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Genetic Analysis of Sporulation in *Magnaporthe grisea* by Chemical and Insertional Mutagenesis

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Chemical and plasmid insertional mutagenesis were used to genetically define critical steps in the sporulation pathway of the rice blast fungus. Six mutants with altered conidiogenesis and spore morphology were genetically and phenotypically characterized. Two mutations, designated *con5*$^-$ and *con6*$^-$, completely abolish conidial production. A series of mutations (*con1*$^-$, *con2*$^-$, *con4*$^-$, and *con7*$^-$) downstream from *con5*$^-$ and *con6*$^-$ affect the development of conidia and reduce sporulation. The *con1*$^-$ and *con2*$^-$ mutations block early steps in conidiogenesis, resulting in >90% reduction in sporulation and the production of abnormally shaped conidia. The *con2*$^-$ mutant is completely aconidial in the dark but produces mostly nonseptate or two-celled conidia under continuous illumination. An independent mutation (*con3*$^-$) that regulates light response for sporulation was isolated from the *con2*$^-$ mutant. The *con4*$^-$ and *con7*$^-$ mutants produce conidia of abnormal cell shape and reduce sporulation by approximately 35%. Formation of appressoria, the infection structure required for penetration of plant cells, is blocked in the *con1*$^-$ and *con7*$^-$ mutants, and reduced by 70 and 22% in the *con2*$^-$ and *con4*$^-$ mutants, respectively. Pathogenicity on rice is lost in the *con1*$^-$ and *con7*$^-$ mutants and significantly reduced in the *con2*$^-$ and *con4*$^-$ mutants. Five mutations (*con1-B*$^-$, *con4*$^-$, *con5*$^-$, *con6*$^-$, and *con7*$^-$) derived from plasmid transformation showed perfect cosegregation with hygromycin B resistance, indicating that the mutations are caused by insertional inactivation. Pairwise crosses between mutants suggested linkages between *CON2* and *CON1* (19 cM apart), and between *CON5* and *CON6*. A schematic sporulation pathway is deduced based on mutant phenotypes and the epistatic relationships among different mutations.

Additional keywords: fungal development, *Magnaporthe grisea*, pleiotropy, *Pyricularia*.

Magnaporthe grisea (Hebert) Barr comb. nov. (anamorph *Pyricularia grisea*, synonym *P. oryzae*) is an ascomycetous fungus that causes the rice blast disease (Ou 1985). In nature, the fungus propagates almost exclusively by the production of asexual spores (conidia). Conidiogenesis in *M. grisea* is holoblastic (Cole 1986) such that conidia are formed by a "blowing-out" of the conidiophore apex. A septum is then formed to delimit the conidium. The growing apex moves to the side to produce the next conidium, resulting in 3 to 5 conidia borne sympodially on a mature conidiophore. Each conidium is a three-celled structure, and each cell contains an identical, haploid nucleus derived from a common mother nucleus. A single conidium produces conidiophores and a new crop of conidia approximately 48 h and 60 h after germination, respectively.

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In the field, infection is initiated when a conidium contacts the leaf surface of a rice plant, then produces a germ tube that forms an appressorium at its end. The appressorium produces an infection peg that penetrates the epidermis (Howard 1994), and subsequent colonization of host cells results in the development of a necrotic lesion. The time between infection and the production of secondary inoculum (latent period) varies from 6 to 8 days depending on the level of compatibility between the fungus and the host. Under 100% relative humidity, the fungus sporulates profusely for the next 3 to 4 days, providing abundant inoculum for the next disease cycle. The severity of disease epidemics is therefore proportional to the amount of colonization of plant tissue and the quantity of spores produced in the lesion (Teng et al. 1991).

Despite the significance of sporulation in plant diseases, few studies have investigated the genetics of sporulation in phytopathogenic fungi (Allermann et al. 1983; Turian 1974; Yang et al. 1991). Most of our understanding of the genetics and molecular biology of sporulation comes from two experimental fungi, *Aspergillus nidulans* (reviewed by Miller 1990; Timberlake 1990; Clutterbuck and Timberlake 1992) and *Neurospora crassa* (reviewed by Springer 1993). Without a direct analysis of the sporulation pathway in a plant pathogen, however, much remains unknown concerning the relationship between sporulation and parasitism. As a prerequisite to developing a sporulation-specific control strategy for rice blast, we began to delineate the sporulation pathway of *M. grisea*. We previously reported a single-gene mutation, designated *con1*$^-$, which blocks the development of normal conidia and reduces sporulation by about 97% (Shi and Leung 1994). In this paper, we applied chemical and plasmid insertional mutagenesis to generate additional mutations affecting the sporulation pathway. We have genetically and phenotypically characterized these mutants and propose a genetic pathway of sporulation based on the phenotypes of the mutants and the epistatic relationships among different mutations.

RESULTS

Isolation and phenotypic characterization of mutants.

The origins and relevant characteristics of strains used for mutagenesis are shown in Table 1. Approximately 7,000 survivors obtained after diepoxyoctane mutagenesis were examined using a stereomicroscope. Mutations affecting both the amount of sporulation and vegetative growth were commonly observed; however, only mutants showing near-normal vegetative growth in culture but defective in some aspects of sporulation were examined in detail. The Con1$^-$ mutant phenotype has been previously described (Shi and Leung 1994) and is included here for comparison purposes (Fig. 1B).

The first mutant isolated from diepoxyoctane mutagenesis was designated Con2$^-$. This mutant produced conidiophores with swollen apices but no mature conidia under normal incubation conditions (i.e., on the laboratory bench without direct illumination) (Fig. 1C). When incubated under continuous light (see Materials and Methods), the Con2$^-$ mutant produced conidia that were either single-celled or two-celled (Fig. 1D) in contrast to the three-celled conidia of the wild type (Fig. 1A). Since the Con2$^-$ mutant appeared aconidial in the dark, we screened for mutations that would restore sporulation in the dark. Mycelial fragments of the original Con2$^-$ mutant (Guy11-con2) and 23 ascospore progeny of Con2$^-$ phenotype were spread onto oatmeal agar and incubated in the dark for 4 days. Seven patches that produced abundant conidia were found on the plates of ascospore progeny 1313-64. Single spores were reisolated from these sporulating patches and subcultured to determine if the restored sporulation phenotype was stable. All single-spore cultures showed an identical phenotype. The conidia produced by the reversion mutants were identical to those produced by the Con2$^-$ mutant under continuous light (Fig. 1E). Thus, the mutation appeared to have changed the ability of the Con2$^-$ mutant to produce conidia in the dark but not the morphology of the conidia. Single-spore cultures derived from three separate sporulating patches were subjected to genetic analysis (see below).

Table 1. Characteristics and origins of *Magnaporthe grisea* strains

Strain	Characteristics and origin	Source
Guy11	Field strain, *Mat2*, hermaphroditic, pathogenic on rice	Leung et al. 1988
2539	Laboratory strain, *Mat1*, hermaphroditic, pathogenic on weeping lovegrass	Leung et al. 1988
Guy11E46	*Mat2*, *con1-A* mutation	Shi and Leung 1994
GT1	*Mat2*, circular plasmid transformation of Guy11	This study
Guy11-con2	*Mat2*, diepoxyoctane mutagenesis	This study
1313-64-con3-3	*Mat2*, spontaneous mutation derived from a con2 strain	This study
GT20	*Mat2*, circular plasmid transformation of Guy11	This study
GT399	*Mat2*, REMI transformation of Guy11	This study
GT560	*Mat2*, circular plasmid transformation of Guy11	This study
GT562	*Mat2*, circular plasmid transformation of Guy11	This study

A total of 800 hygromycin B resistant transformants generated by plasmid transformation were examined for abnormal sporulation. Approximately half of the transformants were derived from restriction enzyme mediated integration (REMI) transformation and the other half from conventional transformation using circular plasmid. From this collection of transformants, five sporulation mutants were recovered. One mutant (GT1) had a phenotype identical to that of the *con1*$^-$ mutation (now designated as *con1-A*$^-$) previously described (Shi and Leung 1994) and it was designated as Con1-B$^-$. Since the Con1-B$^-$ mutant was independently derived by insertional mutagenesis, it was characterized along with other mutants. The other four mutants, designated Con4$^-$, Con5$^-$, Con6$^-$, and Con7$^-$, caused abnormal development of either the conidiophores or the spores. The Con4$^-$ mutant produced ellipsoid conidia (Fig. 1F) compared with the pyriform conidia of the wild type (Fig. 1A). The conidia appeared slightly elongated due to a narrowing of the width of the basal cell. Unlike the conidia of the Con1-B$^-$ mutant (Fig. 1B), however, the length of the conidia of the Con4$^-$ mutant seldom exceeded the normal length of a conidium (~25 μm). Another common feature of the Con4$^-$ mutant was that one or two cells of the three-celled conidium were often devoid of cytoplasm, though conidial germination was apparently not affected. The Con5$^-$ mutant was completely aconidial; it produced little aerial hyphae and no conidiophores. The Con6$^-$ mutant produced conidiophores but did not bear any conidia (Fig. 1G). Occasionally, swellings at hyphal tips that resembled conidium initials were found (Fig. 1H). The Con7$^-$ mutant produced abundant conidiophores that bore near-normal numbers of conidia. Both wild-type and abnormally shaped conidia were observed in the Con7$^-$ mutant (Fig. 1I–K). The most common type of conidia was slightly elongated with an attenuated tip cell (Fig. 1J). Another unique feature of the Con7$^-$ mutant was the morphology of the germinated conidia. Instead of producing a threadlike germ tube, the conidia gave rise to stout germ tubes (Fig. 1K).

Table 2 contrasts the morphogenetic features of the sporulation mutants isolated from chemical and insertional mutagenesis. The percentages of conidial germination of Con1-B$^-$ and Con3$^-$ were similar to the wild type after 24 h on water agar. Percent germination of the Con2$^-$ and Con4$^-$ mutants was less than that of the wild type at 24 h but reached >80% after 48 h. Thus, the lower germination rate was apparently due to a delay in either germination or germling growth rather than non-viability of the spores. All mutants except Con1-B$^-$ and Con6$^-$ showed similar or slightly reduced radial growth relative to the parental strain Guy11. The radial growth of the Con1-B$^-$ and Con6$^-$ mutants was 73 and 35% of the wild type, respectively.

The effect of light on sporulation varied among the different mutants (Table 2). In general, light is not absolutely required for sporulation in *M. grisea* but the level of sporulation can be increased by 100- to 1,000-fold under continuous light. For example, sporulation in Guy11 was increased by approximately 500-fold after exposure to continuous light for 96 h (Table 2). The Con5$^-$ and Con6$^-$ mutants were completely aconidial regardless of illumination. The Con2$^-$ mutant was aconidial in the dark, but sporulation was restored to about 3% of the wild-type level under continuous illumination. The Con3$^-$ and Con7$^-$ mutants had apparently lost the

response to light stimulation and produced abundant spores without direct illumination.

Despite abnormal spore morphology, conidia of the Con2$^-$ and Con4$^-$ mutants produced appressoria on siliconized slides (Fig. 2C and D). Conidia of the Con3$^-$ mutant behaved similarly to those of the Con2$^-$ mutant in appressorium formation. The percentage of germlings producing appressoria, however, was significantly reduced relative to the wild type (Table 2). Germlings of the Con1-B$^-$ and Con7$^-$ mutants produced no appressoria (Fig. 2B and F). Occasionally, the Con6$^-$ mutant produced appressorium-like structures at the hyphal tips (Fig. 2E), a characteristic not observed in other mutants or the parental strain.

Four mutants that produced spores (Con1-B$^-$, Con2$^-$, Con4$^-$, Con7$^-$) were tested for pathogenicity on rice line 51583 and Maratelli by spray inoculation and leaf sheath injection (Table 3). The two rice lines used for pathogenicity tests differed in their susceptibility to Guy11. The number of

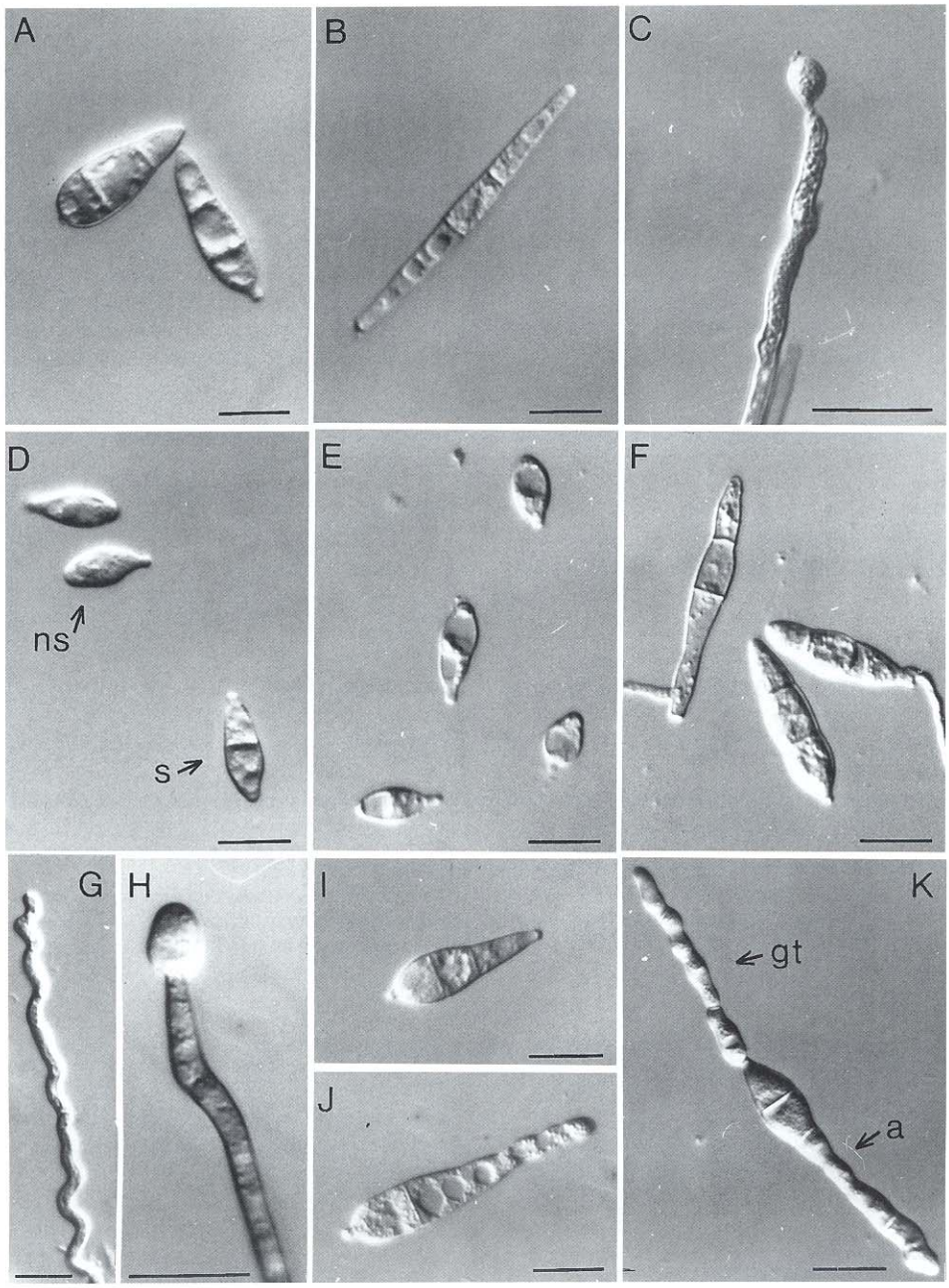

Fig. 1. Morphology of *Magnaporthe grisea* sporulation mutants. **(A)** Wild type. Three-celled, pyriform conidia of wild type. **(B)** Con1$^-$. Elongated conidia. **(C, D)** Con2$^-$. Only conidial initial formed in the absence of direct illumination **(C).** Under continuous illumination, conidia with single (s) or no septum (ns) are formed **(D). (E)** Con3$^-$. Conidia formed in the dark. Conidium morphology identical to that of con2. **(F)** Con4$^-$. Slightly elongated conidia with a narrowing of the basal cell. **(G, H)** Con6$^-$. Conidiophore only **(G).** Occasionally, a swollen hyphal tip resembling a conidium initial **(H)** is formed, but no mature conidia are ever produced. **(I–K)** Con7$^-$. A variety of spore forms are produced: wild-type-like conidium **(I)** and conidum with an attenuated tip (J). Conidium with an attenuated tip (a) produces a stout germ tube (gt) shown in **(K).** Scale bar = 5 μm.

lesions formed on 51583 was consistently higher than that on Maratelli ($P < 0.05$). Six days after spray inoculation, Guy11 produced normal susceptible lesions on the rice plants at two inoculum concentrations (10^4 and 10^5/ml). No lesions were observed on plants inoculated with the Con1-B$^-$ and Con7$^-$ mutants at either spore concentration. At the high concentration (10^5/ml) some lesions were produced by the Con2$^-$ and Con4$^-$ mutants but the number of lesions per leaf was significantly less than that produced by Guy11. Furthermore, the development of a mature lesion was delayed about 2 days relative to the wild type. Lesions produced by the Con2$^-$ mutant were approximately the same size as those caused by the wild type (5 to 6 mm long) whereas those produced by the Con4$^-$ mutant were smaller (~3 mm long 7 days after inoculation). Leaf-sheath injection was performed to determine whether pathogenicity of the mutants could be enhanced if the penetration step was bypassed by artificial wounding. The results were similar to those obtained with spray inoculation; both the Con1-B$^-$ and Con7$^-$ mutants were nonpathogenic whereas the Con2$^-$ and Con4$^-$ mutants caused the same type of lesions as in spray inoculation (Table 3). Reduced number of lesions were produced on Maratelli by the Con2$^-$ and Con4$^-$ mutants, reflecting the high level of resistance in Maratelli as observed in the assay with Guy11.

Genetic analysis of mutant phenotypes.

The inheritance of the mutant phenotypes was demonstrated by random spore segregation in the F_1, sib-cross, and backcross progeny (Table 4). The segregation patterns of the Con1-B$^-$, Con2$^-$, Con4$^-$, and Con5$^-$ phenotypes fitted the 1 wild type/1 mutant ratio, indicating that the mutant phenotypes were under single-gene control. Tetrad analysis conducted for crosses with the Con1-B$^-$, Con2$^-$, Con4$^-$, and Con5$^-$ mutants all confirmed single-gene inheritance (data not shown). In crosses that involved the Con6$^-$ and Con7$^-$ mutants, significant deviations from the expected 1:1 ratio were observed. However, single-gene inheritance was supported by the cosegregation of the mutant phenotypes with hygromycin B resistance (see Table 5 below).

The genetic control of light-sensitivity in the Con3$^-$ mutant was determined by backcrossing the mutant to the wild type and to the Con2$^-$ mutant (Table 6). The Con3$^-$ mutant was distinguished from the Con2$^-$ mutant by the production of abnormal spores without light stimulation. When the Con3$^-$ mutant was crossed to wild type 2539, phenotypes of the random ascospore progeny fell into three categories: wild type, Con2$^-$, and Con3$^-$ (Table 6, cross no. 1323), suggesting that the Con3$^-$ phenotype was not a reversion of the *con2* locus but controlled by mutation at an independent locus. To dem-

Table 2. Comparison of radial growth, germination, appressorium formation, and sporulation among mutants of *Magnaporthe grisea*

Strain	Phenotype	Spore morphology[u]	Radial growth[v]	Germination %[w]	Appressorium formation %[x]	Sporulation (10^3/ml)[y]	
						Dark	Light
GT1	Con1-B$^-$	Elongated	28.5 ± 0.6 (c)	80.6 ± 6.3 (b)	0	0	3.4 ± 1.2 (d)
Guy11-con2	Con2$^-$	Single or two-celled	37.6 ± 0.9 (ab)	62.6 ± 6.4 (d)	44.4 ± 5.3 (c)	0	9.1 ± 5.5 (d)
1313-64-con3-3	Con3$^-$	Single or two-celled	37.5 ± 1.7 (ab)	75.8 ± 5.1 (bc)	46.0 ± 7.1 (c)	121.6 ± 29.0 (a)	215.6 ± 32.4 (a)
GT20	Con4$^-$	Three-celled, ellipsoid	36.3 ± 1.5 (b)	68.6 ± 5.8 (cd)	66.7 ± 16.1 (b)	0.13 ± 0.02 (b)	107.5 ± 5.4 (c)
GT399	Con5$^-$	Aconidial	39.5 ± 0.6 (a)	NA[z]	NA	0	0
GT560	Con6$^-$	Aconidial	13.6 ± 1.3 (d)	NA	NA	0	0
GT562	Con7$^-$	Mixture of abnormal spores	36.3 ± 1.5 (b)	80.8 ± 4.4 (ab)	0	113.7 ± 31.2 (a)	110.0 ± 10.6 (c)
Guy11	Wild type	Three-celled, pyriform	39.3 ± 0.9 (a)	87.8 ± 7.8 (a)	89.7 ± 7.5 (a)	0.28 ± 0.18 (b)	164.4 ± 38.4 (b)

[u] See Figure 1 for details.
[v] Radial growth (mm) was measured 9 days after incubation on oatmeal agar at 26°C. Average of four replicates ± standard deviation. Values with same letter in parentheses are not significantly different at $P = 0.05$ level.
[w] Germination percent was average of six microscope views (about 50 conidia examined per view) 24 h after spreading conidia on 3% water agar ± standard deviation. Values with same letter in parentheses are not significantly different at $P = 0.05$ level.
[x] Percent appressorium formation was average of appressoria formed per germlings observed in three slide preparations. Approximately 100 germlings observed per slide ± standard deviation. Values with same letter in parentheses are not significantly different at $P = 0.05$ level.
[y] Sporulation assay was described in detail in the text. Values with same letter in parentheses are not significantly different at $P = 0.05$ level.
[z] Not applicable.

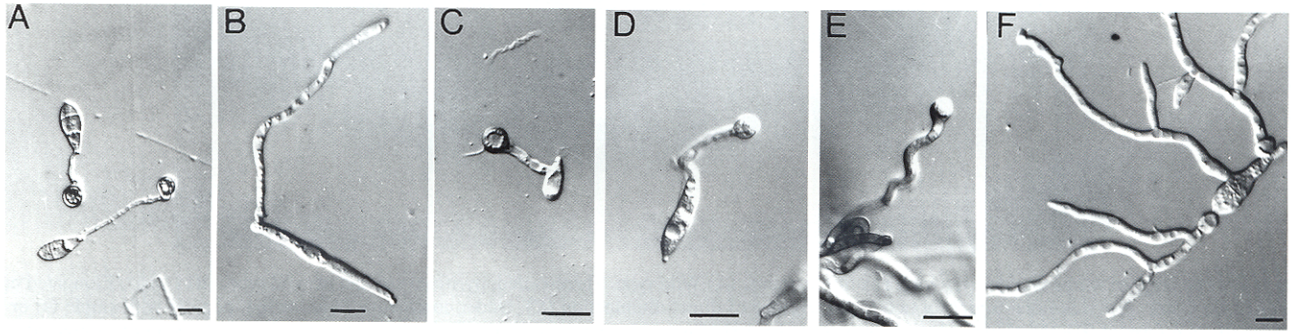

Fig. 2. Appressorium formation in spore morphology mutants of *Magnaporthe grisea*. **(A)** Appressoria produced by the wild-type strain. **(B)** No appressorium produced by the Con1$^-$ mutant. **(C)** The Con2$^-$ mutant produces appressorium. **(D)** Appressorium produced by the Con4$^-$ mutant. **(E)** Appressorium-like structure at hyphal tip of the Con6$^-$ mutant. **(F)** No appressorium formed by the Con7$^-$ mutant. Scale bar = 5 μm.

onstrate that the Con3⁻ phenotype was a result of double mutations (*con2⁻/con3⁻*), a cross was made between the Con3⁻ mutant and a Con2⁻ strain. A 1:1 segregation of Con2⁻ and Con3⁻ phenotypes in random ascospore progeny was observed (Table 6, cross no. 1327), confirming that the Con3⁻ phenotype involves a locus different from the *con2⁻* gene.

To determine whether the loss of light sensitivity in the Con7⁻ mutant was controlled by the *con7⁻* gene, progeny from cross 1540 (GT562 × 2539 in Table 4) were assayed for sporulation with and without light stimulation. Clear segregation of light sensitivity was not observed due to the variability in sporulation among the segregating progeny (data not shown).

Evidence that mutant phenotypes were caused by insertional inactivation.

The mutations obtained from transformation might have arisen either by insertion of pAN7-2 into the corresponding loci or by spontaneous mutations of these genes during or after the transformation process. Cosegregation between hygromycin B resistance and a mutant phenotype would indicate that the mutation was caused by insertion of pAN7-2 into the corresponding loci. It is possible to have hygromycin B resistance associated with wild-type progeny in the case of multiple plasmid integrations. Perfect cosegregations of mutant phenotypes with hygromycin B resistance were observed for *con1-B⁻*, *con4⁻*, *con5⁻*, *con6⁻*, and *con7⁻* mutations, indicating that all five genes were caused by the insertion of pAN7-2 (Table 5). No wild-type progeny with hygromycin B resistance were recovered from these crosses, suggesting that only a single site was inserted in each of the mutant transformants.

Southern blot analysis was conducted to determine the integration pattern in each of the five insertional mutants. Genomic DNA of the mutant parents was digested with *Apa*I and *Eco*RI. Since there is no *Apa*I recognition site within pAN7-2, the number of hybridization bands would indicate the number of integration sites. When *Apa*I-digested DNA was probed with pAN7-2, a single hybridization band was detected for each insertional mutant (Fig. 3A, lanes 1–5), thus confirming our genetic interpretation that all five mutants were caused by single-site integrations. The sizes of the bands were different for all five insertional mutants, providing evidence that the inactivated genes are at different loci of the genome. Southern analysis of *Eco*RI-digested DNA further indicated that all mutants were caused by single-copy insertions (Fig. 3B). Since there are two *Eco*RI sites in pAN7-2, *Eco*RI-digested DNA gave the internal fragments of either 2.4 kb (*con5⁻*, *con4⁻*, and *con1-B⁻* in lanes 3, 4, and 5, respectively) or 6.8 kb (*con7⁻* and *con6⁻* in lanes 1 and 2, respectively). The additional bands of varying sizes in each mutant were flanking hybrid fragments. The *con5⁻* mutant gave only one flanking fragment of approximately 1 kb, suggesting a possible deletion of a portion of the inserted plasmid; alternatively, small fragments may have run off the gel. Cosegregations between plasmid integrations and mutant phenotypes were demon-

Table 3. Pathogenicity of sporulation mutants of *Magnaporthe grisea* on rice

Strain	Phenotype	Rice line	Spray inoculation[w] 10^4	Spray inoculation[w] 10^5	Leaf sheath injection[x]	Latent period[y]
Guy11	Wild type	51583	3.8 (a)	17.8 (a)	+	6
		Maratelli	1.7 (b)	6.9 (b)	+	6
GT1	Con1-B⁻	51583	0	0	0	NA[z]
		Maratelli	0	0	0	NA
Guy11 -con2	Con2⁻	51583	0.3 (c)	1.2 (c)	+	8
		Maratelli	0	0.3 (c)	0	8
GT20	Con4⁻	51583	0.3 (c)	3.3 (bc)	+	8
		Maratelli	0.1 (c)	0.1 (c)	0	8
GT562	Con7⁻	51583	0	0	0	NA
		Maratelli	0	0	0	NA

[w] Spray inoculation done at two concentrations (spores/ml). Average number of lesions per inoculated leaf. Data pooled from three experiments. About 18 plants were scored per mutant. Values with same letter in parentheses are not significantly different at $P = 0.05$ level.
[x] Injected with a 10^5 spore/ml suspension. Only qualitative assessment of lesions was made: + = presence of lesions, – = no lesion. Experiments were repeated three times.
[y] Days from inoculation to the appearance of a mature lesion (~50 mm long).
[z] Not applicable.

Table 4. Inheritance of sporulation mutations of *Magnaporthe grisea*

Mutation	Cross no.	Cross type (mutant × WT)[w]	Random spore progeny WT	Mutant	X^{2x}
Con1-B⁻	1521	GT-1 × 2539	43	30	2.32
Con2⁻	1313	Guy11-con2 × 2539	86	72	1.24
	1318	1313-45 × 2539	33	38	0.35
	1319	1313-37 × 2539	44	32	1.89
	1320	1313-16 × Guy11	28	36	1.00
Total			191	178	0.46
Con4⁻	1522	GT-20 × 2539	30	30	0.00
	1525	1522-12 × 2539	20	14	1.06
	1526	1522-14 × 2539	10	6	1.00
	1527	1522-22 × 1522-27	7	5	0.33
Total			67	55	1.18
Con5⁻	1531	GT-399 × 2539	55	34	4.96[y]
	1534	1531-71 × 1537-66	13	14	0.04
	1535	1531-71 × 1531-68	21	20	0.02
	1537	1531-78 × 2539	12	42	16.67[z]
Total			101	110	0.38
Con6⁻	1539	GT560 × 2539	50	14	20.25[z]
Con7⁻	1540	GT562 × 2539	24	44	5.88[z]

[w] WT = wild type.
[x] Tested against 1:1 ratio.
[y] Significant at $P = 0.05$.
[z] Significant at $P = 0.01$

Table 5. Cosegregation between hygromycin B resistance and mutant phenotypes in progeny of tagged mutants of *Magnaporthe grise*

Mutant	No. of crosses	Number of ascospore progeny[z] Hyg R/Mutant	Hyg R/WT	Hyg S/Mutant	Hyg S/WT
Con1-B⁻	1	30	0	0	43
Con4⁻	6	94	0	0	99
Con5⁻	7	177	0	0	101
Con6⁻	6	136	0	0	50
Con7⁻	5	126	0	0	24

[z] Hyg R = hygromycin B resistant, Hyg S = hygromycin B sensitive, WT = wild type.

onstrate that the Con3⁻ phenotype was a result of double mutations (*con2⁻/con3⁻*), a cross was made between the Con3⁻ mutant and a Con2⁻ strain. A 1:1 segregation of Con2⁻ and Con3⁻ phenotypes in random ascospore progeny was observed (Table 6, cross no. 1327), confirming that the Con3⁻ phenotype involves a locus different from the *con2⁻* gene.

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GT562	Con7⁻	51583	0	0	0	NA
		Maratelli	0	0	0	NA

[w] Spray inoculation done at two concentrations (spores/ml). Average number of lesions per inoculated leaf. Data pooled from three experiments. About 18 plants were scored per mutant. Values with same letter in parentheses are not significantly different at $P = 0.05$ level.
[x] Injected with a 10^5 spore/ml suspension. Only qualitative assessment of lesions was made: + = presence of lesions, – = no lesion. Experiments were repeated three times.
[y] Days from inoculation to the appearance of a mature lesion (~50 mm long).
[z] Not applicable.

Table 4. Inheritance of sporulation mutations of *Magnaporthe grisea*

Mutation	Cross no.	Cross type (mutant × WT)[w]	Random spore progeny WT	Mutant	X^{2x}
Con1-B⁻	1521	GT-1 × 2539	43	30	2.32
Con2⁻	1313	Guy11-con2 × 2539	86	72	1.24
	1318	1313-45 × 2539	33	38	0.35
	1319	1313-37 × 2539	44	32	1.89
	1320	1313-16 × Guy11	28	36	1.00
Total			191	178	0.46
Con4⁻	1522	GT-20 × 2539	30	30	0.00
	1525	1522-12 × 2539	20	14	1.06
	1526	1522-14 × 2539	10	6	1.00
	1527	1522-22 × 1522-27	7	5	0.33
Total			67	55	1.18
Con5⁻	1531	GT-399 × 2539	55	34	4.96[y]
	1534	1531-71 × 1537-66	13	14	0.04
	1535	1531-71 × 1531-68	21	20	0.02
	1537	1531-78 × 2539	12	42	16.67[z]
Total			101	110	0.38
Con6⁻	1539	GT560 × 2539	50	14	20.25[z]
Con7⁻	1540	GT562 × 2539	24	44	5.88[z]

[w] WT = wild type.
[x] Tested against 1:1 ratio.
[y] Significant at $P = 0.05$.
[z] Significant at $P = 0.01$

Table 5. Cosegregation between hygromycin B resistance and mutant phenotypes in progeny of tagged mutants of *Magnaporthe grise*

Mutant	No. of crosses	Number of ascospore progeny[z] Hyg R/Mutant	Hyg R/WT	Hyg S/Mutant	Hyg S/WT
Con1-B⁻	1	30	0	0	43
Con4⁻	6	94	0	0	99
Con5⁻	7	177	0	0	101
Con6⁻	6	136	0	0	50
Con7⁻	5	126	0	0	24

[z] Hyg R = hygromycin B resistant, Hyg S = hygromycin B sensitive, WT = wild type.

Table 6. Inheritance of Con3⁻ mutation recovered from a Con2⁻ strain of *Magnaporthe grisea*

Cross number	Parents	Predicted genotype	Random spore progeny			Expected ratio	X^2
			WT[z]	Con2⁻	Con3⁻		
1323	1313-64-con3-3 × 2539	*con2⁻/con3⁻* × *CON2⁺/CON3⁺*	34	24	22	2:1:1	1.90
1327	1313-64-con3-3 × 1313-21	*con2⁻/con3⁻* × *con2⁻/CON3⁺*	0	41	44	0:1:1	0.11

[z] Wild type.

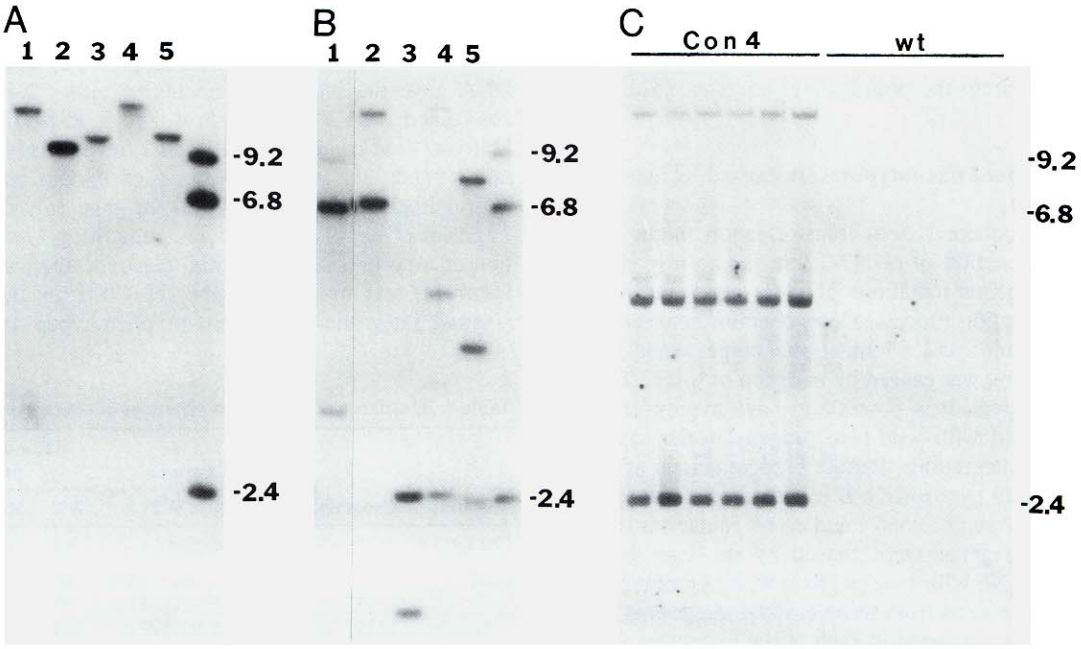

Fig. 3. Southern analysis of insertional mutants of *Magnaporthe grisea*. **(A)** *Apa*I-digested DNA from five mutants. Lanes 1, 2, 3, 4, and 5 contain DNA of *con7⁻*, *con6⁻ con5⁻*, *con4⁻*, and *con1-B⁻* mutants, respectively. **(B)** *Eco*RI-digested DNA of the same mutants as in **(A)**. **(C)** Cosegregation between integration pattern with mutant phenotype. *Eco*RI-digested DNA from hygromycin B resistant/*con4⁻* progeny and hygromycin B sensitive/wild type (wt) progeny from a cross between the *con4⁻* mutant and Guy11. pAN7-2 is used as hybridization probe in all cases. The right-hand most lanes in panels A and B contain linearized pAN7-2, and the 6.8 and 2.4 kb *Eco*RI fragments as size markers.

strated in all mutant crosses (data not shown). As an illustration, Southern analysis of progeny from a *con4* × wild type cross is shown in Fig. 3C.

Epistatic relationship among mutations.

All identified mutants were intercrossed to determine the epistatic relationships among the loci. However, not all crosses were fertile, and hence the order of some of the genetic blocks was inferred based on the phenotypes of the mutants.

Repeated attempts to cross GT1 (*con1-B⁻*) with Guy11-con2 (*con2⁻*) were not successful; hence an analysis of crosses between Guy11E46 (*con1-A⁻*) and two *con2⁻* strains was conducted, recognizing that the allelic relationship between *con1-A⁻* and *con1-B⁻* has not been genetically proven. Progeny obtained from crosses between *con1-A⁻* and *con2⁻* strains gave three phenotypes: wild type, Con1-A⁻, and Con2⁻. Since the *con1-A⁻* (as well as *con1-B⁻*) strain exhibited a light pigmentation on agar medium (Shi and Leung 1994), this provided an additional marker to identify the double mutants (*con1-A⁻/con2⁻*). All double mutants showed the Con2⁻ phenotype suggesting that *con2⁻* was epistatic to *con1-A⁻*. However, in both crosses (Table 7, crosses 1325 and 1326), the ratio of wild type/Con1-A⁻/Con2⁻ progeny deviated significantly from the expected 1:1:2 segregation ratio. The deviation was caused by a deficit in wild-type recombinants and an excess of the parental types, suggesting possible linkage between the two loci (see linkage relationship below). Using the same approach, the epistatic relationships among *con5⁻*, *con6⁻*, and *con7⁻* were determined (Table 7). Similar to the *con1-A⁻* × *con2⁻* crosses, an excess of parental types was observed in progeny of *con5⁻* × *con6⁻* crosses, presumably due to linkage between the two genes (see below). Since the *con5⁻* mutant lacks conidiophores, it is almost certain that *CON5* functions upstream of the other genes. The interrelationships among *con1⁻*, *con4⁻*, and *con7⁻* remain unclear due to infertility of the crosses between these mutants.

Linkage relationship.

Although a systematic linkage analysis of all the mutations was not made, pairwise crosses conducted for the epistasis analysis allowed us to detect linkages in two pairs of loci. Crosses between *con1-A⁻* and *con2⁻* (crosses 1325 and 1326 in Table 7) yielded significantly fewer wild-type progeny than expected if the two genes were unlinked. The segregation data suggested that the *CON1* and *CON2* loci were about 19 cM apart. To further determine whether *CON1* and *CON2* were linked, a random amplified polymorphism DNA (RAPD) marker L-20 that was previously mapped 20 cM from *CON1*

Table 7. Epistatic relationships between sporulation mutations of *Magnaporthe grisea*

Locus pair	Cross no.	Parents	Genotypes	No. of random spore progeny			Expected ratio	X^2 [r]
				WT[q]	Mutant			
con2/con1	1325	1313-16 × Guy11E46[s]	*con2⁻/CON1⁺* × *CON2⁺/con1⁻*	8	45[t]	35[u]	1:2:1	16.61[v]
	1326	1313-36 × Guy11E46	*con2⁻/CON1⁺* × *CON2⁺/con1⁻*	2	12[t]	12[u]		7.85[w]
con5/con6	1543	1539-50 × GT399	*CON5⁺con6⁻* × *con5⁻/CON6⁺*	6	22[x]	26[y]	1:2:1	16.67[v]
	1548	1539-42 × GT399	*CON5⁺/con6⁻* × *con5⁻/CON6⁺*	0	24[x]	23[y]		22.53[v]
con5/con7	1544	1540-22 × GT399	*CON5⁺/con7⁻* × *con5⁻/CON7⁺*	10	21[x]	7[z]	1:2:1	0.89
con6/con7	1542	1539-5- × GT562	*con6⁻/CON7⁺* × *CON6⁺/con7⁻*	9	19[y]	11[z]	1:2:1	0.33
	1547	1540-22 × GT560	*CON6⁺/con7⁻* × *con6⁻/CON7⁺*	17	13[y]	5[z]		9.78[v]
	1549	1539-42 × GT562	*con6⁻/CON7⁺* × *CON6⁺/con7⁻*	18	33[y]	10[z]		2.51

[q] Wild type.
[r] Tested against 1:2:1 ratio, 2 df.
[s] Guy11E46 carries the *con1-A* allele.
[t] Con2⁻.
[u] Con1⁻.
[v] Significant at 0.01.
[w] Significant at 0.05.
[x] Con5⁻.
[y] Con6⁻.
[z] Con7⁻.

(Shi and Leung 1994) was used to test for linkage with the *CON2* locus. Based on analysis of 70 progeny, the L-20 marker was found to be 1.7 cM from *CON2*, confirming the linkage between *CON1* and *CON2*. Segregation patterns from crosses between *CON5* and *CON6* also suggested linkage between the two loci but the genetic distance could not be estimated because the proportion of *con5⁻/con6⁻* mutants was not known. This linkage relationship is considered tentative until additional markers are available to confirm the linkage.

DISCUSSION

Since the development of fertile mating strains in *M. grisea* (Kato and Yamaguchi 1982; Kolmer and Ellingboe 1988; Leung et al. 1988; Valent et al. 1991; Yaegashi 1977), the rice blast fungus has attracted much attention as a model organism for investigating host-pathogen interactions (Valent and Chumley 1991). *Magnaporthe grisea* not only has the advantages of a genetic system similar to *Neurospora crassa* but offers opportunities for the investigation of pathogenesis not possible in experimental fungi. Thus far, most work has focused on the early infection and recognition events (Lee and Dean 1993; Leong et al. 1994; Valent and Chumley 1994). A number of genes controlling cultivar and host specificity have been cloned (Valent and Chumley 1994); however, limited work has been done on other developmental processes that may have an important bearing on pathogenesis. Hamer et al. (1989) identified the first spore morphogenesis mutation called *smo* (for spore morphology). Mutation at the *SMO* locus causes irregular spore shape and reduces pathogenicity (Hamer and Givan 1990). Because of the importance of sporulation in a disease epidemic, we began investigating the genes controlling the sporulation pathway as a first step toward identifying target genes for intervention (Leung and Shi 1994; Shi and Leung 1994). In this study, we applied chemical and plasmid insertional mutagenesis to identify six genes controlling morphogenetic development and one gene controlling the response of sporulation to light.

Each sporulation mutant described in this study has a characteristic profile of morphogenetic defects (Fig. 4). The *con5⁻* mutation causes no reduction in radial growth but produces sparse aerial hyphae and completely blocks the formation of conidiophores. Though reduced in radial growth, the *con6⁻* mutant produces abundant conidiophores and aerial hyphae. The *con7⁻* mutation differs from *con1⁻* in that the development of the spores appears to be more advanced in the *con7⁻* mutant. Sporulation is only reduced by 35% in the *con7⁻* mutant relative to the wild type and some normally shaped spores are formed. Regardless of spore shape, conidia of both *con7⁻* and *con1⁻* mutants do not form appressoria. The *con4⁻* mutant produces ellipsoid conidia but retains the ability to form appressoria. Conidia of the *con2⁻* mutant are morphologically deformed and the ability to form appressoria is significantly reduced. Unlike others who have studied the *smo⁻* mutations that also affect ascus cell shape (Hamer et al. 1989), we have not observed any deformation of asci in crosses involving any two mutants generated in this study.

We have genetically demonstrated that *CON5* is epistatic to *CON6* and *CON7*. For unknown reasons, we were unable to

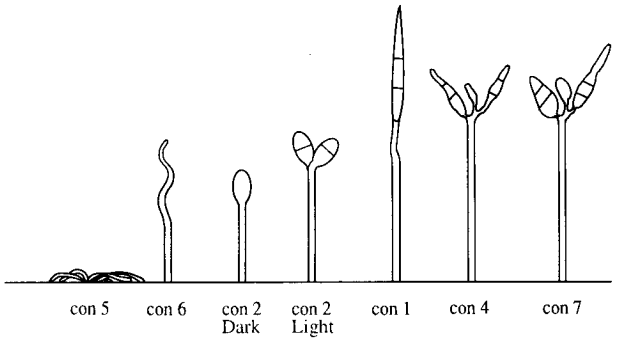

Fig. 4. Schematic diagram to summarize the developmental defects of different sporulation mutations of *Magnaporthe grisea*. The phenotype of the *con2⁻* mutant is light dependent. The mutant is acondial in the dark (only conidial initials formed) but form single-celled or two-celled conidia under continuous light. Genetic analysis suggests two sequences of epistatic relationship: *CON5* > *CON6* > *CON7* and *CON2* > *CON1*. Epistatic interactions among the other genes are not yet determined due to infertility of mutant × mutant crosses.

cross the *con1-B*$^-$ mutant to a large number of *con2*$^-$ strains. Based on analysis of the *con1-A*$^-$ mutant, and assuming allelism between *con1-A* and *con1-B*, we infer that *CON2* is epistatic to *CON1*. Thus, the available genetic data suggest two sequences of mutational blocks: *con5*$^-$ > *con6*$^-$ > *con7*$^-$, and *con2*$^-$ > *con1*$^-$. Since the mutation at *CON6* blocks the production of conidia, we infer *CON6* to be upstream of *CON2*. Judging from the relative degree of conidial development, we would predict *CON2* to be upstream of *CON4* and *CON7*. However, it is possible that conidiogenesis is controlled by more than a single linear pathway, in which case interactions between two mutant genes may produce novel phenotypes. Indeed, the lack of sexual fertility between these mutants suggests that interactions between these mutant genes may affect normal sexual development. Thus, without genetic data, it is not possible to predict the interactions among *con1*$^-$, *con2*$^-$, *con4*$^-$, and *con7*$^-$, which all affect similar stages in spore morphogenesis. The interactions between these genes are being investigated by gene replacement to bypass the infertility barrier.

It is interesting to note that the mutations that yield the Con1-A$^-$ phenotypes have been isolated on several occasions. An ascospore culture having the Con1-A$^-$ spore morphology was recovered among progeny derived from a sexual cross (T. Chow and A. Ellingboe, personal communication). This mutant does not produce appressoria and is nonpathogenic to rice by either spray or wound inoculation (H. Leung, unpublished data). Insertional mutagenesis of strain 2539 also yielded a tagged mutant with phenotypes and pleiotropy identical to the *con1-A*$^-$ and *con1-B*$^-$ mutations (Y. Shi and H. Leung, unpublished data). It is possible that the *CON1* locus is situated in a region of the genome that is inherently unstable. Linkage analysis showed that four RAPD markers and the *CON2* locus mapped to one side of *CON1* (Leung and Shi 1994). Whether *CON1* is located near a telomere has yet to be determined by additional mapping. The available restriction fragment length polymorphism map made from a cross of the same parentage (Guy11 × 2539) will facilitate mapping of the *CON1* locus (Skinner et al. 1993).

Pathogenicity analysis of the morphogenesis mutants further demonstrates the critical role of appressoria in the infection process (see review by Howard 1994). Howard et al. (1991) showed that the build-up of turgor pressure in an appressorium was essential for the penetration of synthetic membranes and host epidermis. It is possible that even minor defects in the appressorial structure could lead to loss of turgor pressure and consequently a reduced ability to penetrate the host. The spore morphology (*smo*$^-$) mutants analyzed by Hamer and Givan (1990) exhibited a reduction in pathogenicity that was attributed to the formation of abnormal appressoria. Talbot et al. (1993) isolated a gene, designated *MPG-1* (*Magnaporthe* pathogenicity gene), responsible for the production of hydrophobins. Mutation at *MPG-1* results in deficiency in appressorium formation and an 80% reduction in lesion formation on rice. Gene expression analysis showed that *MPG-1* was expressed during appressorium formation and during the later stage of pathogenesis when lesions were developed. Talbot et al. (1993) suggested that the MPG1 protein plays an important role in the early elaboration of the infection structure. Recently, Mitchell et al. (1994) showed that *M. grisea* can be rendered nonpathogenic when appressorium formation was abolished by the inactivation of a cAMP-dependent protein kinase that is presumably responsible for signaling appressorium formation. In this study, we found that appressorium formation was closely correlated with the level of pathogenicity. The *con2*$^-$ and *con4*$^-$ mutants both exhibited a reduced ability to produce appressorium. A corresponding reduction in pathogenicity was manifested as a low number of lesions and a slow rate of lesion development (Table 3). As predicted by the inability to form appressoria, both the *con1*$^-$ and *con7*$^-$ mutants were nonpathogenic to rice as assayed by spray inoculation. Pathogenicity, however, was not restored when spores were injected into the leaf sheath (i.e. inoculation by wounding). This suggests that the loss of pathogenicity in the *con1*$^-$ and *con7*$^-$ mutants is not only caused by the lack of appressorium formation but by other processes of fungal development after penetration. The accumulated evidence suggests that mutations affecting spore morphogenesis in general have a strong pleiotropic effect on pathogenesis. The sporulation mutants analyzed in this study exhibit normal growth in culture media but a total loss of, or a significant reduction in, pathogenicity. We expect that the morphogenetic defects are greatly exacerbated in planta when the mutant confronts host defenses and the physical constraints inside plant cells. In a broad sense, the morphogenetic genes are pathogenicity factors as they are essential for normal fungal development and subsequent propagation in the plant. The available series of mutants will allow us to systematically evaluate the functional relationships between morphogenetic development and pathogenesis.

In characterizing the sporulation mutants, we have identified two mutations that result in an altered response to light. The *con3*$^-$ mutation was recovered as a reversion to a high level of sporulation in a *con2*$^-$ genetic background. The *con3*$^-$ mutant produces a large quantity of spores in the dark (Table 2). Genetic analysis showed that *con3*$^-$ is not a suppressor mutation affecting morphogenesis but a gene regulating sporulation in response to light. The *con3*$^-$ gene in *M. grisea* appears to be analogous to the *velvet* (*ve*) gene in *A. nidulans,* in which a mutation in the *ve* gene (*veA1*) results in sporulation without light induction (Mooney and Yager 1990). Like the *con3*$^-$ mutation, the *con7*$^-$ mutant produces similar amount of spores in the dark and in the light. We were unable to demonstrate clear cosegregation between *con7*$^-$ and loss of light responsiveness because of the variability of sporulation in a segregating population. However, results from gene replacement experiments suggested that the altered light response observed in the mutant is an inherent property of the *con7*$^-$ gene. When the retrieved mutant gene was introduced into wild-type strains, the gene-replaced mutants produced as many spores in the dark as in the light (H. Leung, Z. Shi, and D. Christian, unpublished data). Delineation of the regulatory role of the *CON7* gene on morphogenesis and responsiveness to light is in progress.

Since the demonstration of insertional mutagenesis by REMI transformation in *Dictyostelium discoideum* (Kuspa and Loomis 1992), applications of this gene tagging approach have been reported in some filamentous fungi (Lu et al. 1994; Valent et al. 1994). From a collection of 1,310 transformants of *Cochliobolus heterostrophus*, Lu et al. (1994) recovered two toxin nonproducing mutants in which the *Tox1* locus was tagged by plasmid insertions. Valent et al. (1994) reported that four pathogenicity genes in *M. grisea* have been cloned

by insertional mutagenesis. From a collection of 800 transformants, we identified five transformants defective in sporulation as a result of insertional inactivation by pAN7-2. Southern analysis showed that these mutations are caused by single-site insertions and cosegregation between integration patterns with morphogenetic phenotypes was confirmed in the progeny. Thus, the overall efficiency of identifying tagged mutants involved in the sporulation pathway is approximately 0.6% (5/800). This is similar to the frequency (0.4%) observed in the isolation of developmental mutations in *D. discoideum* (Kuspa and Loomis 1992) and to the frequency of recovering pathogenic mutants in *M. grisea* (J. Sweigard and B. Valent, DuPont Co., personal communication).

Insertional mutagenesis offers a number of advantages over conventional mutagenesis. In contrast to chemical mutagenesis, in which multiple hits leading to secondary mutations are common, insertional mutants are often caused by single-site integrations. In all the primary mutants examined in this study, the mutant phenotypes were caused by single-gene inactivations. Since mutant phenotypes can be precisely confirmed by cosegregation with hygromycin B resistance, insertional mutagenesis allows for the detection of mutations that have small or subtle effects on the target phenotype. Using this approach, we have tagged mutations that have quantitative effects on pathogenicity (Y. Shi and H. Leung, unpublished data). Such mutations would have been difficult to define without the aid of cosegregation analysis with hygromycin B resistance. The most important advantage of insertional mutagenesis is the ease with which tagged genes can be retrieved from the mutant genome by plasmid rescue. We have retrieved plasmids containing the tagged sequences of the *con4*$^-$ and *con7*$^-$ genes and confirmed the identity of the cloned sequences by gene replacement (H. Leung and Z. Shi, unpublished data).

Sporulation in phytopathogenic fungi has long been considered a fitness trait measured quantitatively as an epidemiological parameter (Teng et al. 1991). We have identified a series of mutations that block critical steps in the sporulation pathway (*con5*$^-$ and *con6*$^-$) and/or reduce the sporulation capacity (*con1*$^-$, *con2*$^-$, *con4*$^-$, and *con7*$^-$). Most of the sporulation mutants are deficient in other attributes important in pathogenesis (e.g., appressorium formation, ability to colonize host cells, and rate of lesion development). These genetic defects are epidemiologically significant as they affect the capacity of the pathogen to sustain multiple infection cycles in an epidemic. Thus, mutational analysis has enabled us to define an important component of parasitic fitness in precise genetic terms. It is known that rice varieties with partial resistance to blast show a reduction in the number of lesions, lesion size, and sporulation (Yeh and Bonman 1986). Recently, Wang et al. (1994) showed that quantitative loci in the rice plant may affect lesion size and number and thus influence the overall sporulation of the fungus. Our practical objective in delineating the sporulation pathway in *M. grisea* is to interfere with the sporulation process as a means of reducing the disease below an economic threshold. The distinct developmental defects exhibited by the mutants offer opportunities to selectively block critical steps in the pathogenesis pathway. Future work will elucidate the structure and function of these sporulation genes so that their products can be used as targets for suppressing disease.

MATERIALS AND METHODS

Strains and media.

The origins and characteristics of parental strains and mutants used in this study are listed in Table 1. Cultures of the fungus were routinely maintained on oatmeal agar (50 g oatmeal per liter of water). For long-term preservation, pieces of Whatman No. 1 filter paper (4 mm^2) colonized by mycelia were kept in a desiccator at –20°C. For DNA extraction, cultures were grown in liquid complete medium (Crawford et al. 1986) with constant shaking at ambient temperature (24 to 26°C) for 5 days. Mycelia 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.

Chemical mutagenesis.

Conidia of Guy11 were harvested from oatmeal plates by washing with 0.06 M phosphate buffer, pH 7.0. The conidial suspension (0.5 to 1 × 10^7 spore/ml) was incubated in 100 mM diepoxyoctane (Aldrich Chemical, Milwaukee, WI) in potassium phosphate buffer for 60 to 120 min (Chumley and Valent 1990). Approximately 10% survival was obtained after 70 to 75 min incubation. Conidia were spun down by centrifugation and washed two times with phosphate buffer. The conidial suspension was plated onto complete medium and surviving colonies were transferred to oatmeal agar for examination using a stereomicroscope.

Insertional mutagenesis by plasmid transformation.

The basic principle of insertional mutagenesis by integrative transformation has been described by Kuspa and Loomis (1992). The transformation procedure for *M. grisea* was as described by Leung et al. (1990) except that 20% sucrose instead of 1.2 M sorbitol was used as osmoticum. pAN7-2, a 9.2-kb integrative plasmid containing the *Escherichia coli* hygromycin B phosphotransferase gene linked to *Aspergillus nidulans* regulatory sequences (Punt et al. 1987), was used in transformation. To enhance transformation frequency, the method of REMI as described by Schiestl and Petes (1991) and Kuspa and Loomis (1992) was followed. We have previously shown an increase in transformation frequency by using pAN7-2 linearized with a restriction enzyme and incorporating the same enzyme into the transformation mixture (Shi et al. 1995). In REMI transformation, pAN7-2 was linearized with *Bam*HI. Depending on the experiments, approximately 10 to 20 units of *Bam*HI was mixed with the linearized plasmid in 125-µl osmotic buffer, and added to the protoplast suspension to a final concentration of 40 to 80 units of *Bam*HI per ml of transformation mixture. Transformants obtained from circular plasmid or REMI transformation were screened for developmental mutations using a stereomicroscope.

Crossing and genetic analysis.

Crosses were made by pairing two strains on oatmeal agar in a 6-cm-diameter petri plate, incubated at 20°C in the dark for 20 to 25 days. Random ascospores and complete tetrads were obtained as described (Leung and Williams 1985). Random ascospore progeny were described by a cross number followed by an ascospore number. Crosses were made between mutants and wild-type 2539. Segregation ratios ob-

served in F_1 progeny were confirmed by backcrosses and sibcrosses. Genetic nomenclature followed that recommended by Yoder et al. (1986) and Chumley and Valent (1990). Sporulation mutants and the loci defined by genetic analyses were numbered using the prefix con (for *con*idiation) following the sequence of discovery of the respective mutants. Genotypes were described by italics (uppercase for wild type and lowercase for mutants) and phenotypes were described by roman type with the first letter capitalized.

Assay of growth, sporulation, appressorium formation, and pathogenicity.

The morphology of the primary mutants and their progeny was examined under differential interference contrast optics using an Olympus BHS research microscope. Photomicrographs were taken with Kodak Technical Pan film 2415. The relative radial growth of the mutants and the parental strain were measured on oatmeal agar after incubation for 9 days at 26°C. Sporulation was assayed in cultures of synchronous growth. Plates were inoculated by spreading 50 µl of spore suspension (approximately 10^3 spores/ml) of a mutant strain evenly onto 6-cm-diameter oatmeal plates. For aconidial mutants, a suspension of mycelial fragments was used to inoculate the plates. After 48 h incubation in the dark, one set of plates was incubated under continuous light (cool white light 40 w, 130 µE s^{-1} m^{-2}) while another set was incubated in the dark by wrapping with aluminum foil. All cultures were kept at 26°C. After 5 days incubation, conidia were harvested from a plate by washing with 1 ml of sterile water and the number of conidia counted with a hemacytometer. Four plates per culture were measured for growth and sporulation. To determine whether conidia of mutants were able to produce appressoria, conidia were germinated on a microscope slide coated with Sigmacote (Sigma, St. Louis, MO). Approximately 500 conidia in 20 µl were placed onto the siliconized slide, covered with a coverslip, and incubated at 26°C under 100% humidity. Growth behavior of conidia was observed 24 h after incubation using differential interference contrast microscopy. The percentages of germlings that produced appressoria were recorded for three slides (approximately 100 germlings scored per slide).

Primary mutants were tested for pathogenicity on rice variety 51583 and Maratelli (kindly provided by the International Rice Research Institute, Los Banos, Philippines). Rice plants were grown in potting soil (55% peat moss, 30% pumice, and 15% sand, Soil Incorporated, Puyallup, WA) under continuous light (1 cool white fluorescent/1 Grolux light, 140 µE s^{-1} m^{-2}) at ambient room temperature in the laboratory. Plants were fertilized with ferric sulfate and soluble fertilizer Peters (20-20-20) weekly. Inoculation by spraying and injection of the leaf sheath was as previously described (Shi and Leung 1994). For each mutant, approximately five plants were inoculated per pathogenicity test. The number of lesions per leaf and the time taken to develop a mature sporulating lesion (~50 mm long) were recorded for each inoculated plant. Inoculation experiments were conducted three times.

All experiments on radial growth, germination of conidia, appressorium formation, sporulation, and pathogenicity were done in a completely randomized design. Differences between mutants were tested by analysis of variance and the Fisher least significant difference method.

DNA manipulations and RAPD mapping.

DNA from mutants was isolated as described by Borromeo et al. (1993). Approximately 5 µg of DNA was digested with *Eco*RI and *Apa*I, electrophoresed in 0.7% agarose gel and blotted to N-plus Hybond membrane (Amersham, Chicago, IL) following the manufacturer's instructions. pAN7-2 was labeled with ^{32}P-ATP using the Decaprime II DNA Labeling Kit (Ambion, Austin, TX). Blots were probed with pAN7-2 and the hybridization signal was detected by autoradiography as recommended by the manufacturer. RAPD markers linked to the *CON1* locus were used to detect linkages in crosses that involved *CON1* as described (Shi and Leung 1994; Williams et al. 1990).

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