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<sup>−</sup> and con6<sup>−</sup>, completely abolish conidial production. A series of mutations (con1<sup>−</sup>, con2<sup>−</sup>, con4<sup>−</sup>, and con7<sup>−</sup>) downstream from con5<sup>−</sup> and con6<sup>−</sup> affect the development of conidia and reduce sporulation. The con1<sup>−</sup> and con2<sup>−</sup> mutations block early steps in conidiogenesis, resulting in >90% reduction in sporulation and the production of abnormally shaped conidia. The con2<sup>−</sup> mutant is completely asexual in the dark but produces mostly monoseptate or two-celled conidia under continuous illumination. An independent mutation (con3<sup>−</sup>) that regulates light response for sporulation was isolated from the con2<sup>−</sup> mutant. The con4<sup>−</sup> and con7<sup>−</sup> 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<sup>−</sup> and con7<sup>−</sup> mutants, and reduced by 70 and 22% in the con2<sup>−</sup> and con4<sup>−</sup> mutants, respectively. Pathogenicity on rice is lost in the con1<sup>−</sup> and con7<sup>−</sup> mutants and significantly reduced in the con2<sup>−</sup> and con4<sup>−</sup> mutants. Five mutations (con1<sup>B−</sup>, con4<sup>−</sup>, con5<sup>−</sup>, con6<sup>−</sup>, and con7<sup>−</sup>) 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.


*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 asxexual 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.

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; Clutterback 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<sup>−</sup>, 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 acondial 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 on 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 resolated 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).

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 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 acondial; 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 1. Characteristics and origins of Magnaporthe grisea strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics and origin</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guy11</td>
<td>Field strain, Mat2, hermaphroditic, pathogenic on rice</td>
<td>Leung et al.</td>
</tr>
<tr>
<td>2539</td>
<td>Laboratory strain, Mat1, hermaphroditic, pathogenic on</td>
<td>Leung et al.</td>
</tr>
<tr>
<td></td>
<td>weeping lovegrass</td>
<td>1988</td>
</tr>
<tr>
<td>Guy11E46</td>
<td>Mat2, con1-A mutation</td>
<td>Shi and Leung</td>
</tr>
<tr>
<td>GT1</td>
<td>Mat2, circular plasmid transformation of Guy11</td>
<td>This study</td>
</tr>
<tr>
<td>Guy11-con2</td>
<td>Mat2, diepoxyoctane mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>1313-64-con3-3</td>
<td>Mat2, spontaneous mutation derived from a con2 strain</td>
<td>This study</td>
</tr>
<tr>
<td>GT20</td>
<td>Mat2, circular plasmid transformation of Guy11</td>
<td>This study</td>
</tr>
<tr>
<td>GT399</td>
<td>Mat2, REMI transformation of Guy11</td>
<td>This study</td>
</tr>
<tr>
<td>GT560</td>
<td>Mat2, circular plasmid transformation of Guy11</td>
<td>This study</td>
</tr>
<tr>
<td>GT562</td>
<td>Mat2, circular plasmid transformation of Guy11</td>
<td>This study</td>
</tr>
</tbody>
</table>
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 germings producing appressoria, however, was significantly reduced relative to the wild type (Table 2). Germings 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

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**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 conidium 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 F3, sib-cross, and backcross progeny (Table 4). The segregation patterns of the Con1-B^+, Con2^+, Con4^+, and Con5^+ phenotypes fitted the 1:1 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 Con^- and Con^- 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 Con^- mutant was determined by backcrossing the mutant to the wild type and to the Con^- mutant (Table 5). The Con^- mutant was distinguished from the Con^- mutant by the production of abnormal spores without light stimulation. When the Con^- 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 Con^- phenotype was not a reversion of the con2 locus but controlled by mutation at an independent locus. To dem-

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pheno-type</th>
<th>Spore morphology*</th>
<th>Radial growth*</th>
<th>Germination %*</th>
<th>Appressorium formation %*</th>
<th>Sporulation (10^3/ml)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT1</td>
<td>Con1-B^-</td>
<td>Elongated</td>
<td>28.5 ± 0.6 (c)</td>
<td>80.6 ± 6.3 (b)</td>
<td>0</td>
<td>3.4 ± 1.2 (d)</td>
</tr>
<tr>
<td>Guy11-con2</td>
<td>Con2^-</td>
<td>Single or two-celled</td>
<td>37.6 ± 0.9 (ab)</td>
<td>62.6 ± 6.4 (d)</td>
<td>44.4 ± 5.3 (c)</td>
<td>9.1 ± 5.5 (d)</td>
</tr>
<tr>
<td>1313-64-con3-3</td>
<td>Con3^-</td>
<td>Single or two-celled</td>
<td>37.5 ± 1.7 (ab)</td>
<td>75.8 ± 5.1 (bc)</td>
<td>46.0 ± 7.1 (c)</td>
<td>121.6 ± 29.0 (a)</td>
</tr>
<tr>
<td>GT20</td>
<td>Con4^-</td>
<td>Three-celled, ellipsoid</td>
<td>36.3 ± 1.5 (b)</td>
<td>66.6 ± 5.8 (cd)</td>
<td>66.7 ± 16.1 (b)</td>
<td>107.5 ± 5.4 (a)</td>
</tr>
<tr>
<td>GT399</td>
<td>Con5^-</td>
<td>Aconidal</td>
<td>39.5 ± 0.6 (a)</td>
<td>NA^</td>
<td>NA^</td>
<td>0.13 ± 0.02 (b)</td>
</tr>
<tr>
<td>GT560</td>
<td>Con6^-</td>
<td>Aconidal</td>
<td>13.6 ± 1.3 (d)</td>
<td>NA</td>
<td>NA</td>
<td>107.5 ± 5.4 (a)</td>
</tr>
<tr>
<td>GT562</td>
<td>Con7^-</td>
<td>Mixture of abnormal spores</td>
<td>36.3 ± 1.5 (b)</td>
<td>80.8 ± 4.4 (ab)</td>
<td>0</td>
<td>113.7 ± 31.2 (a)</td>
</tr>
<tr>
<td>Guy11</td>
<td>Wild type</td>
<td>Three-celled, pyriform</td>
<td>39.3 ± 0.9 (a)</td>
<td>87.8 ± 7.8 (a)</td>
<td>89.7 ± 7.5 (a)</td>
<td>0.28 ± 0.18 (b)</td>
</tr>
</tbody>
</table>

* See Figure 1 for details.

* 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.

* 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.

* 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.

* Sporulation assay was described in detail in the text. Values with same letter in parentheses are not significantly different at P = 0.05 level.

* Not applicable.

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**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 inser-
tional 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 ApaI and EcoRI Since there is no ApaI recognition site within pAN7-2, the number of hybridization bands would indicate the number of integration sites. When ApaI-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 EcoRI-digested DNA further indicated that all mutants were caused by single-copy insertions (Fig. 3B).

Since there are two EcoRI sites in pAN7-2, EcoRI-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 4. Inheritance of sporulation mutations of Magnaporthe grisea |  |
|------------------------|-----------------|-----------------|-----------------|-----------------|
| Mutation | Cross no. | Random spor | Sparse type | Progeny |
| | | (mutant × WT)* | WT | Mutant | X²x |
| 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 | 4.96x |
| | 1534 | 1531-71 × 1537-66 | 13 | 14 | 0.04 |
| | 1535 | 1531-71 × 1531-68 | 21 | 20 | 0.02 |
| | 1537 | 1531-78 × 2539 | 12 | 42 | 16.67x |
| Total | | | 101 | 110 | 0.38 |
| Con6' | 1539 | G560 × 2539 | 50 | 14 | 20.25x |
| | 1540 | GT562 × 2539 | 24 | 44 | 5.88x |

*WT = wild type.
# Tested against 1:1 ratio.
$ Significant at P = 0.05.
$ Significant at P = 0.01.

| Table 5. Cosegregation between hygromycin B resistance and mutant phenotypes in progeny of tagged mutants of Magnaporthe grisea |  |
|------------------------|-----------------|-----------------|-----------------|-----------------|
| Mutant | No. of crosses | 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 |

* Hyg R = hygromycin B resistant, Hyg S = hygromycin B sensitive, WT = wild type.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Rice line</th>
<th>Spry inoculation*</th>
<th>Leaf sheath invasion*</th>
<th>Latent period*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guy11</td>
<td>Wild type</td>
<td>51583</td>
<td>3.8 (a)</td>
<td>17.8 (a)</td>
<td>+</td>
</tr>
<tr>
<td>GT1</td>
<td>Con1-B'</td>
<td>51583</td>
<td>1.7 (b)</td>
<td>6.9 (b)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maratelli</td>
<td>0</td>
<td>0</td>
<td>NA*</td>
</tr>
<tr>
<td>Guy11</td>
<td>Con2'</td>
<td>51583</td>
<td>0.3 (c)</td>
<td>1.2 (c)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maratelli</td>
<td>0</td>
<td>0</td>
<td>NA*</td>
</tr>
<tr>
<td>GT20</td>
<td>Con4'</td>
<td>51583</td>
<td>0.3 (c)</td>
<td>3.3 (bc)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maratelli</td>
<td>0.1 (c)</td>
<td>0.1 (c)</td>
<td>+</td>
</tr>
<tr>
<td>GTS62</td>
<td>Con7'</td>
<td>51583</td>
<td>0</td>
<td>0</td>
<td>NA*</td>
</tr>
</tbody>
</table>

* 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.
# Injected with a 10⁶ spore/ml suspension. Only qualitative assessment of lesions was made: + = presence of lesions, − = no lesion. Experiments were repeated three times.
$ Days from inoculation to the appearance of a mature lesion (~50 mm long).
$ Not applicable.
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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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 con1-1, 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 ApaI and EcoRI. Since there is no ApaI recognition site within pAN7-2,

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

| Strain | Phenotype | Rice line | Spray inoculation* | Leaf sheath injection* | Latent period | WT | Mutant | X2
|--------|-----------|-----------|-------------------|-----------------------|--------------|----|--------|-----
| Guy1   | Wild type | 51583     | 3.8 (a) 17.8 (a) | 6                     |              |    |        |     
| GT1    | Con1-1    | 51583     | 1.7 (b) 6.9 (b) | 6                     |              |    |        |     
| Guy1   | Con2-2    | 51583     | 0.3 (c) 1.2 (c)  | 8                     |              |    |        |     
| GT20   | Con4-     | 51583     | 0.3 (c) 3.3 (bc) | 8                     |              |    |        |     
| GT562  | Con7-     | 51583     | 0.1 (c) 0.1 (c) | 8                     |              |    |        |     

* 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.
* Injected with a 107 spore/ml suspension. Only qualitative assessment of lesions was made: + = presence of lesions, − = no lesion. Experiments were repeated three times.
* Days from inoculation to the appearance of a mature lesion (-50 mm long).

Table 4. Inheritance of sporulation mutations of Magnaporthe grisea

| Mutation | Cross no. | Cross type | Random spor | Mutant | X2
|----------|-----------|------------|-------------|--------|-----
| Con1-1   | 1521      | GT-1 × 2539| WT 43       | 30     | 2.32
| Con2-2   | 1313      | Guy1-1 × 2539| 86          | 72     | 1.24
| Con1-1   | 1318      | 1313-45 × 2539| 33          | 38     | 0.35
| Con1-1   | 1319      | 1313-37 × 2539| 44          | 32     | 1.89
| Con1-1   | 1320      | 1313-16 × 1311| 28          | 36     | 1.00
| Total    |           |            | 191         | 178    | 0.46
| Con1-1   | 1522      | GT-20 × 2539| 30          | 30     | 0.00
| Con1-1   | 1525      | 1522-12 × 2539| 20          | 14     | 1.06
| Con1-1   | 1526      | 1522-14 × 2539| 10          | 6      | 1.00
| Con1-1   | 1527      | 1522-22 × 1522-27| 7           | 5      | 0.33
| Total    |           |            | 67          | 55     | 1.18
| Con1-1   | 1531      | GT-399 × 2539| 55          | 34     | 4.96
| Con1-1   | 1534      | 1531-71 × 1537-66| 13          | 14     | 0.04
| Con1-1   | 1535      | 1531-71 × 1531-68| 21          | 20     | 0.02
| Con1-1   | 1537      | 1531-78 × 2539| 12          | 42     | 16.67
| Total    |           |            | 101         | 110    | 0.38
| Con1-1   | 1539      | GT560 × 2539| 50          | 14     | 20.25
| Con1-1   | 1540      | GT562 × 2539| 24          | 44     | 5.88

* WT = wild type.
* Tested against 1:1 ratio.
* Significant at P = 0.05.
* Significant at P = 0.01.

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

<table>
<thead>
<tr>
<th>Mutant</th>
<th>No. of crosses</th>
<th>Hyg R/Mutant</th>
<th>Hyg R/WT</th>
<th>Hyg S/Mutant</th>
<th>Hyg S/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con1-1</td>
<td>1</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>Con2-2</td>
<td>6</td>
<td>94</td>
<td>0</td>
<td>0</td>
<td>99</td>
</tr>
<tr>
<td>Con1-1</td>
<td>7</td>
<td>177</td>
<td>0</td>
<td>0</td>
<td>101</td>
</tr>
<tr>
<td>Con6-2</td>
<td>6</td>
<td>136</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Con7-2</td>
<td>5</td>
<td>126</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th>Cross number</th>
<th>Parents</th>
<th>Predicted genotype</th>
<th>Random spore progeny</th>
<th>Expected ratio</th>
<th>X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1323</td>
<td>1313-64-con3-3 × 2539</td>
<td>con2+/con3+ × CON2*/CON3*</td>
<td>WT²</td>
<td>Con2-</td>
<td>Con3-</td>
</tr>
<tr>
<td>1327</td>
<td>1313-64-con3-3 × 1313-21</td>
<td>con2'/con3- × con2'/CON3*</td>
<td>0</td>
<td>41</td>
<td>44</td>
</tr>
</tbody>
</table>

* Wild type.

Fig. 3. Southern analysis of insertional mutants of Magnaporthe grisea. (A) ApcI-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) EcoRI-digested DNA of the same mutants as in (A). (C) Co-segregation between integration pattern with mutant phenotype. EcoRI-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 EcoRI fragments as size markers.

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*

<table>
<thead>
<tr>
<th>Locus pair</th>
<th>Cross no.</th>
<th>Parents</th>
<th>Genotypes</th>
<th>No. of random spore progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>7*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>con2/con1</td>
<td>1325</td>
<td>1313-16 × Guy11E46*</td>
<td>con2*/CON1* × CON2*/con1*</td>
<td>WT 4</td>
</tr>
<tr>
<td></td>
<td>1326</td>
<td>1313-36 × Guy11E46</td>
<td>con2*/CON1* × CON2*/con1*</td>
<td>Mutant 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Expected ratio 1:2:1</td>
</tr>
<tr>
<td>con5/con6</td>
<td>1543</td>
<td>1539-50 × GT399</td>
<td>CON5*/con6* × con5*/con6*</td>
<td>16.61*</td>
</tr>
<tr>
<td></td>
<td>1548</td>
<td>1539-42 × GT399</td>
<td>CON5*/con6* × con5*/con6*</td>
<td>7.85*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>con5/con7</td>
<td>1544</td>
<td>1540-22 × GT399</td>
<td>CON5*/con7* × con5*/con7*</td>
<td>16.67*</td>
</tr>
<tr>
<td></td>
<td>1542</td>
<td>1539-5 × GT562</td>
<td>CON5*/con7* × con5*/con7*</td>
<td>22.53*</td>
</tr>
<tr>
<td></td>
<td>1547</td>
<td>1540-22 × GT560</td>
<td>CON6*/con7* × con6*/con7*</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>1549</td>
<td>1539-42 × GT562</td>
<td>CON6*/con7* × con6*/con7*</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

q Wild type.
* Tested against 1:2:1 ratio, 2 df.
* Guy11E46 carries the con1-A allele.
* Con2*.
* Con5*.
* Con6*.
* 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 asci cell shape (Hamer et al. 1989), we have not observed any deformation of ascus 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

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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 conidiospore 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, all of which affect similar stages in sporophores. 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- sporophore 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 sporophore morphology (smpo) 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 also 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 con-3- mutation was recovered as a reversion to a high level of sporulation in a con2- genetic background. The con-3- 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 (veAl) 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 Dictyocteliun discoideum (Kuspa and Loomis 1992), applications of this gene tagging approach have been reported in some filamentous fungi (Lu et al. 1994; Valient 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. Valient 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 con\(^4\) and con\(^7\) 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 (con\(^5\) and con\(^6\)) and/or reduce the sporulation capacity (con\(^{1-3}\), con\(^{4}\), and con\(^{7}\)). 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\(^{4}\) 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 BamHI. Depending on the experiments, approximately 10 to 20 units of BamHI 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 BamHI 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 F1 progeny were confirmed by backcrosses and sib-
crosses. 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 conidiation) following the
sequence of discovery of the respective mutants. Genotypes
were described by italics (uppercase for wild type and lower-
case 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. Photomicro-
graphs 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 10^7 spores/ml) of a mutant strain
evenly onto 6-cm-diameter oatmeal plates. For conidial mu-
nants, a suspension of mycelial fragments was used to inocu-
late 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 deter-
mine whether conidia of mutants were able to produce ap-
pressoria, 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 mi-
acroscopy. The percentages of germlings that produced appre-
soria were recorded for three slides (approximately 100
germlings scored per slide).

Primary mutants were tested for pathogenicity on rice vari-
ety 51583 and Maratelli (kindly provided by the Interna-
tional 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) un-
der 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 injec-
tion 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 be-
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
EcoRI and Apal, 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 32P-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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