leung (Wash. St. Univ.), type 5 on C039
4360-15-1	2	⚥, M	Laboratory strain, type 4 on C039 (B. Valent, unpublished)
4360-19-1	1	⚥, M	Laboratory strain, type 5 on C039 (B. Valent, unpublished)

[a] Strains designated by the symbol "⚥" mate as males or as females. Strains designated by the symbol "♂" mate only as males. The designations H, M and L (for high, medium and low) indicate relative fertility of hermaphroditic strains. Relative assessments of fertility are somewhat subjective. In crosses with most partners, highly fertile strains produce many perithecia, each containing hundreds of viable ascospores.

[b] Among rice pathogens, lesion type on cultivar C039 is listed. Strains 4321-R-14 and 4321-R-37, which are type 0 on C039, due to the presence of *Avr1-C039*, are virulent on other cultivars. None of the progeny of cross 4349 carry *Avr1-C039*. Therefore, these type 1 strains appear to lack minor pathogenicity factors for infecting C039.

of water at 70° for 1 hr. The suspension was then filtered through cheesecloth and the volume of the filtrate was adjusted to 1 liter with water. 2YEG medium, used for checking germination of conidia and for isolation of ascospores, contained 0.2% yeast extract and 1% glucose. Unless otherwise stated solid media contained 1.5% agar.

Strains were stored dried and frozen in cellulose filter paper discs (13 mm diameter, Schleicher and Schuell, #597). The fungus was allowed to grow at 25° until it permeated filter paper discs placed on agar plates. The discs were then dried in a desiccator for two weeks before being frozen at −20° or at −70° in sterile glassine stamp envelopes. Strains were also stored by drying harvested infected plant tissue for two weeks in a desiccator and freezing as above. Selected strains were also stored frozen at −70° as mycelial suspensions in 15% glycerol.

Genetic crosses: Crosses were performed by placing strains of opposite mating type on oatmeal agar and allowing them to grow together at 20° under continuous fluorescent light. Tetrads or random ascospores could be isolated at 15–21 days. Ascospores were isolated by hand on 2YEG medium solidified with 4% agar using a finely drawn glass needle and a 50× Wild stereomicroscope. Perithecia were opened by squeezing with fine tweezers (Dumont, Style 5, Biological) and the released asci were separated from one another. After incubation on the agar plate for approximately 30 min, ascospores could be released by rubbing the ascus gently with the glass needle. Mature perithecia oozed loose ascospores through their necks, and some retained loose ascospores in their bulbs. In particularly fertile crosses, where ascospores were released from many hundreds of asci, ascospores were spread and isolated at random. If germination was low, random ascospores were isolated by separating the spores from individual asci and taking only one to insure random sampling. Great care was taken not to confuse crescent-shaped ascospores with the slightly larger teardrop-shaped conidia (Yaegashi and Udagawa 1978).

DNA preparation, blotting and hybridization: Small amounts of total *M. grisea* DNA (≈50 μg) were prepared as described (Hamer *et al.* 1989). DNA was digested with restriction endonucleases, fractionated on 0.8% agarose gels, and transferred to Amersham Hybond-N membranes as specified by the manufacturer. Hybridization was performed as specified by Amersham using probes radioactively labeled by the random oligonucleotide primer method (Feinberg and Vogelstein 1983).

Infection assay: Seed of the indica rice cultivar CO39

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was obtained from the International Rice Research Institute (IRRI) in Los Baños, Philippines. Seed of the japonica cultivar Yashiro-mochi was obtained from DR. HAJIME KATO, Kobe University, Kobe, Japan. Seed of cultivar M201 was obtained from the California Cooperative Rice Research Foundation in Biggs, California. Weeping lovegrass seed was obtained from Valley Seed Service in Fresno, California.

Seven rice seeds or approximately 30 weeping lovegrass seeds were planted in vermiculite (W. R. Grace and Co.) in 4 inch diameter plastic pots and incubated in Conviron CG108 growth chambers. Two rice cultivars (seven seeds each) were sometimes included in a single pot. Plants were watered automatically three times per day with Hoagland's nutrient solution (HOAGLAND and ARNON 1950) modified by increasing the ammonium nitrogen to 18%. Light from cool white fluorescent and tungsten incandescent bulbs ranged in intensity from 400 to 500 $\mu E/m^2/sec$, measured at pot height, 46 inches from the bulbs. Plants were incubated using a daily cycle of 14 hr light (27°, 85% relative humidity (RH)) followed by 10 hr of darkness (21°, 85% RH). Plants were inoculated about two weeks after germination, when the fourth foliar leaf was half emerged.

Inoculation: Conidia were collected from cultures growing on oatmeal agar plates by washing with 0.25% gelatin (Sigma, No. G-0510). Samples from all conidial suspensions inoculated on plants were spotted on 2YEG medium to check for germination. The concentration of conidia was adjusted to a known titer ranging from 10,000 to 500,000 conidia per ml in different experiments. The optimal inoculum concentration resulted in frequent individual lesions. This concentration appeared to be a function of both the rice cultivar and the fungal strain under investigation.

Three to four ml of inoculum were applied per pot using an artist's air brush (Paasche H No. 1) connected to compressed air at 20 psi. Plants were inoculated inside a plastic bag which was then sealed to maintain the high humidity required for the fungus to penetrate the cuticle of the host plant (HOWARD and FERRARI 1989). After 20–24 hr of incubation in low light, the plants were removed from the bags and placed in a growth chamber. Controls in each experiment included plants sprayed with gelatin solution as well as plants inoculated with standard pathogenic and nonpathogenic *M. grisea* strains. Infected plants did not produce conidia in the growth chamber when the relative humidity was maintained below 90%. Thus, the nonconidiating lesions produced represented a single round of infection. The pathogen was reisolated from diseased plants by placing excised tissue on 4% water agar. Tissues containing type 2, 3, 4 or 5 lesions (see Figure 1) conidiated within 12 to 24 hr. Conidia were isolated axenically by touching conidiophores with the sharp point of a 4% water agar sliver (LATTERELL and ROSSI 1986) or with a small wire loop containing gelatin solution.

Scoring infection: The infection assay, designed to mimic natural infection conditions as closely as possible, resulted in lesions that resemble those seen in the field. Seven days after inoculation, symptoms were scored by examining the youngest leaf that was expanded at the time of inoculation. This is the most susceptible plant tissue (LATTERELL 1975). Fungal strains typically form lesions that are relatively uniform in size on this tissue. Although variation in lesion size appeared to be continuous when considering the whole range of strains available, a scale of five landmark lesion types was established for scoring purposes (see Figure 1). Rarely, mesothetic reactions were seen in which several types of lesions were found intermingled on single leaves. In such cases, the occurrence of a mixed response was recorded, and lesions types were plotted according to the predominant type. Some infections that were not truly mesothetic still produced abundant dark brown necrotic flecks (type 1) in addition to larger lesions; these were assigned lesion type designations according to the larger lesions.

All progeny sets were assayed at least twice. Selected progeny sets as well as selected progeny strains were assayed three or more times as indicated to firmly establish the reproducibility of the assay under the controlled environmental regime in the growth chamber. The maximum lesion size obtained with standard control strains varied slightly, expanding or compressing the lesion type scale. However, the relative ranking of strains on the lesion type scale remained constant from assay to assay. Results from infection assays were documented by taping diseased leaf tissue onto index cards using Scotch Brand (3MM Co.) transparent tape. These cards, when stored dry in the dark, maintain a reliable record of symptoms (LATTERELL 1975).

Nomenclature: We reserve the terms "virulence" and "avirulence" for interactions that are demonstrated to be cultivar specific. "Avirulence genes," alleles of which determine virulence or avirulence on specific rice cultivars, are named "*Avr-*," and a code following the hyphen refers to the cultivar on which the gene is effective (*e.g.*, "*Avr-CO39*"). Genes that have not been demonstrated to have cultivar specific effects are termed "pathogenicity genes," even though specificity may eventually be demonstrated. Pathogenicity genes therefore include genes that confer host species specificity, genes that determine the extent of lesion development and genes that encode functions generally necessary for infecting any plant. Pathogenicity (or virulence) and nonpathogenicity (or avirulence) are defined as described in the legend to Figure 1. Other aspects of genetic nomenclature have been described (CRAWFORD *et al.* 1986; YODER, VALENT and CHUMLEY 1986).

The naming of cultivar specificity determinants in our system as "avirulence genes" does not imply dominance of the avirulence phenotype as described by FLOR (1956, 1971). We have no information on dominance because the fungus does not form stable vegetative diploids that would permit dominance tests of virulence phenotypes (CRAWFORD *et al.* 1986). We have chosen to name these genes "*Avr-*" for consistency with the bacterial and fungal literature on genes that control cultivar specificity (CRUTE 1986; TAMAKI *et al.* 1988).

RESULTS

Backcross scheme: Laboratory strain 4091-5-8, a highly fertile pathogen of weeping lovegrass, was crossed with field isolate O-135, a female sterile pathogen of rice and weeping lovegrass. This cross produced many perithecia that each contained numerous asci (cross 4274). Approximately 50% of the ascospores produced were viable. This cross was repeated several times with similar results. We reasoned that a series of backcrosses in which highly fertile progeny were mated with O-135 should eventually yield rice pathogens with the high fertility characteristic of 4091-5-8. Backcrosses were conducted as shown in Figure 2. For each of these crosses, random ascospore progeny were checked twice for fertility in mating with O-135. Strains that exhibited a level of fertility comparable to that of 4091-5-8 were chosen as parents for the backcross generations. All strains, except for

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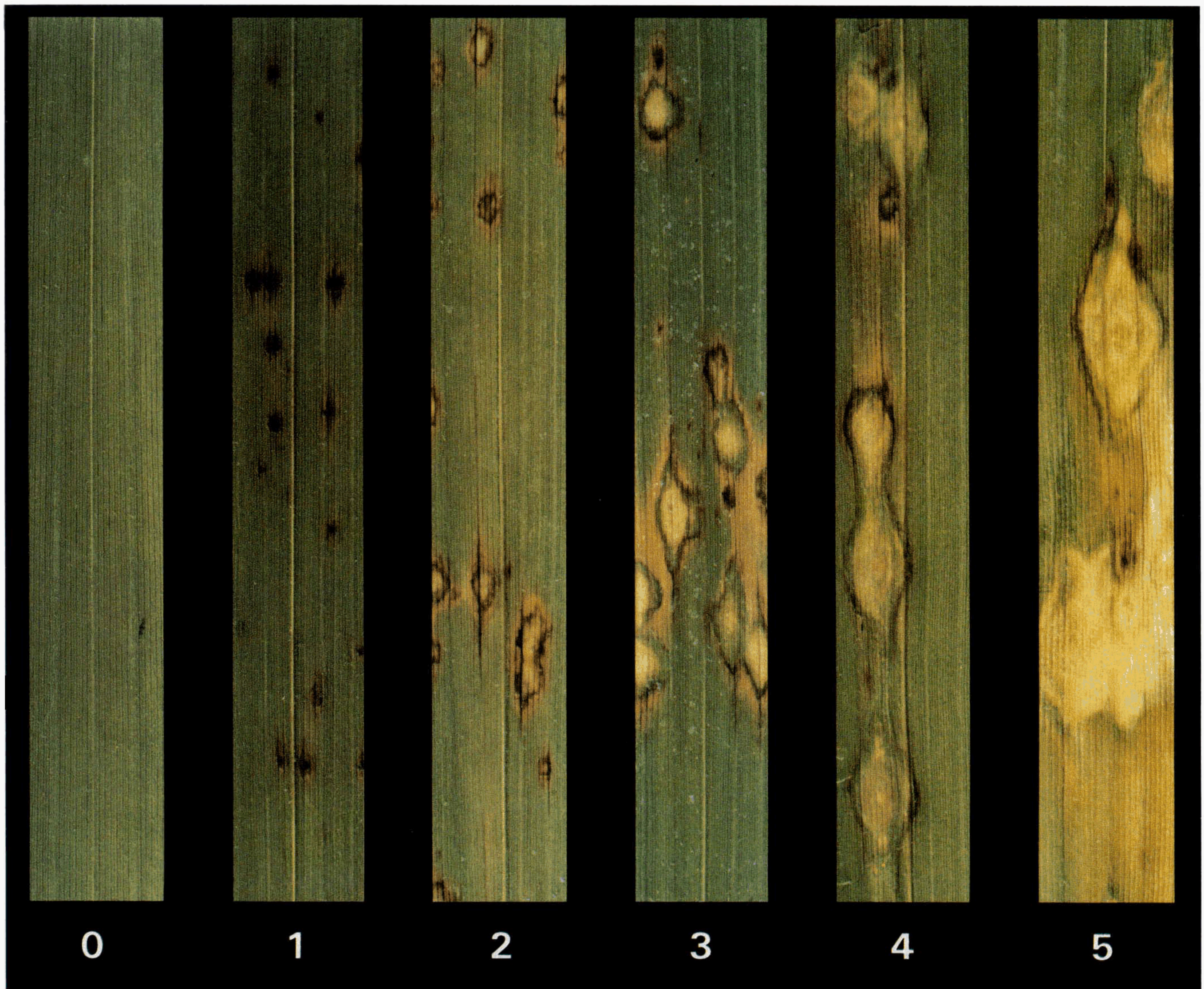

FIGURE 1.—Leaf segments (cultivar CO39) showing standard lesion types. Six lesion types have been defined as follows for rice seedlings inoculated under conditions described in MATERIALS AND METHODS. It is important to note that, under these conditions, lesions do not conidiate *in situ* and are thus devoid of the gray spore mass that would otherwise appear on lesion types 2 to 5. Type 0, no visible evidence of infection; type 1, uniform dark brown pinpoint lesions without visible centers. These lesions typically are barely visible, but can reach 0.5 mm in diameter in some fungus-plant combinations, as shown; type 2, small lesions with distinct tan centers surrounded by a darker brown margin (the lesions shown are approximately 1 mm in diameter); type 3, small eyespot lesions approximately 2 mm in length with tan centers surrounded by dark brown margins; type 4, intermediate size eyespot lesions, approximately 3–4 mm in length; type 5, large eyespot lesions that attain the maximum size seen for a particular cultivar (approximately 5 mm in length for CO39). Types 0 and 1 were considered nonpathogenic, or avirulent, interactions because affected tissue did not produce conidia under high humidity conditions. The fungus in these interactions thus failed to complete the disease cycle. Types 2, 3, 4 and 5 were considered pathogenic, or virulent, because conidia were produced from such infected tissues under high humidity conditions. A similar progression of lesion types was observed on rice cultivars M201 and Yashiro-mochi.

4314-R-98, were chosen without regard for pathogenicity phenotype.

Segregation of mating type and pathogenicity among first generation progeny: Mating type segregated 1:1 among 71 random progeny tested from Cross 4274 (Figure 2); 33 were *Mat1-1* and 38 were *Mat1-2*. Fifty-nine of these first generation progeny were inoculated on weeping lovegrass. Both parents and all 59 progeny tested were successful pathogens of weeping lovegrass, as expected. In contrast, only 6 of 59 progeny were pathogens of rice cultivar CO39 (Figure 3A), and 4 of these 6 progeny produced limited type 2 lesions. These results suggest that strains 4091-5-8 and O-135 differ by several genes that determine the ability to infect rice and that these genes do not appear to play a role in pathogenicity toward weeping lovegrass.

Segregation of pathogenicity and virulence toward CO39 through backcross generations: Parents for the fourth and fifth-A generations and random

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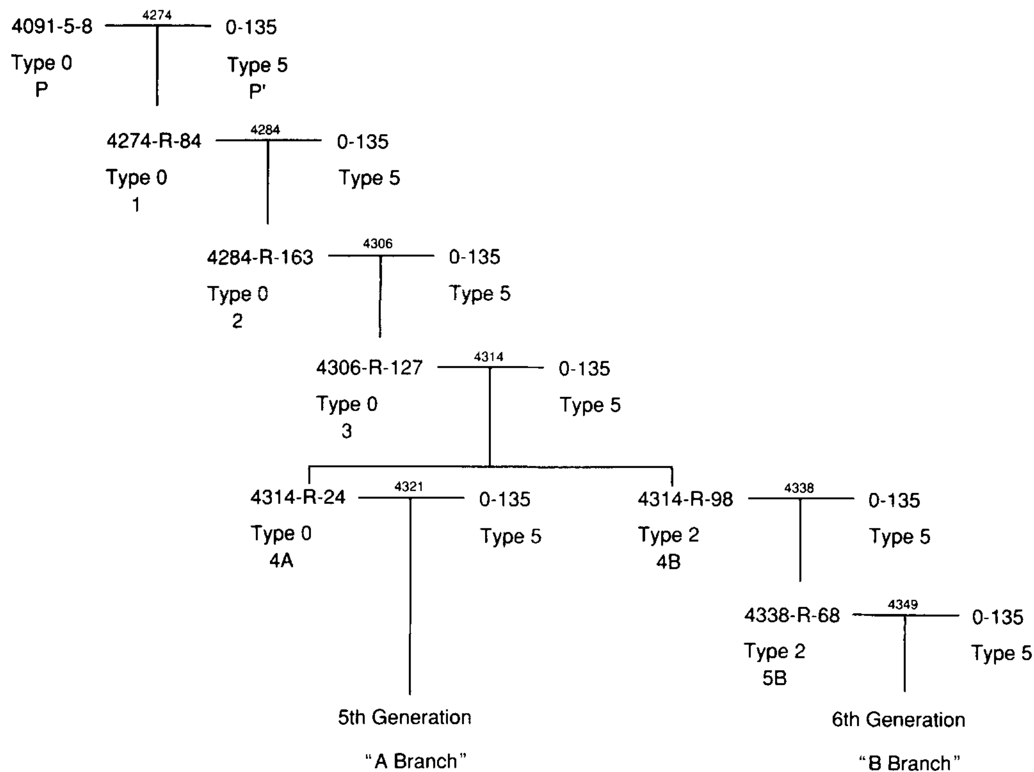

FIGURE 2.—Backcrossing scheme. Pairs of strains that were crossed are connected by horizontal lines. The serial number assigned to each cross is shown on the horizontal line. Strain 4091-5-8 (*Mat1-2*) was crossed with the rice pathogenic field isolate O-135 (*Mat1-1*). *Mat1-2* hermaphroditic progeny with wild-type gray pigment were selected as parents for backcross generations. Lesion type on CO39 is listed for each strain. All *Mat1-2* hermaphrodites from these crosses produced no visible symptoms (type 0) on cultivars Yashiro-mochi and M201. Note that in all cases tested (crosses 4314, 4321, 4338 and 4349), progeny pathogenic on CO39 appeared in equal frequencies in each mating type. First generation: strain 4274-R-84 was chosen from 143 random ascospore progeny to be the second generation parent. Second generation: strain 4284-R-163 was chosen from 187 random ascospores. Third generation: strain 4306-R-127 was chosen from 163 random ascospores. Fourth generation: two progeny from 188 random ascospores were chosen as parents for the two branches at the fourth generation, designated "A" and "B" as shown. Strain 4314-R-24 produced type 0 symptoms and strain 4314-R-98 produced type 2 symptoms on cultivar CO39. Strain 4314-R-98 retained the high fertility of 4091-5-8 in crosses with O-135, producing comparable numbers of perithecia and ascospores with 50% viability. Strain 4314-R-24 mated with O-135 to produce ascospores with 50% viability, but produced significantly fewer perithecia. Fifth-B generation: strain 4338-R-68 was chosen from 187 random ascospores.

ascospore progeny from each cross (crosses 4314 and 4321, respectively) were tested for the ability to infect rice cultivar CO39. Among the fourth generation progeny tested (Figure 3B), 28 were pathogens and 28 were nonpathogens. Looking simply at this segregation ratio, there appeared to be a single gene difference between the parents with a major ("all-or-nothing") effect on the ability to infect cultivar CO39. Among the 28 fourth generation pathogens, however, there was a reproducible normal distribution of lesion types 2–5. The fifth-A generation showed a similar result (Figure 3C). Among 71 progeny examined, 38 were pathogens and 33 were nonpathogens of cultivar CO39, suggesting segregation of a single major gene. Once again, among the 38 pathogenic progeny, a normal distribution of lesion types was observed. The ranking of pathogenic progeny relative to one another in the scale of lesion types was highly reproducible for both the fourth and fifth-A generations, suggesting segregation of "minor genes." That is, several genes of individually small effect appeared to determine the extent to which a pathogenic strain colonizes host tissue. Comparison of the lesion type profiles of the pathogenic progeny for both generations suggested that lesion size factors accumulated as the backcrosses progressed.

Segregation of pathogenicity and virulence toward cultivar M201: Progeny from the backcross generations were inoculated on a second cultivar, M201 (Figure 4), to investigate cultivar specificity of the genes identified above. As with CO39, very few first generation progeny produced lesions on M201. In the third, fourth and fifth-A generations increasing numbers of progeny were pathogenic on M201. In the fourth generation 31 of the 56 progeny tested produced type 0 or type 1 symptoms and the remaining 25 produced type 2 through type 5 symptoms suggesting segregation of a single gene with a major effect on the ability to infect M201 ($\chi^2 = 0.64$, $P > 0.1$). The ratio of 42 nonpathogens to 29 pathogens

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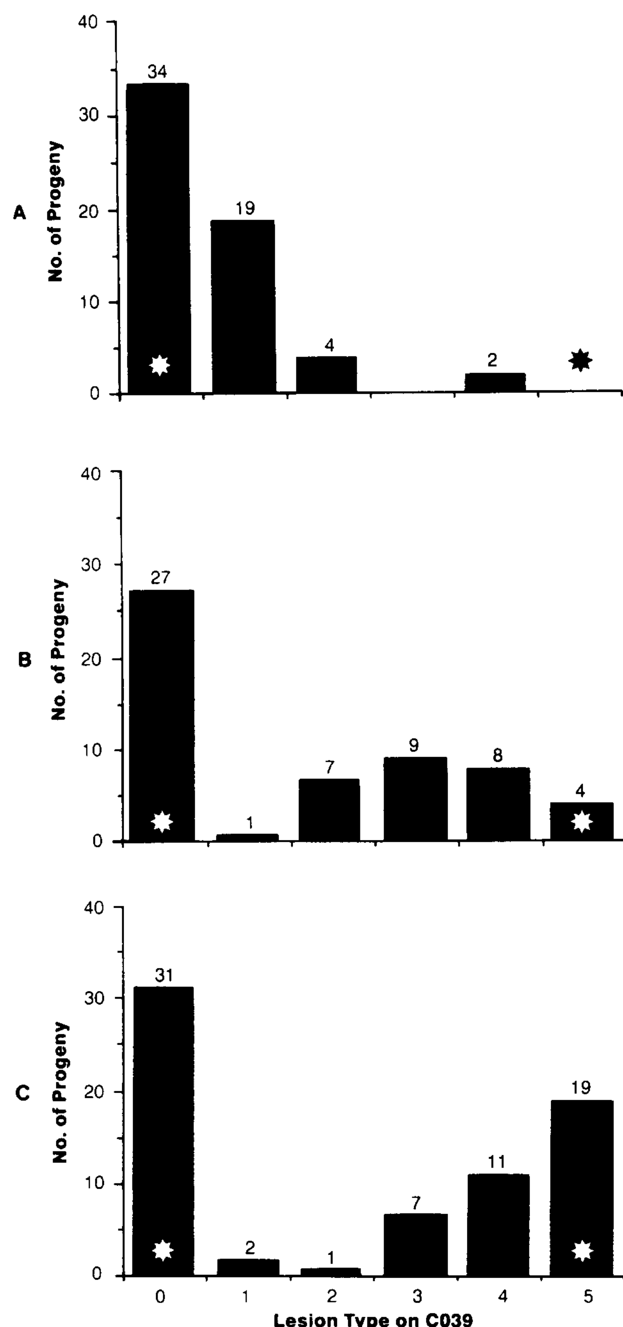

FIGURE 3.—Segregation of lesion types resulting from infection of CO39 with progeny from first, fourth and fifth-A generation crosses. Conidia derived from random ascospore progeny from each cross were inoculated onto CO39 as described in MATERIALS AND METHODS. The number of progeny represented is listed above each bar. The asterisks indicate the lesion types produced by the parents. (A) First generation progeny (cross 4274) assayed at 1×10^4 conidia/ml. Type 0 and type 1 progeny also failed to produce type 2–5 lesions when inoculated at 5×10^5 conidia/ml. Some strains that produced type 0 symptoms at the lower inoculum concentration produced type 1 lesions at the higher concentration. (B) Fourth generation progeny (cross 4314) assayed at 1×10^4 conidia/ml. Two additional assays confirmed these results: one assay was inoculated at 5×10^4 conidia/ml, and another at 5×10^5 conidia/ml. (C) Fifth generation progeny (cross 4321) assayed at 1×10^4 conidia/ml. Two additional assays were inoculated at 5×10^4 conidia/ml. Additional assays were performed on selected progeny from this cross.

among the fifth-A generation progeny (Figure 4D) is also suggestive of the segregation of a single gene that is critical for infection of M201 ($\chi^2 = 2.4$, $P > 0.1$). On cultivar M201, the distribution of lesion types produced by the pathogenic progeny in the fourth and fifth-A generations (Figure 4, C and D) does not resemble a simple normal distribution, in contrast to the result on CO39 (Figure 3, B and C).

Unlike the result on cultivar CO39, several progeny strains produced an extreme type 1 flecking response on cultivar M201. These progeny produced abundant uniformly dark brown flecks with diameters up to 1 mm. The relatively large number of type 1 progeny and the general shape of the pathogenicity profile led to the hypothesis that some of these type 1 progeny actually carried the virulence allele of the hypothetical major gene, but failed to produce lesions capable of sporulation as a consequence of inheriting several nonpathogenicity alleles of "minor genes" required for colonizing rice tissue. To test this hypothesis, one fifth generation progeny (strain 4321-R-28), which produced extreme type 1 flecks on M201, was crossed with strain 4360-15-1, which produced type 4 lesions on M201 (cross 4378). The progeny segregated 0 type 0:17 type 1:21 type 2:17 type 3:6 type 4:0 type 5. The normal distribution of lesion types obtained, and the absence of type 0 progeny indeed suggests that 4321-R-28 should be grouped among the progeny of cross 4321 that carry the virulence allele of the proposed major gene. Categorizing some type 1 progeny of cross 4321 among the pathogens of M201 improves the fit of the data to the 1:1 hypothesis. This result illustrates the difficulty in interpreting the genotype of progeny strains with lesion types 0 and 1. A failure to infect may be due to the action of a major gene for avirulence or to the cumulative effects of minor genes for small lesion size; the possibilities can only be distinguished by further genetic analysis.

Segregation of pathogenicity and virulence toward cultivar Yashiro-mochi; identification of cultivar specificity determinants: First and fifth-A generation progeny were assayed for pathogenicity on a third rice cultivar, Yashiro-mochi. Fifty-six of 59 first generation progeny from Cross 4274 were nonpathogens (type 0 or type 1) of Yashiro-mochi, even when inoculated at the high concentration of 500,000 conidia/ml. The remaining three progeny produced type 4 symptoms. However, the fifth-A generation progeny showed a clear bimodal distribution of lesion types (Figure 5), suggesting single gene segregation of the ability to infect Yashiro-mochi. Among the progeny of Cross 4321 that successfully infected Yashiro-mochi, almost all gave Type 5 lesions, unlike the results on CO39 and M201 (Figures 3C and 4D). The six progeny that formed smaller lesions (Types 2–4) did so reproducibly.

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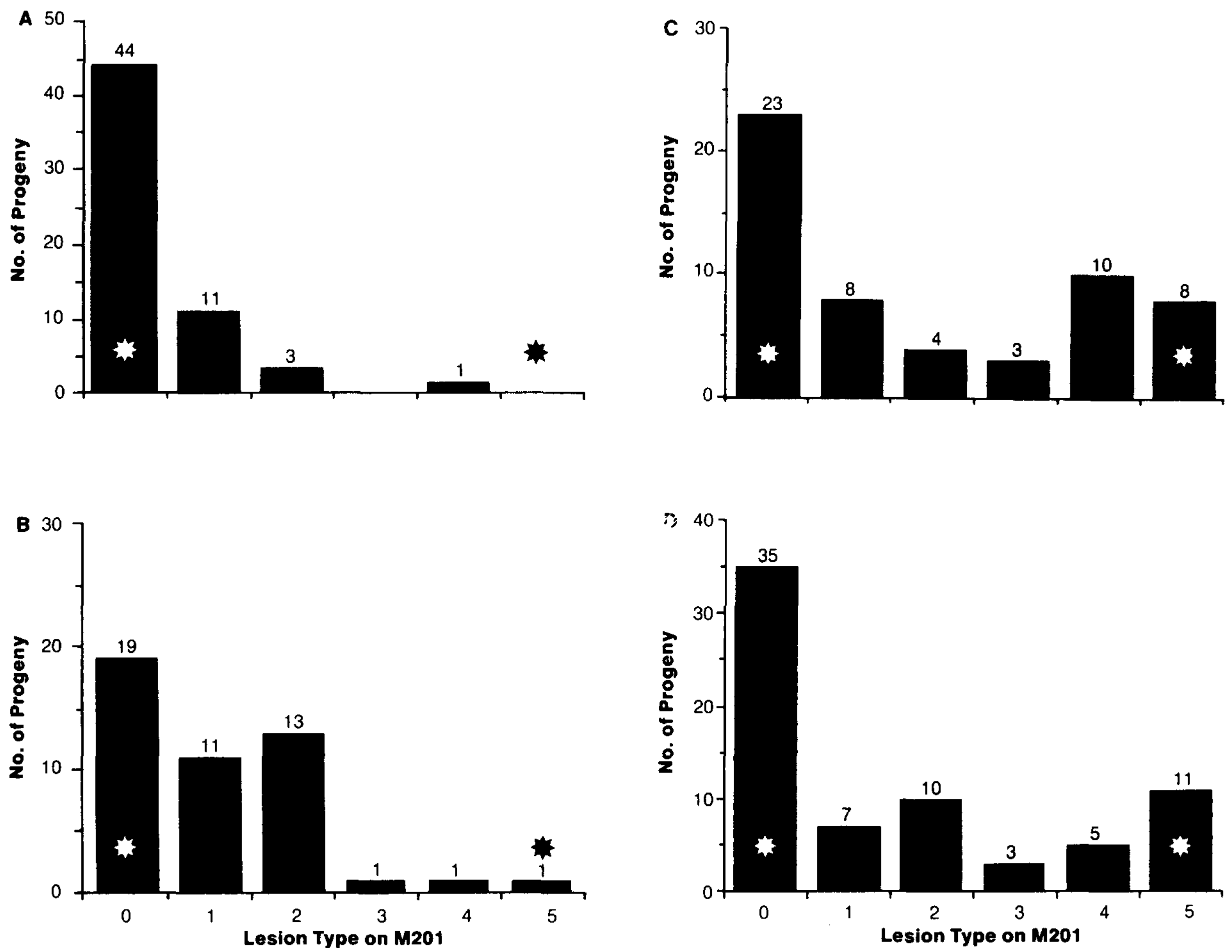

FIGURE 4.—Segregation of lesion types resulting from infection of M201 with progeny from genetic crosses. The asterisks indicate the lesion types of the parents. Progeny were inoculated on M201 at an inoculum concentration of 5 × 10^5 conidia/ml. (A) First generation progeny (cross 4274). (B) Third generation progeny (cross 4306). (C) Fourth generation progeny (cross 4314). (D) Fifth generation progeny (cross 4321, A branch). All inoculations were repeated at least once at 5 × 10^4 conidia/ml.

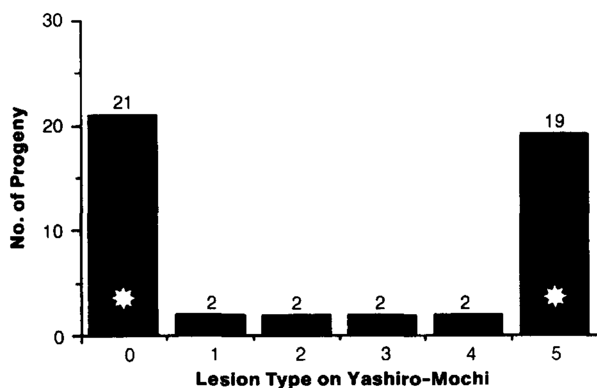

FIGURE 5.—Lesion types produced by fifth generation progeny (cross 4321, A branch) on cultivar Yashiro-mochi. Progeny of cross 4321 were inoculated on Yashiro-mochi at 1 × 10^4 conidia/ml. The results were confirmed by inoculation with 5 × 10^5 conidia/ml. Many type 0 strains produced type 1 flecking at this high inoculum concentration. Selected individuals were re-inoculated one or more times at 5 × 10^4 conidia/ml.

Comparisons of lesion types produced by individual progeny on the three cultivars CO39, M201, and Yashiro-mochi indicate that the single genes with major effects identified in Figures 3C, 4D, and 5 are cultivar specific. Table 2 shows cultivar specificity for selected progeny from Cross 4321. Therefore, the major genes identified in this study are "avirulence

TABLE 2

Cultivar specificity differences among fifth generation progeny (cross 4321, A branch)

Strain	Mating type	Lesion type		
		C039	Yashiro-mochi	M201
4321-R-11	1	5	5	5
4321-R-25	2	5	5	5
4321-R-37	1	0	5	5
4321-R-39	2	5	5	0
4321-R-14	2	0	5	0
4321-R-63	2	5	0	0
4321-R-13	2	0	0	0

Progeny from cross 4321 (see Figures 3C, 4D and 5) show differences in ability to infect rice cultivars. Lesion types are defined in Figure 1. Two strains in this table (4321-R-63 and 4321-R-13) are hermaphrodites.

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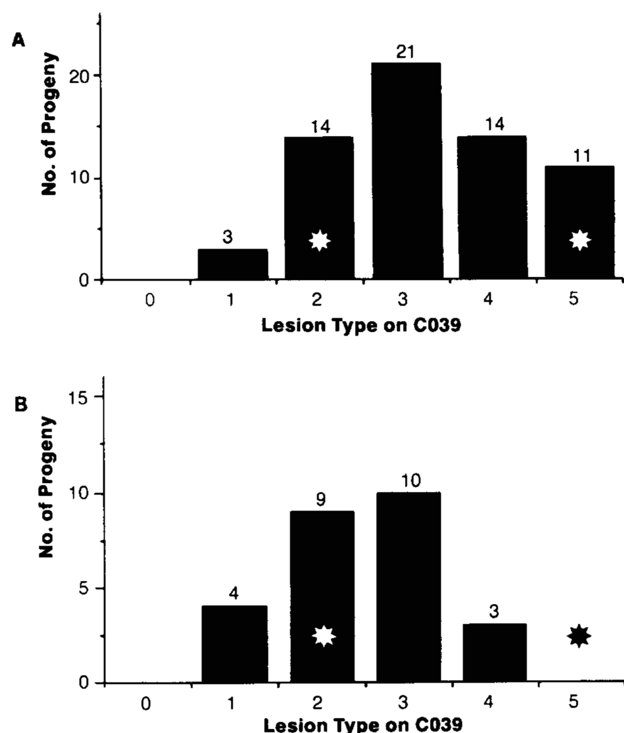

FIGURE 6.—(A) Pathogenicity of random fifth generation progeny from cross 4338 (B branch) on cultivar CO39. The fourth generation progeny strain 4314-R-98 (type 2 on CO39) was crossed with O-135 to check the prediction that no type 0 progeny should arise. Sixty three of 187 progeny isolated were chosen at random for pathogenicity testing. The assay was conducted twice at 1×10^4 conidia/ml. (B) Pathogenicity of *Mat1-2* hermaphrodites from cross 4338 on CO39. Twenty-six of 187 progeny produced perithecia when mated with O-135. These *Mat1-2* hermaphrodites were tested for pathogenicity on CO39 at an inoculum concentration of 1×10^4 conidia/ml. Note that only 7 of the progeny strains in (B) are also included in (A).

genes," alleles of which determine virulence or avirulence on specific rice cultivars.

Progeny in two complete tetrads from Cross 4321 were inoculated on rice cultivars CO39, M201 and Yashiro-mochi. Data from both tetrads are consistent with the conclusion that single gene differences between the parents control cultivar specificity (data not shown). The alleles of these genes that confer avirulence must have been derived from strain 4091-5-8 because strain O-135 is virulent on all three rice cultivars.

Crosses in which the virulence allele of the CO39 avirulence gene is homozygous: We hypothesized that the fifth-A generation parent 4314-R-24 cannot infect rice cultivar CO39 due to the presence of the avirulence allele of an "avirulence gene." We therefore predicted virulent siblings of 4314-R-24 must contain the virulence allele. In crosses between such progeny and O-135, the virulence allele should be homozygous, and no class of avirulent progeny should arise. This prediction was confirmed with two crosses shown in Figure 2: cross 4338 (see Figure 6A) and

TABLE 3

Polygenic inheritance of factors that determine lesion type on three rice cultivars

Cultivar	Parents' lesion type A	Parents' lesion type B	No. of progeny with lesion type 0	1	2	3	4	5
C039	5	3	0	0	9	27	16	7
Yashiro-mochi	5	2	7	5	25	15	5	2
M201	5	2	3	13	14	14	13	2

Lesion types produced by the parents and 59 progeny of cross 4363 inoculated at 5×10^4 conidia/ml. Parent A (the female parent) was strain 6043 and parent B was strain 4321-R-20. Mating type segregated normally (35 *Mat1-1* strains:24 *Mat1-2* strains).

cross 4349 (1 type 0:7 type 1:12 type 2:13 type 3:18 type 4:7 type 5 progeny). A third cross, number 4337, between 4314-R-39 (type 2) and O-135, produced 0 type 0:7 type 1:17 type 2:15 type 3:17 type 4:4 type 5 progeny. Thus, all three crosses lacked the avirulent progeny class and showed a normal distribution of lesion types consistent with the segregation of minor genes controlling lesion size.

As further confirmation of segregation in the absence of a major avirulence allele, a cross was conducted between two strains that are virulent on cultivars CO39, M201 and Yashiro-mochi, but which differ in the extent of colonization achieved (Table 3). Most of the progeny were virulent, falling into a normal distribution of lesion types on each of the three rice cultivars. This result supports the hypothesis of polygenic inheritance of pathogenicity toward rice. In general, a pathogenic progeny strain that was less successful on one cultivar also was less successful on the two other cultivars. This observation suggests that at least some of the polygenic pathogenicity factors may not be cultivar specific; rather, they may be host species specificity determinants.

Crosses to confirm single gene segregation of specificity toward cultivars CO39, M201, and Yashiro-mochi: Three crosses support the existence of avirulence genes identified in the backcrossing scheme. Table 4A shows the results of a cross in which the parents were heterozygous for virulence on CO39 and homozygous for virulence on M201 and on Yashiro-mochi. Single gene segregation for virulence on cultivar CO39 is clear, and all the progeny in the cross were virulent on M201 and Yashiro-mochi. No progeny virulent on CO39 were obtained in a cross where both parents were avirulent on CO39 (Table 4B). In a cross where both parents were virulent on CO39 (Table 4C), all progeny were virulent. We named the avirulence gene that has been identified in this series of crosses *Avr1-CO39*.

Additional evidence for the existence of the avirulence gene corresponding to cultivar M201 is also seen in Table 4. Progeny from a cross between a virulent and an avirulent parent (Table 4C), showed single

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TABLE 4

Crosses to confirm the segregation of cultivar specificity determinants

Cultivar	Parents' lesion type		No. of progeny with lesion type						AV:V	Ratio	χ^2
	A	B	0	1	2	3	4	5			
A. Cross 4386: 6043 × 4321-R-37											
C039	5	0	29	0	0	2	8	19	29:29	1:1	
Yashiro-mochi	5	5	0	0	0	0	17	41	0:58	0:1	
M201	5	5	0	0	0	0	6	52	0:58	0:1	
B. Cross 4365: 4314-R-24 × 4321-R-37											
C039	0	0	55	0	0	0	0	0	55:0	1:0	
Yashiro-mochi	0	5	27	0	2	1	3	22	27:28	1:1	0.02
C. Cross 4399: 4360-19-1 × 4321-R-63											
C039	5	5	0	0	19	5	15	24	0:63	0:1	
Yashiro-mochi	5	0	21	7	1	2	2	30	28:35	1:1	0.78
M201	5	0	31	1	12	3	3	13	32:31	1:1	0.02

Lesion types of parents and progeny. The first parent listed is parent A and the second parent is B. Parent A was the female strain. The inoculum concentration was 5×10^4 conidia/ml in all assays for the crosses in A and B. In C, one inoculation was done at 5×10^5 conidia/ml and one was done at 5×10^4 conidia/ml. The predicted ratio is avirulent (AV):virulent (V). All χ^2 values listed are not significant ($P > 0.05$).

gene segregation for virulence on M201. The virulent progeny in this cross gave a distribution of lesion types similar to that of Cross 4321 (Figure 4D). A cross between two virulent parents gave only virulent progeny as expected (Table 4A). The avirulence gene corresponding to cultivar M201 identified here has been named *Avr1-M201*.

Finally, all three crosses in Table 4 gave results consistent with the presence of an avirulence gene corresponding to Yashiro-mochi. Two crosses between virulent and avirulent parents (Table 4, B and C) show the predicted single gene segregation and the cross between two virulent parents (Table 4A) yielded only virulent progeny. These crosses thus define an avirulence gene, *Avr1-YAMO*, corresponding to cultivar Yashiro-mochi.

Preliminary evidence suggests that the three avirulence genes are not linked to one another (Table 5). The genes *Avr1-CO39* and *Avr1-YAMO* do not show linkage to *Mat1*. Table 5 also shows that although *Avr1-M201* appears to be linked to mating type in Cross 4321, no linkage was detected in Cross 4399. Therefore, the linkage between *Avr1-M201* and *Mat1* remains uncertain.

Segregation of fertility through the backcross generations: One goal of the genetic crosses diagrammed in Figure 2 was to introduce the high fertility of strain 4091-5-8 into the genetic background of O-135. For this reason, all random ascospore progeny were tested specifically for ability to mate with O-135. In crosses 4274, 4284, 4306, 4314 and 4338, approximately 15% of the random ascospore progeny produced perithecia with O-135 and were therefore *Mat1-2* hermaphrodites. In crosses with standard laboratory tester strains, there were fewer *Mat1-1* hermaphrodites than *Mat1-2* hermaphrodites, suggesting that O-135 may carry one or more genes linked to mating type that contribute to female sterility. In cross 4321, only 6% of the progeny produced perithecia with O-135. In all of the crosses in Figure 2, approximately 50% of the ascospores were viable. Through the backcrossing generations, fertility, as measured by ability to cross with O-135, did not improve. That is, no progeny were recovered that mated with O-135 better than 4091-5-8 did, and many progeny were less fertile than 4091-5-8. Many progeny that mated with O-135 produced a lower percentage of viable ascospores or fewer perithecia or both.

As with random progeny shown in Figures 3 and 6A, the ability of hermaphroditic progeny to infect cultivar CO39 increased through the stages of the backcross scheme. However, as illustrated for Cross 4338, most hermaphroditic strains derived in these crosses showed less than average pathogenicity (compare Figure 6, A and B). No type 5 progeny appeared among the fifth or sixth generation *Mat1-2* hermaphrodites. Nine of the type 4 progeny shown in Figure 6A were *Mat1-2* and five were *Mat1-1*. Likewise, seven of the type 5 progeny were *Mat1-2* and four were *Mat1-1*. The equal distribution of mating types among the type 4 and type 5 rice pathogens suggests that mating type segregated independently from factors controlling pathogenicity toward CO39. Altogether, these observations suggest that female fertility did not segregate independently from rice pathogenicity factors.

All fifth and sixth generation *Mat1-2* hermaphrodites were inoculated on cultivars Yashiro-mochi and M201. All of these progeny were avirulent on both cultivars.

Accumulation of MGR repetitive sequences through backcross generations: Strain O-135 con-

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TABLE 5

Segregation of mating type and genes controlling cultivar specificity

Gene pair	Cross	Phenotype	No. of progeny	χ^{2a}
Avr1-C039/Mat1	4321	Avr/Mat1-1	14	2.4
		Vir/Mat1-1	12	
		Avr/Mat1-2	14	
		Vir/Mat1-2	20	
Avr1-C039/Mat1	4386	Avr/Mat1-1	15	2.1
		Vir/Mat1-1	10	
		Avr/Mat1-2	12	
		Vir/Mat1-2	17	
Avr1-M201/Mat1	4321	Avr/Mat1-1	5	20.4[b]
		Vir/Mat1-1	21	
		Avr/Mat1-2	26	
		Vir/Mat1-2	8	
Avr1-M201/Mat1	4399	Avr/Mat1-1	15	2.3
		Vir/Mat1-1	11	
		Avr/Mat1-2	17	
		Vir/Mat1-2	19	
Avr1-YAMO/MAT1	4321	Avr/Mat1-1	8	2.5
		Vir/Mat1-1	15	
		Avr/Mat1-2	13	
		Vir/Mat1-2	10	
Avr1-C039/Avr1-M201	4321	Avr/Avr[c]	20	2.2
		Avr/Vir	17	
		Vir/Avr	13	
		Vir/Vir	21	
Avr1-C039/Avr1-YAMO	4321	Avr/Avr	14	6.0
		Avr/Vir	8	
		Vir/Avr	8	
		Vir/Vir	18	
Avr1-M201/Avr1-YAMO	4321	Avr/Avr	15	3.2
		Avr/Vir	10	
		Vir/Avr	8	
		Vir/Vir	15	

The parents for cross 4321 are identified in Figure 2, for cross 4386 in Table 4A and for Cross 4399 in Table 4C.

[a] The values of χ^2 presented test the hypothesis of no linkage (*i.e.*, 1:1:1:1 for the four progeny classes).

[b] This χ^2 value is significant ($P < 0.001$). All others are not significant ($P > 0.05$).

[c] The progeny classes indicated here and in the remainder of the table refer first to the gene listed first in the column, "gene pair."

tains many MGR sequences, in contrast to strain 4091-5-8 (HAMER *et al.* 1989). MGR "bands" from O-135 detected by Southern hybridization were faithfully transmitted to progeny, accumulating as the backcross scheme proceeded (see Figure 7). We were not able to detect bands in backcross progeny that could not have arisen from O-135, and no bands could be identified that did not segregate normally. For example, Figure 7 shows MGR hybridization patterns for the backcross parents, for one progeny strain from cross 4321 (5A) and for eight sixth generation progeny strains (6B). After the first generation, most backcross parents acquired small numbers of new MGR bands. The third generation strain showed a MGR pattern identical to the second generation strain, despite the fact that these two strains produced progeny, when crossed with O-135, that had very different lesion type profiles on cultivar M201 (Figure 4, B and C).

Four highly fertile sixth generation progeny, 4349-R-1, -19, -81 and -128, produced type 3, type 2, type 1 and type 1 symptoms on CO39, respectively. As shown in Figure 7, the MGR patterns for these progeny contained significantly fewer bands than the patterns of the four highly pathogenic (lesion type 5) progeny shown, 4349-R-6, -8, -54, and -69. This suggests that progress in introgression of highest fertility into the rice pathogen genetic background was slowed by linkage between fertility factors in 4091-5-8 and pathogenicity factors in O-135.

It is apparent that many MGR bands segregated independently from pathogenicity toward rice. Although all the strains represented in Figure 7 are pathogens of weeping lovegrass, only those shown in the six rightmost tracks are successful pathogens of rice. Clearly, plant pathogenic strains can contain many MGR sequences and yet fail to infect rice. MGR sequences thus are not sufficient to confer pathogenicity toward rice, and the studies reported here provide no support for any role of MGR sequences in pathogenicity toward any host.

The smallest MGR band shown in Figure 7 (approximately 0.8 kb in length) appears to be linked to *Avr1-CO39*. The backcross parents labelled 1, 2, 3 and 4A contain the avirulence allele of *Avr1-CO39* and lack the 0.8 kb band. The parent of the fifth-B generation, 4314-R-98 (4B), lacks the avirulence allele and has gained the band. All subsequent progeny in the B-branch lack the avirulence allele as expected. Strain 4321-R-317 (5A) from the A-branch has also lost the avirulence allele of *Avr1-CO39* and gained the 0.8-kb band. Examination of the phenotypes of 20 more progeny of cross 4321 yielded only two recombinants. One recombinant was virulent, but lacked the 0.8 kb band, and the other was avirulent, but contained the band.

Frequent occurrence of melanin-deficient progeny in crosses involving some rice pathogens: Crosses shown in Figure 2 that involved O-135 as a parent illustrate a characteristic common to many *M. grisea* crosses of normally pigmented field isolates that infect rice. That is, Buf⁻ mutants occurred frequently among the meiotic progeny (B. VALENT, unpublished results; CHUMLEY and VALENT 1990). The *BUF1*$^+$ gene encodes an enzyme involved in the biosynthesis of the dark gray pigment, DHN-melanin (CHUMLEY and VALENT 1990; HOWARD and FERRARI 1989). Even though all parents in the crosses shown in Figure 2 produce the DHN-melanin typical of wild-type *M. grisea*, high frequencies of Buf$^-$ progeny were obtained from most of these crosses: 4% in cross 4274, 9% in cross 4284, 5% in cross 4314; 12% in cross

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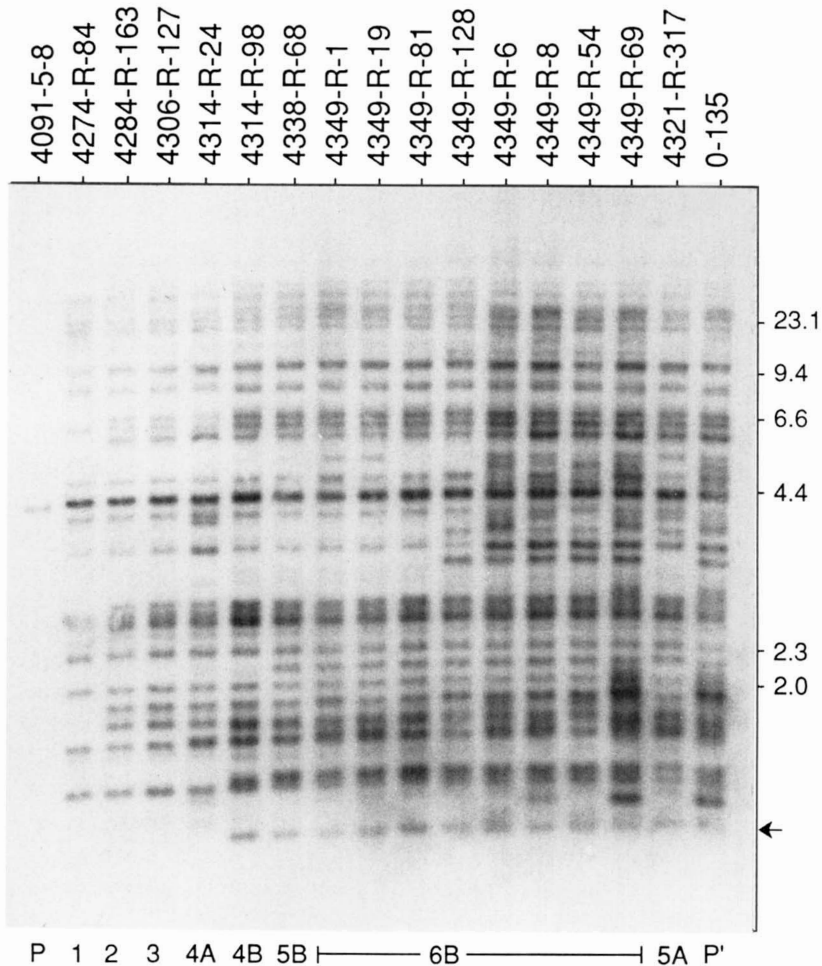

FIGURE 7.—MGR hybridization patterns of parents and selected progeny strains. Genomic DNA was digested with *Eco*RI, electrophoresed, and transferred to a hybridization membrane (Amersham Hy-Bond N). Blots were probed with ^{32}P-labeled pCB586, which carries a MGR sequence component (HAMER *et al.* 1989). The parents of the original cross are labeled "P." The backcross parents are labeled "1," "2,", "3", "4A," "4B," and "5B" as in Figure 2. The analysis also included one hermaphroditic rice pathogen from cross 4321 (5A) and eight progeny strains from the sixth generation cross 4349 (6B). Major differences are apparent in the MGR patterns for the four progeny of cross 4349 to the left, chosen for high fertility (see Table 1 for pathogenicity), and the four progeny of cross 4349 to the right, chosen for high pathogenicity. The positions of DNA size standards, *Hin*dIII fragments of bacteriophage Lambda DNA, are marked. More than one band may correspond to a single MGR element, because *Eco*RI sites are present in some MGR elements. The smallest MGR band (indicated by the arrow) appears to be linked to *Avr1-CO39*.

4321; 5% in cross 4338; 7% in cross 4349 (none were obtained in cross 4306). Some of the subsequent crosses described here produced frequent Buf$^-$ progeny (for example, cross 4386 in Table 4A) and some did not (for example, cross 4363 in Table 3).

This meiotic mutator effect appears to be specific for the *BUF1*$^+$ gene. Mutations in two other melanin biosynthetic genes, *ALB1*$^+$ and *RSY1*$^+$ (CHUMLEY and VALENT 1990), were not detected. In addition, mating type segregated normally in all crosses tested.

DISCUSSION

The genetic basis for differences in cultivar specificity between races of the rice blast pathogen has been previously unknown due to the infertility of field isolates of *M. grisea* that infect rice. Plant pathologists have expected, however, that analysis of the fungus would reveal the action of dominant avirulence genes in determining cultivar specificity (KIYOSAWA 1976; KIYOSAWA 1980). This expectation was based on the overall resemblance of the interaction between rice cultivars and *M. grisea* races to "gene-for-gene" systems of race-cultivar specificity (DAY 1974; CRUTE 1986). That is, like other gene-for-gene systems, rice blast is characterized by numerous races of the fungus and by the presence of dominant blast resistance genes in cultivars of rice.

Classic studies of race-cultivar specificity that defined the gene-for-gene relationship were conducted by HAROLD FLOR, who analyzed the interactions between flax (*Linum usitatissimum*) and flax rust (*Melampsora lini*) (FLOR 1956, 1971). FLOR's studies revealed that a flax cultivar is resistant to a particular race of the pathogen only if the cultivar carries a dominant major resistance gene that corresponds to a dominant gene for avirulence carried by the pathogen. Thus, there is a one-to-one ("gene-for-gene") functional correlation between resistance genes in the host and avirulence genes in the pathogen. Flor's results showed that the flax genome contains a number of genes that can independently express rust resistance, while the genome of the pathogen contains a similar array of genes that can independently express avirulence. A strain of *M. lini* is capable of infecting a particular flax cultivar only if the pathogen lacks all avirulence genes that correspond to the host's resistance genes.

We report here the identification of three unlinked

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major genes that govern cultivar specificity in races of the rice blast fungus. We call these genes "avirulence genes" for consistency with the literature on cultivar specificity, even though we cannot yet determine dominance relationships (see MATERIALS AND METHODS). The three avirulence genes were derived from a nonpathogen of rice through a series of backcrosses to a rice pathogen that is virulent on all three rice cultivars. Other avirulence genes, at least one of which appears to be derived from a rice pathogen, have been identified in a separate series of crosses (B. VALENT, L. FARRALL and F. G. CHUMLEY, unpublished results). The identification of avirulence genes derived both from pathogens and nonpathogens of rice is analogous to results in bacterial host-pathogen systems (TAMAKI *et al.* 1988; WHALEN, STALL and STASKAWICZ 1988; KOBAYASHI, TAMAKI and KEEN 1989).

The segregation in the fourth and fifth-A generation crosses of an avirulence gene specific for each of the first three rice cultivars tested was surprising, because the backcross parents were chosen on the basis of fertility alone (specifically, on the basis of being *Mat1-2* hermaphrodites that mate well with O-135). The avirulence genes may have been retained through the early backcross generations due to linkage to genes required for fertility. The fact that all fifth and sixth generation *Mat1-2* hermaphroditic progeny were avirulent on cultivars M201 and Yashiro-mochi gives this idea some support. In addition, *Avr1-M201* appeared to be linked to *MAT1*. There also may have been other avirulence genes for cultivars CO39, M201 and Yashiro-mochi segregating in the original cross that were lost before they could become apparent in the fourth generation. Because studies with three randomly chosen cultivars led to the identification of avirulence genes, it seems extremely likely that strain 4091-5-8 carries avirulence genes corresponding to other rice cultivars. YAEGASHI and ASAGA (1981) reported circumstantial evidence that another nonpathogen of rice, the field isolate finger millet pathogen WGG-FA40 (one parent of strain 4091-5-8), carries an avirulence gene corresponding to the rice resistance gene *Pi-a*. The suggestion that avirulence genes for rice are common in nonpathogens as well as pathogens of rice makes the mode of action of avirulence genes even more intriguing.

If rice blast is a gene-for-gene system as hypothesized, the identification of avirulence genes predicts the existence of corresponding major resistance genes in rice cultivars. *Avr1-CO39* and *Avr1-M201* are effective against rice cultivars CO39 and M201, which have not been shown to contain blast resistance genes. Inoculations with rice pathogen field isolates from diverse geographic locations suggest that avirulence toward CO39 or M201 is relatively rare. All 19 rice pathogen field isolates we tested were virulent on CO39, and all 34 rice pathogen field isolates we tested were virulent on M201. In contrast to *Avr1-CO39* and *Avr1-M201*, *Avr1-YAMO* is effective toward a rice cultivar with a known blast resistance gene [Yashiro-mochi, which carries the blast resistance gene *Pi-ta* (YAMADA *et al.* 1976)]. Avirulence toward Yashiro-mochi is relatively common among rice pathogen field isolates (J. L. NOTTEGHEM, personal communication; B. VALENT, unpublished results). We have identified a second avirulence gene for Yashiro-mochi, *Avr2-YAMO*, which appears to be derived from a rice pathogen field isolate and is unlinked to *Avr1-YAMO* (B. VALENT, L. FARRALL and F. G. CHUMLEY, unpublished results). A functional correspondence between any of these pathogen avirulence genes and host resistance genes must be tested by crossing the appropriate rice cultivars and inoculating the progeny with fungal strains described in this paper.

Preliminary reports of identification of *M. grisea* cultivar specificity determinants have appeared. LEUNG *et al.* (1988) reported identification of four major genes, and ELLINGBOE, WU and ROBERTSON (1990) reported identification of seven. LEUNG *et al.* (1988) tested the model of single gene segregation of host specificity by a single cross for one of the genes. An attempt to confirm segregation of a second gene led to an unpredicted result, and no attempt was reported to confirm segregation of the two remaining genes. In addition, there appeared to be problems with reproducibility of infection types. Genes were identified by ELLINGBOE, WU and ROBERTSON (1990) on the basis of a single genetic cross, with no attempt to confirm segregation in subsequent crosses. These reports thus remain preliminary, awaiting further genetic analysis.

A major feature of the crosses described in this study was a range of lesion sizes among progeny that were pathogens of rice. Multiple infection assays demonstrated that individual strains were consistently ranked relative to other strains in the progeny sets when comparing lesion types. This reproducibility suggests environmental variation was not the major factor determining lesion size in these experiments. Instead, we suggest that the distributions of lesion types obtained in numerous crosses were due to the segregation of minor genes that determine lesion size on rice. The segregation of minor genes would account for our inability to observe the segregation of avirulence genes until the fourth and fifth generations of the backcrossing program. Only then were numerous progeny that produced type 4 or 5 lesions obtained (Figures 3 and 4). Subsequent crosses demonstrated segregation of major genes for cultivar specificity and minor genes for lesion size in isolation from one another.

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In general, progeny that showed reduced lesion size on one cultivar also showed reduced lesion size on the other cultivars. This observation suggests minor genes may not have cultivar specific effects. Other observations seem to contradict this hypothesis, however. A large increase in lesion size appeared between the fourth and fifth-A generations on CO39 (Figure 3, B and C); 14% of the virulent progeny produced maximum-size type 5 lesions in the fourth and 50% produced type 5 lesions in the fifth-A generations. This result contrasts with a large increase in lesion size on cultivar M201 between the third and the fourth generations (Figure 4); 6% of the virulent progeny produced type 5 lesions in the third, 32% of the virulent progeny produced type 5 lesions in the fourth and 36% produced type 5 lesions in the fifth-A generations. Seventy-six percent of the virulent progeny in the fifth-A generation produced type 5 lesions on Yashiro-mochi. The sparsity of progeny with intermediate lesion types on Yashiro-mochi might suggest that the minor genes have less effect on infection of this cultivar than on CO39 and M201 (Figure 5).

Partial resistance to plant pathogens among crop plants is an essential component to controlling disease in the field (PARLEVLIET 1985). This incomplete resistance is effective in reducing disease, and it is often polygenic. There would be considerable interest in determining if partial resistance is race specific (PARLEVLIET and ZADOKS 1977). It is interesting to speculate that the minor pathogenicity genes identified in this study may correspond functionally to quantitatively inherited "minor resistance genes" in the host. Minor pathogenicity genes could thus exhibit cultivar specific effects depending on the complement of minor resistance genes present in a cultivar. The *M. grisea* system holds much potential for addressing these types of questions.

We initially hypothesized that differences in genome organization between rice pathogens and nonpathogens were responsible for the low viability of ascospores in these crosses. If this had been true, the viability of ascospores should have improved through the backcrossing scheme we pursued. No improvement in the viability of ascospores in crosses with O-135 was observed. To the contrary, only a small fraction of the backcross progeny retained the ability of the laboratory strain 4091-5-8 to mate with O-135 and produce ascospores with 50% viability. Further investigations concerning genome organization differences between pathogens and nonpathogens of rice are being pursued by establishing electrophoretic karyotypes (ORBACH *et al.* 1988; M. J. ORBACH, F. G. CHUMLEY and B. VALENT, unpublished results).

The $BUF1^+$ gene appears to be especially mutable during genetic crosses involving rice pathogen field isolates and some of their progeny. In these same crosses, MGR physical markers resolved as single bands in Southern hybridization behaved as simple Mendelian markers. MGR bands have proven to be valuable physical markers in a RFLP mapping project under way in our laboratory (J. A. SWEIGARD, A. WALTER, B. VALENT and F. G. CHUMLEY, unpublished results).

A major goal of this work was to develop laboratory strains that infect rice, are hermaphrodites, and cross with other rice pathogens to yield many viable ascospores. Hermaphroditic laboratory strains that produce type 4 or type 5 lesions on rice cultivar CO39 were obtained among the progeny in the backcrossing scheme (for example, 4349-R-8 and 4321-R-317 in Table 1). Although some of these strains are interfertile, they show a much lower level of fertility than the best crosses involving nonpathogens of rice. Rare hermaphrodites with the highest level of fertility (see legend to Figure 7 and Table 1) produce type 3 lesions on CO39. Figure 7 also demonstrates that even by the sixth generation, the most fertile hermaphrodites accumulated far fewer MGR bands than the most successful rice pathogens. After the third generation, selection of strains on the basis of highest fertility led to four crosses with very similar lesion type profiles on CO39. These crosses were the fourth generation cross 4314 (Figure 3B), the fifth generation crosses 4338 (Figure 6A) and 4337, and the sixth generation cross 4349. A likely explanation for this apparent block in further improving the fertility of rice pathogen progeny is that the two strains 4091-5-8 and O-135 differ at several loci that determine maximum fertility and at several loci that determine full pathogenicity, and that in some cases these loci are linked in repulsion.

This study has brought the classical genetic basis for *M. grisea* host specificity differences into sharper focus. Cytological studies of fungus-host interactions described here have been undertaken (HEATH *et al.* 1990; R. J. HOWARD and M. A. FERRARI, unpublished results) to aid in determining gene function. The challenge remains to clone the important genes and work towards a biochemical understanding of their modes of action.

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LITERATURE CITED

CATEN, C. E., C. PERSON, J. V. GROTH and S. J. DHAHI, 1984 The genetics of pathogenic aggressiveness in three dikaryons of *Ustilago hordei*. Can. J. Bot. **62:** 1209–1219.

CHUMLEY, F. G., and B. VALENT, 1990 Genetic analysis of melanin deficient, nonpathogenic mutants of *Magnaporthe grisea*. Mol. Plant Microbe Interact. **3:** 135–143.

CRAWFORD, M. S., F. G. CHUMLEY, C. G. WEAVER and B. VALENT, 1986 Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. Genetics **114:** 1111–1129.

CRUTE, I. R., 1986 The genetic basis of relationships between microbial parasites and their hosts, pp. 80–142 in *Mechanisms of Resistance to Plant Diseases*, edited by R. S. S. FRASER. Martinus Nijhoff and W. Junk, Dordrecht.

DAY, P. R., 1974 *Genetics of Host-Parasite Interaction*. W. H. Freeman, San Francisco.

ELLINGBOE, A. H., B.-C. WU and W. ROBERTSON, 1990 Inheritance of avirulence/virulence in a cross of two isolates of *Magnaporthe grisea* pathogenic to rice. Phytopathology **80:** 108–111.

FEINBERG, A. P., and B. VOGELSTEIN, 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13.

FLOR, H. H., 1956 The complementary genetic systems in flax and flax rust. Adv. Genet. **8:** 29–54.

FLOR, H. H., 1971 Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. **9:** 275–296.

HAMER, J. E., L. FARRALL, M. J. ORBACH, B. VALENT and F. G. CHUMLEY, 1989 Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. Proc. Natl. Acad. Sci. USA **86:** 9981–9985.

HEATH, M. C., B. VALENT, R. J. HOWARD and F. G. CHUMLEY, 1990 Interactions of two strains of Magnaporthe grisea with rice, goosegrass, and weeping lovegrass. Can. J. Bot. **68:** 1627–1637.

HOAGLAND, D. R., and D. I. ARNON, 1950 The water-culture method for growing plants without soil. Univ. Calif. Agric. Exp. Stn. Cir. 347 (Rev. Ed.).

HOWARD, R. J., and M. A. FERRARI, 1989 The role of melanin in appressorium function. Exp. Mycol. **13:** 403–418.

KATO, H., 1978 Biological and genetic aspects in the perfect state of rice blast fungus *Pyricularia oryzae* Cav. and its allies, pp. 1–19 in *Mutation Breeding for Disease Resistance*. (Gamma Field Symposia No. 17).

KATO, H., and T. YAMAGUCHI, 1982 The perfect state of *Pyricularia oryzae* Cav. from rice plants in culture. Ann. Phytopathol. Soc. Jpn. **48:** 607–612.

KIYOSAWA, S., 1976 Pathogenic variations of *Pyricularia oryzae* and their use in genetic and breeding studies. SABRAO J. **8:** 53–67.

KIYOSAWA, S., 1980 On the virulence analysis of pathogen race frequencies. Ann. Phytopathol. Soc. Jpn. **46:** 582–593.

KOBAYASHI, D. Y., S. J. TAMAKI and N. T. KEEN, 1989 Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. *tomato* confer cultivar specificity on soybean. Proc. Natl. Acad. Sci. USA **86:** 157–161.

LATTERELL, F. M. 1975 Phenotypic stability of pathogenic races of *Pyricularia oryzae*, and its implication for breeding blast resistant rice varieties, pp. 199–234 in *Proceedings of the Seminar on Horizontal Resistance to Blast Disease of Rice. Colombia Series CE-No. 9*. Centro Internacional de Agricultura Tropical, Cali.

LATTERELL, F. M., and A. E. ROSSI, 1986 Longevity and pathogenic stability of *Pyricularia oryzae*. Phytopathology **76:** 231–235.

LEUNG, H., E. S. BORROMEO, M. A. BERNARDO and J. L. NOTTEGHEM, 1988 Genetic analysis of virulence in the rice blast fungus *Magnaporthe grisea*. Phytopathology **78:** 1227–1233.

MACKILL, A. O., and J. M. BONMAN, 1986 New hosts of *Pyricularia oryzae*. Plant Dis. **70:** 125–127.

ORBACH, M. J., D. VOLLRATH, R. W. DAVIS and C. YANOFSKY, 1988 An electrophoretic karyotype of *Neurospora crassa*. Mol. Cell. Biol. **8:** 1469–1473.

OU, S. H., 1985 Blast, pp. 109–201 in *Rice Diseases*. Commonwealth Agricultural Bureaux, Slough, U.K.

PARLEVLIET, J. E., 1985 Resistance of the non-race-specific type, pp. 501–525 in *The Cereal Rusts, Volume 2*, edited by A. P. ROELFS and W. R. BUSHNELL. Academic Press, New York.

PARLEVLIET, J. E., and J. C. ZADOKS, 1977 The integrated concept of disease resistance; a new view including horizontal and vertical resistance in plants. Euphytica **26:** 5–21.

ROSSMAN, A. Y., R. J. HOWARD and B. VALENT, 1990 *Pyricularia grisea*, the correct name for the rice blast disease fungus. Mycologia **82:** 509–512.

TAMAKI, S., D. DAHLBECK, B. STASKAWICZ and N. T. KEEN, 1988 Characterization and expression of two avirulence genes cloned from *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **170:** 4846–4854.

VALENT, B., and F. G. CHUMLEY, 1987 Genetic analysis of host species specificity in *Magnaporthe grisea*. UCLA Symp. Mol. Cell. Biol. (New Ser.) **48:** 83–93.

VALENT, B., M. S. CRAWFORD, C. G. WEAVER and F. G. CHUMLEY, 1986 Genetic studies of fertility and pathogenicity in *Magnaporthe grisea*. Iowa State J. Res. **60:** 569–594.

WHALEN, M. C., R. E. STALL and B. J. STASKAWICZ, 1988 Characterization of a gene from a tomato pathogen determining hypersensitive resistance in non-host species and genetic analysis of this resistance in bean. Proc. Natl. Acad. Sci. USA **85:** 6743–6747.

YAEGASHI, H., and K. ASAGA, 1981 Further studies on the inheritance of pathogenicity in crosses of *Pyricularia oryzae* with *Pyricularia* sp. from finger millet. Ann. Phytopathol. Soc. Jpn. **47:** 677–679.

YAEGASHI, H., and S. UDAGAWA, 1978 The taxonomical identity of the perfect state of *Pyricularia grisea* and its allies. Can. J. Bot. **56:** 180–183.

YAEGASHI, H., and M. YAMADA, 1986 Pathogenic race and mating type of *Pyricularia oryzae* from Soviet Union, China, Nepal, Thailand, Indonesia and Colombia. Ann. Phytopathol. Soc. Jpn. **52:** 225–234.

YAMADA, M., S. KIYOSAWA, T. YAMAGUCHI, T. HIRANO, T. KOBAYASHI, K. KUSHIBUCHI and S. WATANABE, 1976 Proposal of a new method for differentiating races of *Pyricularia oryzae* Cavara in Japan. Ann. Phytopathol. Soc. Jpn. **42:** 216–219.

YODER, O. C., B. VALENT and F. CHUMLEY, 1986 Genetic nomenclature and practice for plant pathogenic fungi. Phytopathology **76:** 383–385.

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