Genetic Analysis and Rapid Mapping of a Sporulation Mutation in *Magnaporthe grisea*

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A developmental mutation causing abnormal conidium morphology and a reduction in sporulation was found in the rice blast fungus, *Magnaporthe grisea*. The conidium mutant, Con$^-$, was recovered among survivors of an electroporation experiment in which germinated conidia of strain Guy11 were subjected to an electrical shock of 3,000 V/cm. Instead of producing a cluster of four or five conidia sympodially borne on a conidiophore, the Con$^-$ mutant produced an aerial hypha bearing an elongated terminal conidium (4 × 50 μm). The growth rate and sporulation of the Con$^-$ mutant on culture media were 73 and 2.3%, respectively, of that of the wild-type Guy11. Pigment formation in the mutant was reduced and delayed for at least 7 days relative to that of the wild-type strain. Random spore and tetrad analysis showed that the Con$^-$ phenotype was controlled by a single gene (*Con1*). Delayed pigment formation co-segregated with the Con$^-$ phenotype. The Con$^-$ strains did not produce normal appressoria on a siliconized slide or onion epidermis and were nonpathogenic on rice lines susceptible to the wild-type parent. Double mutants obtained from crosses between Con$^-$ strains and a previously described spore morphology (Smo) mutant had a Con$^-$ phenotype, indicating that the Con$^-$ mutation is epistatic to Smo$^-$. As a first step toward physical isolation of the *Con1* gene, bulked segregant analysis was employed to rapidly generate DNA markers flanking the *Con1* gene. A linked marker 7 cM away from the *Con1* locus was obtained.

Additional keywords: development, *Pyricularia oryzae*, random amplified polymorphic DNA.

Rice blast, caused by *Magnaporthe grisea* (anamorph *Pyricularia grisea*, synonym *P. oryzae*) is the most important fungal disease of rice worldwide. Although the sexual stage of the fungus can be produced in the laboratory (Kato and Yamaguchi 1982; Yaegashi 1977), the disease in the field is caused exclusively by the asexual phase of the fungus. Asexual reproduction of *M. grisea* involves the production of aerial conidiophores followed by the formation of conidia within 24 hr. Conidiogenesis in *M. grisea* is holoblastic (Cole 1986), with the apex of the conidiophore swelling to produce the first conidium, followed by the formation of a septum to delimit the conidium. The active apical tip moves to the side to produce the next conidium, until four or five conidia are borne sympodially on a mature conidiophore. A mature conidium is a three-celled structure, and each cell contains an identical nucleus derived from a common mother nucleus. Conidia are readily dislodged from the conidiophore and provide the inoculum for multiple cycles of infection on the rice plant. The polycyclic nature of the disease makes sporulation a key determinant of disease severity (Teng *et al.* 1991). Despite the importance of sporulation in rice blast epidemics, little is known about the genetic control of sporulation of the blast fungus.

Successful development of fertile laboratory strains (Kolmer and Ellingboe 1987; Leung *et al.* 1988; Valent *et al.* 1986; Valent and Chumley 1991) and transformation systems (Parsons *et al.* 1987; Daboussi *et al.* 1989; Leung *et al.* 1990) have made the blast fungus amenable to genetic and molecular analysis. The fungus is a heterothallic ascomycete with bipolar mating control (Kato and Yamaguchi 1982; Yaegashi 1977). It produces asci each containing eight unordered ascospores that allow random spore and tetrad analysis. Much of the current genetic work has focused on host-pathogen recognition and the early infection process (Valent and Chumley 1991). In studies of fertile rice-infecting strains of *M. grisea*, a number of genes controlling pathogenicity and specificity on rice varieties have been defined (Leung *et al.* 1988; Ellingboe *et al.* 1990; Valent *et al.* 1991). Genetic and cytological studies of pigment-deficient mutants showed that melanin synthesis is required for infection of the rice plant (Howard and Ferrari 1989; Chumley and Valent 1990). In an attempt to isolate mutants defective in the early infection process, Hamer *et al.* (1989) identified a class of morphological mutants called Smo (for *spore morphology*), which produce irregularly shaped conidia but grow and sporulate normally.

Table 1. *Magnaporthe grisea* strains used in this study

Strain	Description	Source
Guy11	MAT1-2, field strain pathogenic on rice	Leung et al., 1988
2539	MAT1-2, laboratory strain, nonpathogenic on rice	Leung et al., 1988
Guy11E46	MAT1-2, Con$^-$ mutant of Guy11	This study
926-1, 926-13, 926-14, 926-19	MAT1-1, near-isogenic strains of Guy11 backcrossed to Guy11 eight times	This study
4316-R-1	MAT1-2, Smo$^-$	J. Hamer, Purdue University

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We are interested in understanding the genetic control of sporulation in *M. grisea* as a means to identify, in the sporulation pathway, potential targets for intervention in the disease cycle. To determine the genetic bases of sporulation, we searched for mutants with a clear morphogenetic defect in the sporulation apparatus. We describe here a developmental mutation causing abnormal conidium morphology as well as a reduction in sporulation. The mutation is caused by a single-gene defect that acts epistatically to the Smo mutation. As a first step toward physical isolation of the mutant gene, bulked segregant analysis (Michelmore *et al.* 1991) was applied to rapidly identify DNA markers linked to the target gene.

RESULTS

Phenotypic characterization.

The origins and characteristics of the *M. grisea* strains used in this study are described in Table 1. The Con$^-$ mutant, Guy11E46, was isolated from survivors of Guy11 after electroporation treatment (see Materials and Methods). The conidial morphology of the wild-type strain and that of the Con$^-$ mutant are shown in Figure 1. Instead of producing a cluster of three to five conidia sympodially borne on a conidiophore, the mutant Guy11E46 produced a terminal, elongated, three-celled conidium (4 × 50 μm) (Fig. 1B and D). The percentage of germinating conidia and germination patterns were similar for mutant and wild-type strains (85–90%). The elongated conidia produced normal germ tubes from their terminal or basal cells (80%) and occasionally from both terminal cells (19%). Five-day-old cultures of Con$^-$ strains produced approximately the same number of aerial hyphae as the wild-type Guy11 (4.2/mm^2 of agar surface). The proportion of aerial hyphae differentiated into conidiophores was estimated to be 18–29% in wild-type strains, by counting the number of aerial hyphae and conidiophores over a 12-hr period with a stereomicroscope. However, it was not possible to determine the percentage of conidiogenic aerial hyphae in the Con$^-$ mutants, because conidiophores with elongated conidia could not be easily distinguished from undifferentiated hyphae. The growth rate and sporulation (measured in 12-day-old cultures) of Guy11E46 on oatmeal medium were 73 and 2.3%, respectively, of that of the wild-type Guy11 (Fig. 2). Another distinctive feature of the mutant was delayed and reduced pigment formation. Wild-type strains produced dark pigment after 5 days of growth on oatmeal or potato-dextrose media, whereas the mutant produced only a small amount of pigment after 12 days. Thus, 12-day-old Con$^-$ cultures were light gray, in contrast to the dark-colored wild-type cultures.

Observation of appressorium formation on siliconized slides and on onion epidermal cells indicated that the elongated conidia do not form normal appressoria. Conidia of wild-type strains formed appressoria within 24–48 hr after placement on a hydrophobic surface, whereas conidia of Con$^-$ cultures exhibited indeterminate hyphal growth (Fig. 3A and B). The growth behavior of Con$^-$ conidia on onion epidermis was similar to that observed on siliconized slides. Conidia of Guy11 formed appressoria readily and colonized the epidermal cells within 48 hr after inoculation (Fig. 3C and E). In contrast, Con$^-$ conidia produced long germ tubes, resulting in a network of hyphae on the onion cell surface, without form-

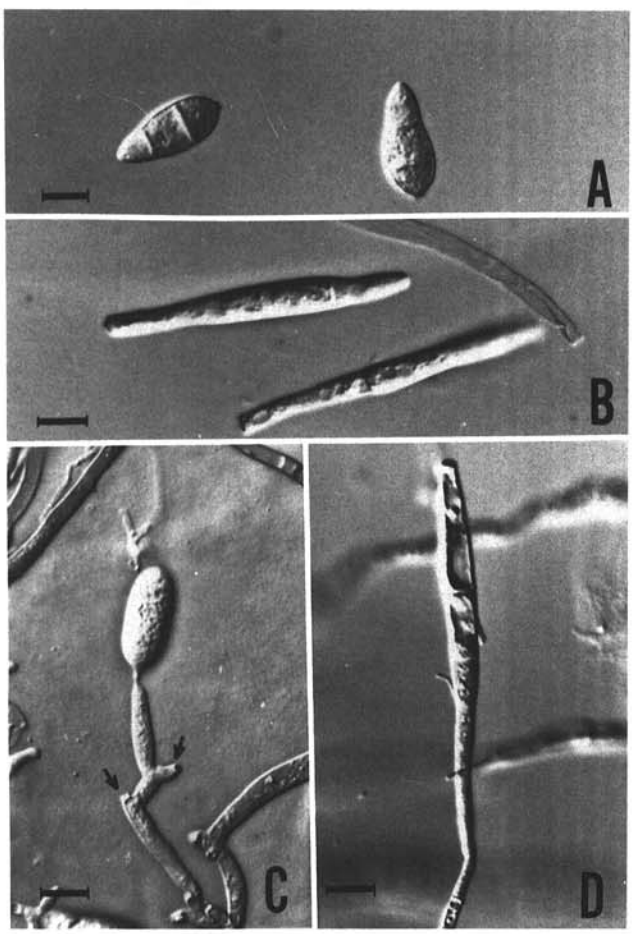

Fig. 1. Morphology of conidia and conidiophores of the wild type and Con$^-$ mutant of *Magnaporthe grisea*. **A,** Three-celled, pear-shaped conidia of the wild-type strain. **B,** Elongated conidia produced by the Con$^-$ mutant. **C,** Wild-type conidiophore with sympodial branching. Mature conidia have dislodged, revealing the denticles (arrows). **D,** Conidiophore of the Con$^-$ mutant, bearing an elongated terminal conidium. Scale bar = 10 μm.

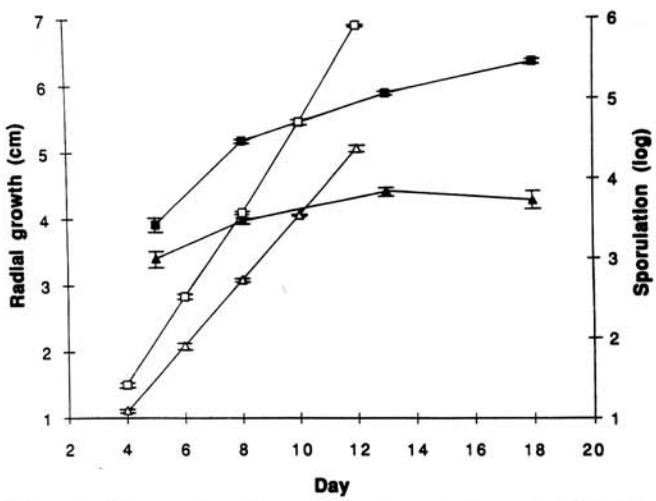

Fig. 2. Radial growth and sporulation of the wild-type strain Guy11 (squares) and the mutant strain Guy11E46 (triangles) of *Magnaporthe grisea*. Radial growth (open symbols) was measured on oatmeal agar. Sporulation (filled symbols) is expressed as the number of conidia per square centimeter of oatmeal agar surface. The data points are averages of three experiments; standard errors are indicated.

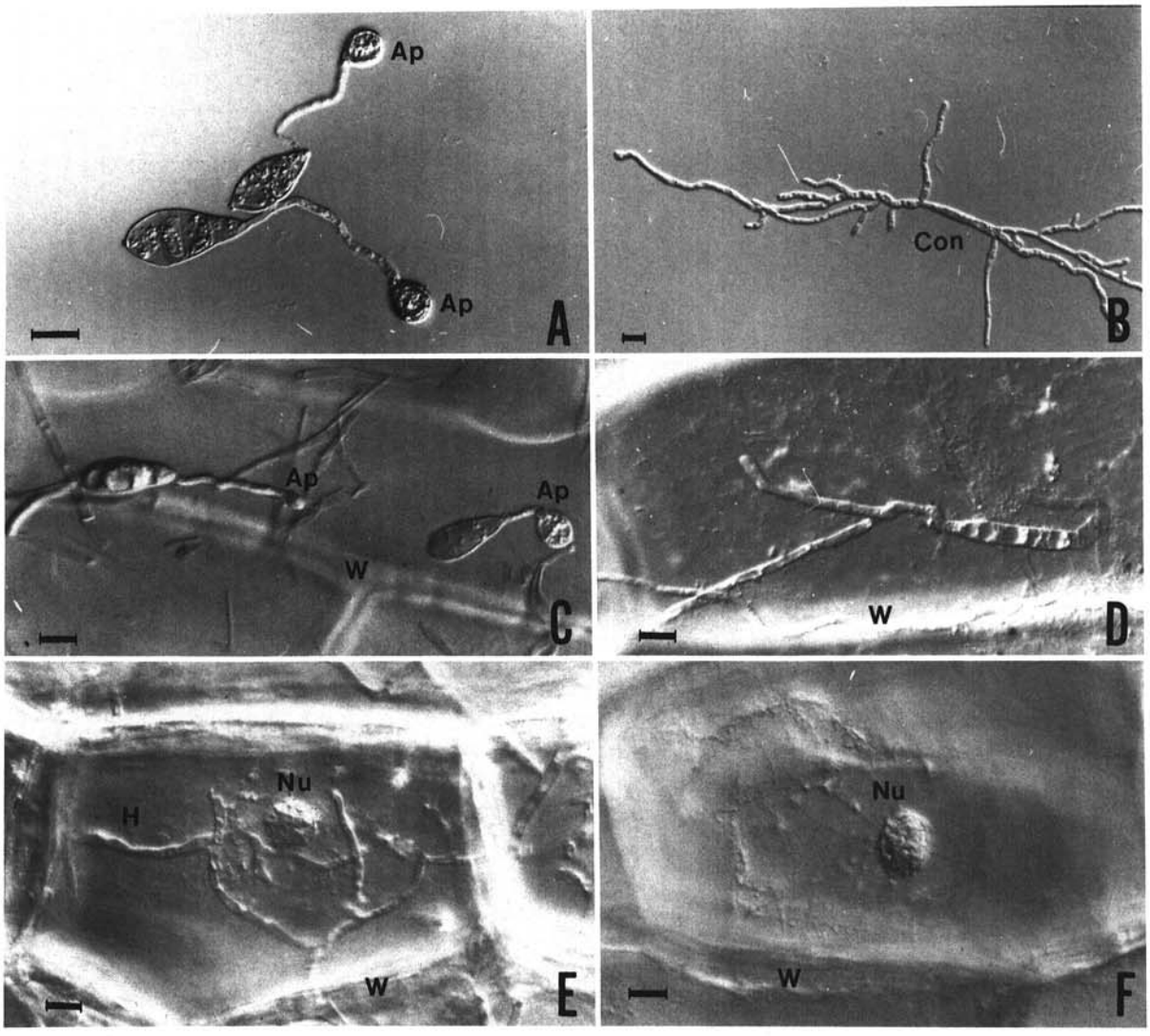

Fig. 3. Formation of appressoria in the wild type and the Con⁻ mutant of *Magnaporthe grisea*. **A,** Wild-type conidia produce normal appressoria (Ap) on a siliconized slide. **B,** A Con⁻ mutant conidium (Con) fails to produce an appressorium and exhibits indeterminate hyphal growth on a siliconized slide. **C,** Wild-type conidia produce appressoria on an onion epidermal strip and penetrate and colonize onion cells 48 hr after inoculation (W = onion cell wall). **D and F,** Con⁻ mutant conidia do not form appressoria and are unable to colonize onion epidermal cells. **E,** Extensive growth of wild-type hyphae (H) inside an onion cell (N = onion cell nucleus). Scale bar = 10 μm.

Table 2. Segregation of random ascospore progeny from crosses between conidium mutants (Con⁻) and wild-type strains (Con⁺) of *Magnaporthe grisea*

Cross number	Cross (Con⁻ × Con⁺)	Generation	No. of progeny		$\chi^2_{1:1}$ [a]
			Con⁻	Con⁺	
1065	Guy11E46 × 2539	F_1	86	122	6.23*
1066	Guy11E46 × 926-14	F_1	11	16	0.93
1070	Guy11E46 × 926-19	F_1	11	7	0.89
1066a	Guy11E46 × 926-1	F_1	10	7	0.53
1068	Guy11E46 × 926-13	F_1	12	18	1.20
1207	1065-12 × 2539	Backcross	23	18	0.61
1206	1066-5 × Guy11	Backcross	46	36	1.22
1246	1066-2 × Guy11	Backcross	30	14	5.82*
1243	1066-4 × Guy11	Backcross	25	21	0.35
1244	1066-12 × Guy 11	Backcross	20	13	1.48
1205	1066-3 × Guy 11	Backcross	29	29	0.00
1245	1066-3 × 1066-21	Sib	19	29	2.08
1241	1066-13 × 1065-23	Sib	15	5	5.00*
1250	1066-1 × 1066-18	Sib	20	12	2.00
Total			357	347	0.14

[a] Asterisk indicates significant deviation from 1:1 ratio at $P = 0.05$.

ing appressoria (Fig. 3D), and no hyphal growth was observed inside the onion cell (Fig. 3F).

Genetic control.

Table 2 shows the segregation of random spores from crosses between Con$^+$ and Con$^-$ strains. The segregation ratios of 11 of the 14 families did not deviate significantly from 1 Con$^-$ to 1 Con$^+$. The pooled segregation ratio, 357 Con$^-$ to 347 Con$^+$, showed a good fit to 1:1 ($\chi^2 = 0.14$, $P > 0.7$), suggesting that the Con$^-$ phenotype is under single-gene control. The skewed segregation observed in three crosses did not appear to be related to the viability of the Con$^-$ phenotype, because excesses of either Con$^-$ or Con$^+$ progeny were observed in all crosses. Single-gene control of the Con$^-$ phenotype was confirmed by tetrad analysis; all eight complete tetrads isolated from a Con$^-$ × Con$^+$ cross (cross 1065) showed a segregation ratio of 4 Con$^-$ to 4 Con$^+$. The wild-type and mutant genes were designated *Con1*$^+$ and *Con1*$^-$, respectively. A reduction in pigment formation was observed in all Con$^-$ strains, and no recombinant was identified in over 1,000 Con$^-$ progeny examined.

An Smo mutation in *M. grisea* was previously described by Hamer *et al.* (1989). The *smo* mutation causes abnormally shaped conidia. The shape of the ascus is also affected when two *smo* mutants are crossed. In contrast to the elongated conidia of Con$^-$ mutants, conidia of Smo$^-$ mutants are short and spherical. To determine the genetic relationship between the *smo* and *Con1*$^-$ mutations, a Smo$^-$ strain was crossed with Con$^-$ strains. Three phenotypes (the wild type, Smo$^-$, and Con$^-$) were observed among the progeny of two Smo$^-$ × Con$^-$ crosses (Table 3). Although the segregation ratios were distorted with an excess of wild-type progeny, the recovery of wild-type progeny indicated that *Smo* and *Con1* are independent genetic loci. To demonstrate the epistatic relationship between the two mutations, complete tetrads were isolated to determine the phenotype of the *smo/Con1*$^-$ double mutant in a nonparental ditype or tetratype tetrad. Of 19 complete and incomplete tetrads isolated, 11 complete tetrads showed a segregation ratio of 2 wild type to 2 Smo$^-$ to 4 Con$^-$, which were interpreted as tetratype tetrads. The tetrad type of the remaining eight incomplete tetrads could not be determined definitively. Attempts to determine the genotype of the Con$^-$ progeny (either *smo/Con1*$^-$ or *Smo/Con1*$^-$) were not successful, because of the infertility of these progeny. Nonetheless, the segregation in the tetratype tetrads showed that *Con1* is epistatic to *Smo*.

Pathogenicity.

To determine whether the Con$^-$ phenotype affects pathogenicity, Guy11E46 was crossed with strain 926-14, a near-isogenic strain of Guy11 developed by backcrossing the F_1 progeny of 2539 × Guy11 to Guy11 eight times. It was necessary to have a near-isogenic Guy11 as a parent so that the segregation of pathogenicity would not be affected by non-pathogenicity or avirulence genes from other strains. This cross (1066 in Table 2) generated progeny with 1 Con$^+$ to 1 Con$^-$ segregation at the *Con1* locus. Progeny from this cross were tested on rice lines Maratelli and 51583 by spray inoculation. All Con$^+$ strains produced normal lesions similar to those caused by Guy11, whereas none of the Con$^-$ strains caused lesions on rice. In this cross, the Con$^-$ phenotype co-segregated with reduced pigmentation and a lack of appressorium formation on a siliconized slide surface (Table 4). To determine whether wounding the plant would compensate for the lack of appressorium formation, conidia of 10 progeny (five Con$^+$ and five Con$^-$) were injected into the leaf sheaths of Maratelli and 51583. As in the spray inoculation tests, normal lesions were produced by the Con$^+$ strains, but not by the Con$^-$ strains.

Genetic mapping by bulked segregant analysis.

Bulked segregant analysis was used to identify random amplified polymorphic DNA (RAPD) markers linked to the *Con1* locus. About 65% of the more than 200 random primers tested gave DNA amplification with genomic DNA of *M. grisea*. Of 125 random primers tested with Con$^+$ and Con$^-$ bulked DNA, 22 showed apparent polymorphisms (Fig. 4A). In the preliminary screen, we included primers that showed absolute differences (absence or presence) or relative differences (in the intensity of amplified bands) between the two bulked DNA samples. The 22 primers were tested with six Con$^+$ and six Con$^-$ progeny to determine the patterns of inheritance of the RAPD markers. Five markers that showed fewer than two recombinants in the 12-progeny sample were further tested with 70 segregants to determine the degree of linkage. Figure 4B shows the banding patterns of a RAPD marker linked to the *Con1* locus. By means of segregation analysis with 70 progeny, four RAPD markers with linkages

Table 3. Segregation of progeny derived from crosses between Con$^-$ and Smo$^-$ strains of *Magnaporthe grisea*

Cross[a]	No. of progeny		
	Con$^-$	Smo$^+$	Wild type
1066-2 × 4316-R-1	12	14	38
1066-3 × 4316-R-1	4	10	23

[a] Parental phenotype Con$^-$ × Smo$^-$.

Table 4. Co-segregation of *Con1* with pathogenicity, appressorium formation, and pigmentation in a cross between strains Guy11E46 (Con$^-$) and 926-14 (Con$^+$) of *Magnaporthe grisea*

Strain	Conidial phenotype	No. of progeny	Pathogenicity[a]		Appressorium formation[b]	Pigment[c]
			Maratelli	51583		
926-14	Con$^+$		+	+	Normal	Normal
Guy11E46	Con$^-$		−	−	None	Reduced
Random ascospore	Con$^+$	10	+	+	Normal	Normal
	Con$^-$	15	−	−	None	Reduced

[a] Pathogenicity on rice lines Maratelli and 51583 assayed by spray inoculation. + = Normal lesions; − = no lesion.
[b] Observed on siliconized slides.
[c] Observed on potato-dextrose agar.

to *Con1* were found. The closest marker was about 7 cM away from the *Con1* gene (Fig. 5). Interestingly, all four markers were on one side of the *Con1* locus. The gene order of the five loci was supported by the recovery of recombinants of double crossovers. There was a close correspondence between the observed and the expected frequencies of double-crossover recombinants between the loci (Table 5), suggesting that little chromosome interference occurred in this region of the chromosome.

DISCUSSION

We have defined a single-gene mutation in *M. grisea* that causes a developmental defect in the formation of conidia. This mutation appears to be stable, since no reversion to wild-type conidia has been observed over many generations of subculturing. The developmental defect in conidial morphology concomitantly reduces sporulation (to 3% of that of the wild type). The Con$^-$ mutation has pleiotropic effects on several characters in the asexual and sexual phases. The primary mutant Guy11E46 has a 27% reduction in growth rate. Growth rate reduction and delayed pigmentation were observed in all Con$^-$ segregants. Although Con$^-$ strains can be crossed with other Con$^+$ strains, we have not succeeded in crossing two Con$^-$ strains of compatible mating type. It is possible that the Con$^-$ mutation also affects certain processes in sexual development. A similar phenomenon of concomitant defects in asexual and sexual phases has been observed in other fungi; for example, strains of *Neurospora crassa* with the aconidial mutation *acon-3* (Matsuyama *et al.* 1974) and strains of *Aspergillus nidulans* with the *stunted* (*stuA*) and *medusa* (*medA*) mutations (Miller 1990) are sexually infertile.

The Con$^-$ mutation is epistatic to the spore morphology mutation Smo previously described (Hamer *et al.* 1989). Although Smo$^-$ strains produce abnormally shaped spores, the development of the conidiophores and conidial production is normal. Conidia of double mutants (*Con1*$^-$/smo) are phenotypically identical to those of the Con$^-$ strains, which indicates that the *Con1* gene controls the formation of conidia before cell shape is affected by the Smo$^-$ mutation. From the phenotypes of Con$^-$ strains, we infer that the defect of the Con$^-$ mutation lies between the development of the conidiophore and the proliferation of conidia at the growing apex. We observed that mutant Guy11E46 produced approximately the same number of aerial hyphae as Guy11. However, we were not able to determine the percentage of conidiogenous hyphae in the Con$^-$ strains, because of difficulty in distinguishing mutant conidiophores from undifferentiated aerial hyphae. It is possible that the reduction in sporulation is due to a combined effect of fewer conidia per conidiophore and fewer conidiogenous hyphae.

Pathogenicity tests on Con$^+$ and Con$^-$ segregants showed that the Con$^-$ phenotype is correlated with a loss of pathogenicity on rice. Analysis of Smo$^-$ strains showed that spore morphology per se is not a primary determinant of pathogenicity, since some *smo* mutants are pathogenic (Hamer *et al.* 1989; Hamer and Givan 1990). Nonetheless, the Smo$^-$ strains obtained from rice-infecting isolates produced fewer and smaller lesions on rice than the parental wild-type strain, which suggests that abnormal cell shape may render the mutant less effective in penetrating and invading the host cells. Nonpathogenicity in Con$^-$ strains, in contrast to strains with the *smo* mutation, appears to be absolute. The lack of pathogenicity is not due to the viability of Con$^-$ conidia, because the percentage of germinating conidia in Con$^-$ strains is similar to that of Con$^+$ strains. Using a siliconized slide and onion epidermis as penetration surfaces, we determined that the abnormally shaped conidia of mutant Guy11E46 do not pro-

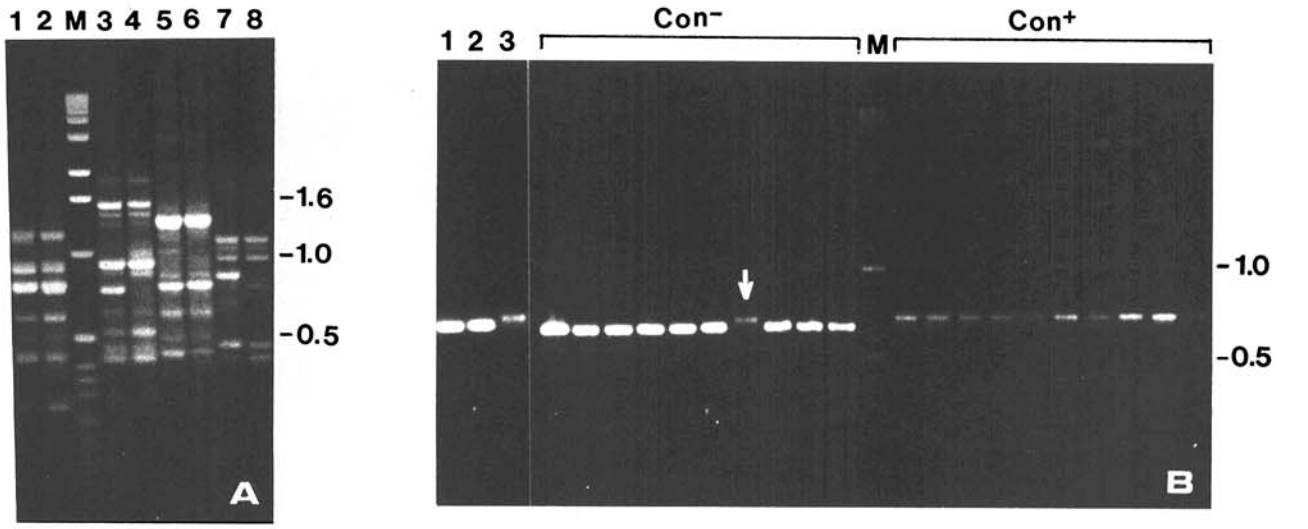

Fig. 4. Ethidium bromide–stained gels illustrating bulked segregant analysis with random amplified polymorphic DNA (RAPD) markers. Samples of genomic DNA of *Magnaporthe grisea* were pooled separately according to phenotype. **A,** RAPD patterns of bulked DNA samples subjected to amplification in a polymerase chain reaction (PCR) with different random primers. In the four pairs of bulked DNA (lanes 1 and 2; lanes 3 and 4; lanes 5 and 6; and lanes 7 and 8), each member of a pair was amplified by the same random primer. Polymorphisms were detected in pairs (3,4) and (7,8). The primers were retested with 70 segregating progeny to determine the linkage between a polymorphic RAPD marker and the *Con1* locus. **B,** Segregation analysis of an RAPD marker linked to the *Con1* locus. Lanes 1, 2, and 3 show the polymorphic marker of the parental strains Guy11, Guy11E46, and 2539, respectively. Lanes Con$^-$ and Con$^+$ contain PCR-amplified products of individual Con$^+$ and Con$^-$ segregants. Note the association between RAPD alleles and the conidium phenotypes. A single recombinant is identified in this sample (arrow). Lane M contains a 1-kb ladder used as a size marker (Gibco BRL, Gaithersburg, MD). Molecular sizes (in kilobases) are indicated on the right.

duce the normal appressoria necessary for penetration (Howard and Ferrari 1989). No colonization of onion cells by Con$^-$ cultures was observed 48 hr after inoculation. The Con$^-$ strains also failed to cause lesions on rice when injected into the leaf sheath, which suggests that the pathogenicity defect or defects cannot be compensated for by wounding. Thus, the lack of the ability to penetrate is only one of the causes of nonpathogenicity. A more extensive co-segregation analysis is needed to determine whether the lack of pathogenicity is a direct result of the mutated *Con1* gene.

Some parallels can be found between the morphogenetic mutants of *A. nidulans* and *M. grisea*. In *A. nidulans*, the *STUNTED* genetic pathway consists of two genetic loci, *stunted* (*stuA*) and *medusa* (*medA*), which modulate the cell type and spatial organization of the conidiophore structure (Miller 1990). The *stuA* mutation blocks the development of medullae and phialides but causes a small number of conidia to be produced directly from the vesicle. The Con$^-$ mutation of *M. grisea* described here appears to be similar to the *stuA* mutation in that normal conidiophore development is blocked. There is no sympodial branching of the conidiophore, and only a terminal conidium is produced on each conidiophore (Figs. 1C and 1D). However, unlike *stuA*, the *Con1*$^-$ mutation also alters the morphology of the conidium. Whether the *Con1* gene is analogous to any of the known sporulation genes in *A. nidulans* can only be addressed by a molecular analysis of the gene.

Gene cloning in *M. grisea* by functional complementation with genomic sequences has been limited by a low transformation frequency (Parsons *et al.* 1987; Leung *et al.* 1990; Valent and Chumley 1991). An alternative strategy is to identify closely linked DNA markers and then approach the target gene by chromosome walking. The development of RAPD markers has greatly improved the efficiency of tagging specific genes. Martin *et al.* (1991) used isogenic tomato lines to identify RAPD markers closely linked to the resistance gene against *Pseudomonas syringae* pv. *tomato*. By combining RAPD markers with bulked segregant analysis, Michelmore *et al.* (1991) identified three RAPD markers closely linked to a gene for resistance to downy mildew in lettuce. Based on restriction fragment length polymorphisms (RFLP), genetic maps of *M. grisea* have been generated using single-copy DNA probes (Skinner *et al.*, in press) and a repetitive DNA sequence (Romao and Hamer 1992). Development of RFLP maps, however, requires substantial time and effort. Furthermore, the application of an RFLP map to locate a gene is often limited to the mapping population derived from two specific parental strains. In contrast, bulked segregant analysis is highly versatile, because it does not require specific breeding lines or genetically characterized mapping populations. The only requirement is a segregating population with sufficient polymorphisms surrounding the target gene.

We have demonstrated that bulked segregant analysis is highly efficient in generating markers closely linked to a specific locus in *M. grisea*. By pooling DNA of 15 progeny of each phenotype, four out of 125 primers yielded genetic markers linked to the *Con1* locus. Using the same procedure, we identified two RAPD markers linked to the mating-type locus of *M. grisea* by screening 60 primers (unpublished data). Thus, the overall percentage of useful primers in bulked segregant analysis of these crosses was about 3% (4/125 and 2/60). The simplicity and speed of bulked segregant analysis allowed a rapid identification of markers linked to the *Con1* gene. Saturation mapping of the *Con1* region with RAPD markers should provide tightly linked markers that could be used to select adjoining sequences in a genomic library. Thus, bulked segregant analysis with RAPD markers alone or used in combination with available RFLP maps removes a major limitation on chromosome walking as a cloning approach in *M. grisea*.

An interesting feature of the Con$^-$ mutation is the extent of pleiotropy. It is possible that *Con1* is a regulatory gene that not only controls the morphogenesis of the conidium, but also plays a role in the pathways of pigment formation, hyphal morphogenesis, appressorium formation, and pathogenicity. We have identified additional sporulation mutations that do not affect vegetative growth but are defective in appressorium formation and pathogenicity (unpublished data). It appears that proper functioning of a set of developmental genes is essential for pathogenesis. Analysis of the *Con1* gene and other sporulation mutations may reveal a link between morphogenetic functions and pathogenesis.

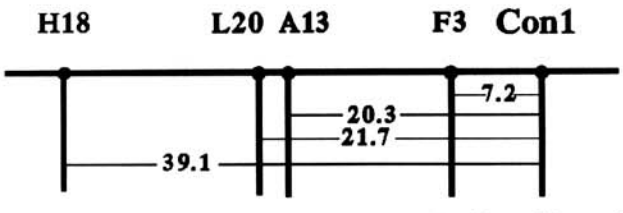

Fig. 5. Linkage map of the region containing the *Con1* locus. The genetic markers are designated by the names of the primers (Operon Technologies, Alameda, CA). Distances are expressed as percentages of recombination.

MATERIALS AND METHODS

Strains and media.

The parental mating strains used for genetic analysis are described in Table 1. Cultures of the fungus were maintained on oatmeal agar (50 g of oatmeal per liter of water). Observations on pigment formation were made on potato-dextrose agar. For culture storage, pieces of Whatman No. 1 filter paper (4 mm^2) colonized by mycelia were kept in a desiccator at –20° C. DNA was extracted from mycelia grown in liquid complete medium (Crawford *et al.* 1986) with constant shaking at ambient temperature (24–26° C) for 5 days. Mycelia

Table 5. Frequencies of observed and expected recombinants resulting from double crossovers around the *Con1* locus

Chromosomal region[a]	Expected	Observed
A13-F3-Con1	0.7	0
L20-F3-Con1	0.9	1
L20-A13-Con1	0.6	1
H18-L20-Con1	4.4	4
H18-A13-Con1	3.9	3
H18-F3-Con1	1.7	1
H18-L20-A13	0.9	2
H18-L20-F3	3.5	4
H18-A13-F3	2.5	2
L20-A13-F3	0.4	0
Total	19.5	18

[a] See Figure 5 for marker designations.

were harvested by filtration through a layer of Miracloth (Calbiochem, La Jolla, CA), washed with distilled water, frozen in a –70° C freezer, and lyophilized.

Isolation of the mutant.

The conidium mutant was recovered among the survivors of an electroporation experiment designed to test the viability of conidia under different intensities of electrical shock. In this instance, germinated conidia of Guy11 were subjected to an electrical shock of 3,000 V/cm from an electroporation device described by Speyer (1990). Surviving cultures were examined under a stereomicroscope for abnormal cultural morphology and growth. One culture, designated Guy11E46, was found to produce abnormally shaped conidia. No similar mutants were recovered in two subsequent electroporation experiments repeated under identical conditions. It was, therefore, not certain whether the sporulation mutant was caused by electric shock or a result of spontaneous mutation.

Crossing and genetic analysis.

Crosses were made by pairing two strains on oatmeal agar in a petri plate 5.6 cm in diameter, incubated at 20° C in the dark for 20–25 days. Random ascospores and ascospores from individual asci were obtained as described previously (Leung and Williams 1985). Random spore progeny were described by a cross number followed by the ascospore number. Tetrad progeny were described by the cross number followed by the ascus number and progeny number from the ascus.

Crosses were made between the mutant strain and the wild-type strain. Segregation ratios observed in F_1 progeny were confirmed by backcrosses and sib-crosses. To generate mutant progeny for pathogenicity tests, the mutant Guy11E46 was crossed with strain 926-14, a near-isogenic strain of Guy11 with the opposite mating type (*MAT-1*). The genetic nomenclature follows that recommended by Yoder *et al.* (1986). Genotypes are described by italics, with the wild-type form in upper case and the mutant form in lower case. Phenotypes are described by Roman type followed by a superscript plus or minus to indicate the wild-type or the mutant phenotype, respectively.

Phenotypic characterization.

The morphology of the primary mutant and its progeny was examined under differential interference contrast optics with an Olympus BHS research microscope. Photomicrographs were taken with Kodak Technical Pan film 2415. Production of aerial hyphae by the mutant and wild-type strains was observed on 3% water agar with an Olympus SZH stereomicroscope at 50×. The relative growth rate and sporulation of the original mutant Guy11E46 and Guy11 were measured on oatmeal agar at 25° C over an 18-day period. Sporulation was estimated by washing conidia from three circular agar plugs (0.8 cm in diameter) in 5 ml of water and counting the conidia with a hemacytometer.

To determine whether the abnormal conidia of Guy11E46 were able to produce normal appressoria, conidia were placed on a siliconized microscope slide that had been coated with Sigmacote (Sigma, St. Louis, MO). Growth behavior of conidia was also examined, by the technique described by Chida and Sisler (1987). Epidermal cells from onion rather than rice were used because of the ease of preparing onion epidermal strips for microscopic examination. This technique has been successfully used to assess the penetration ability of melanin-deficient mutants of the fungus (Chida and Sisler 1987). Onion epidermal strips were treated with chloroform to remove wax and washed thoroughly with water. The inner epidermis was peeled, cut into 5 × 10 mm sections, and floated on sterile distilled water on a microscope slide. Approximately 200 conidia in 10 µl were placed on the epidermal strip and incubated at 24° C. The growth behavior of conidia of the mutant Guy11E46 and the wild-type Guy11 was observed by differential interference contrast microscopy 24 and 48 hr after inoculation.

Mutant derivatives were tested for pathogenicity on two rice lines, Maratelli and IRRI accession 51583, kindly provided by the Rice Germplasm Center of the International Rice Research Institute, Los Baños, Philippines. Rice plants were grown in potting soil containing 55% peat moss, 30% pumice, and 15% sand (Soil Inc., Puyallup, WA) in a growth chamber at 29° C, in a 12-hr photoperiod with irradiance of 250 $\mu E \cdot m^{-2} \cdot sec^{-1}$. Inoculum was prepared by washing conidia from oatmeal agar plates with water plus 0.02% Tween 20 and adjusting to 10^5 spores per milliliter. Two inoculation methods, spray and injection, were used to assay pathogenicity. The spray inoculation method was described by Leung *et al.* (1988). For the injection method, approximately 0.2 ml of the spore suspension was injected into the leaf sheaths of 28-day-old plants with a 6-ml syringe and a 21-gauge needle. The plants were kept at 100% relative humidity for 24 hr at 29° C and then maintained in the growth chamber for another 5 days before disease scoring. Lesions were scored on the spray-inoculated leaves or the new leaf emerged from the inoculated leaf sheath. Types of lesions, as described by Leung *et al.* (1988), were recorded. About five plants were examined per pathogenicity test. All inoculation experiments were conducted twice.

Genetic mapping by RAPD bulked segregant analysis.

The basic principles of using RAPD markers (Williams *et al.* 1990) and bulked segregant analysis (Michelmore *et al.* 1991) have been described. Genomic DNA of *M. grisea* was extracted following the cetyltrimethylammonium bromide procedure of Murray and Thompson (1980). DNA from 15 Con^- and 15 Con^+ F_1 progeny (10 µg of DNA from each) of the cross Guy11E46 × 2539 were pooled separately according to phenotype. The concentration of the bulked DNA was adjusted to 5 ng/µl for use in a polymerase chain reaction. Ten-base random primers were purchased from Operon Technologies (Alameda, CA). DNA amplification was performed in a reaction volume of 12.5 µl containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 5 mM $MgCl_2$; 0.001% gelatin; dATP, dCTP, dTTP, and dGTP, each at 0.1 mM (Sigma); 5 ng of bulked DNA; 2 µM of primer; and 0.3 unit of *Taq* DNA polymerase (Promega, Madison, WI). Amplification was performed in a Perkin-Elmer Model 480 thermocycler programmed for an initial denaturing step of 5 min at 94° C followed by 40 cycles of 1.5 min at 94° C, 1.5 min at 37° C, 2.5 min at 74° C, and 5 min at 74° C. Then 6 µl of the 12.5-µl reaction mix was electrophoresed on 1.5% agarose gel in 0.5× Tris-borate-EDTA buffer at 90 V for 90 min. A 1-kb ladder (Gibco BRL, Gaithersburg, MD) was used as a size marker. After electrophoresis, the gel was stained with ethidium bro-

mide (0.5 μg/ml) for 20 min, and the products were visualized with a UV transilluminator.

Polymorphic loci observed in the two bulked segregant populations were tested for linkages to the *Con1* locus using DNA from 70 individual segregants. Segregation analysis for each RAPD marker was repeated at least twice. Linkage distances between the *Con1* locus and RAPD markers were calculated by the maximum likelihood method (Weir 1989).

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