Interactions Between Spore Morphogenetic Mutations Affect Cell Types, Sporulation, and Pathogenesis in *Magnaporthe grisea*

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We have previously defined four single-gene mutations, *con1*, *con2*, *con4*, and *con7*, that control various stages of spore morphogenesis in the rice blast fungus. To delineate the developmental pathway of spore morphogenesis, we investigated the interactions among these morphogenetic genes by generating strains with double mutations via transformation-mediated gene disruption. Plasmids containing portions of the inactivated *CON4* and *CON7* genes were introduced into strains harboring single mutation to produce double mutants. Interaction between *con1* and *con4* resulted in reduced vegetative growth and suppression of sporulation. Interaction between *con1* and *con7* suppressed spore production but not vegetative growth. The *con2/con4* and *con4/con7* double mutants produced hybrid spore types with characteristic features of both parental mutants. The *con2/con7* mutant produced *con2* type spores, indicating that *con2* is epistatic to *con7* in spore morphogenesis. The epistatic relationship, however, reversed when traits related to pathogenesis were considered. Double mutants harboring the *con7* mutation could not form appressoria or colonize plant tissue, indicating that *con7* is epistatic to *con2* and *con4* in appressorium formation and pathogenesis. Thus, morphogenetic genes interact at multiple levels leading to different epistatic relationships in the pathways of spore morphogenesis, appressorium formation, and pathogenesis.

Additional keywords: conidia, fungal development, pathogenicity, *Pyricularia grisea*.

For many plant diseases caused by phytopathogenic fungi, the production of asexual spores is a primary determinant of disease severity. Fungal spores are the infectious propagules responsible for initiating infection as well as for disease dissemination. Interference with critical steps in spore production could be a useful strategy for reducing primary inoculum and the spread of the disease. However, such a strategy has not been widely exploited due to a limited understanding of the genetic regulation of sporulation in phytopathogenic fungi.

Magnaporthe grisea (anamorph, *Pyricularia grisea*; class: Pyrenomycetes), the causal agent of rice blast disease, has the

genetic attributes that make it highly amenable for genetic and molecular analysis (Valent and Chumley 1991). Genetic crosses can be made readily, and efficient transformation systems are available (Parsons et al. 1987; Leung et al. 1990; Shi et al. 1995). The fungus produces abundant asexual spores (conidia) that are responsible for the multiple infection cycles within a cropping season. The sporulation apparatus consists of a conidiophore bearing sympodially 4 to 5 pyriform, three-celled conidia. On media favoring sporulation, the fungus produces spores approximately 2 days after conidial germination (Leung and Shi 1994). On the rice plant, sporulation begins on susceptible lesions approximately 5 days after inoculation.

Using chemical and insertional mutagenesis, we have identified six genes—*con1*, *con2*, *con4*, *con5*, *con6*, and *con7*—that control various steps in the sporulation pathway (Shi and Leung 1994; Leung and Shi 1994; Shi and Leung 1995). Each of these mutations results in a unique profile of abnormal characteristics in sporulation and spore morphogenesis. Mutation at the *con5* locus completely blocks conidiophore formation whereas mutation at the *con6* locus leads to the production of abundant conidiophores but no conidia. Mutations at the *con1*, *con2*, *con4*, and *con7* loci result in the production of conidia with distinct cell shapes. The *con2* mutant is aconidial in the dark but produces abnormally shaped, one- or two-celled conidia under direct illumination. The *con1* mutant produces elongated conidia that are twice as long (50 µm) and half as wide (8 µm) as the wild-type conidia. Conidia of the *con4* mutant are ellipsoid with a slight narrowing of the basal cell. The *con7* mutant produces a mixture of normally and abnormally shaped conidia. The characteristic type of conidia is slightly elongated, and many have attenuated tip cells. The *con7* mutant produces equally abundant conidia in total darkness as under direct illumination.

A common feature among four spore-producing mutations, *con1*, *con2*, *con4*, and *con7*, is a reduction in pathogenicity on rice. Reduced pathogenicity in the *con2* and *con4* mutants is manifested as a lower infection efficiency and longer latent period relative to the wild type (Shi and Leung 1995). Conidia of both *con2* and *con4* mutants produce fewer appressoria than the wild type and cause few lesions on rice plants inoculated with a high concentration of spores. Conidia of the *con1* and *con7* mutants do not produce appressoria and are nonpathogenic on rice (Shi and Leung 1995). Furthermore, the loss of pathogenicity in the *con1* and *con7* mutants cannot be com-

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compensated by wound inoculation, suggesting that additional developmental defects other than penetration are responsible for the loss of pathogenicity in these mutants. Genetic analyses showed that *con2* is epistatic to *con1*, *con5* epistatic to *con6*, and *con6* epistatic to *con7* (Shi and Leung 1995); however, the interactions between other morphogenetic genes remain unclear due to infertility in most crosses between mutants.

To further delineate the sporulation pathway, we investigated the interactions between morphogenetic loci with transformation-mediated gene disruption. We report here the construction and analyses of a series of mutants carrying double mutations. We found that interactions between the morphogenetic mutations result in hybrid spore types or a complete suppression of spore formation and pathogenesis.

RESULTS

Isolation of inactivated genes by plasmid rescue.

Genetic analyses indicated that both *con4* and *con7* genes in GT20 (*con4*) and GT562 (*con7*) mutants are tagged by inserted plasmid pAN7-2 (see Table 1 for strain designations; Shi and Leung 1995). To retrieve the mutated *CON4* and *CON7* genes, mutant genomic DNA was digested with *ApaI* (no recognition site in pAN7-2) and self-ligated. The ligated DNA was used to transform *Escherichia coli* by electroporation. Approximately 50 ampicillin resistant colonies were recovered by transforming 10^8 *E. coli* cells with 0.1 µg of ligated genomic DNA. The plasmids retrieved from the *con4* and *con7* mutants were named pGT20A and pGT562A to indicate plasmids containing *ApaI*-digested fragments from strains GT20 and GT562, respectively. pGT20A contained approximately 1 kb and 1.2 kb of flanking sequences on either side of the inserted pAN7-2 (D. Fujimoto, Z. Shi, D. Christian, and H. Leung, *unpublished results*). pGT562A contained approximately 2.4 kb of flanking DNA (Z. Shi and H. Leung, *unpublished result*).

To confirm that the retrieved plasmids represent the inactivated *CON4* and *CON7* loci, pGT20A and pGT562A were linearized with *ApaI* and introduced into wild-type strains by transformation. Mutants with *con4* spore morphology were recovered at a high frequency (≈40%) from transformation of Guy11 with pGT20A. The phenotypes of mutant transformants were identical to those of the original GT20 mutant with respect to spore morphology, sporulation, and appressorium formation. When pGT562A was introduced into Guy11, 16% of the transformants (8 out of 51) showed typical *con7* mutant spore and colony morphology. The representative *con4* and *con7* mutant transformants were subjected to Southern analysis with pAN7-2 as a probe. GR17-2 (*con4*) and GR16-35 (*con7*) mutant transformants have patterns identical to the original mutants, GT20 and GT562, respectively (Fig. 1A). These results indicate that pGT20A and pGT562A contain at least a portion of the inactivated *CON4* and *CON7* genes, respectively.

Production of double mutants by gene disruption.

Three strains, Guy11-*con2*, 89-8-1, and GR43-3, carrying the *con2*, *con1-C*, and *con7* mutations, respectively, were used as recipients for transformation. When pGT20A was introduced into Guy11-*con2*, mutant transformants with nonparental spore morphology were recovered. Of 36 transformants obtained, 14

produced spores that were morphologically distinct from those of either the *con2* or *con4* mutants. The new spore type had morphological features intermediate between the parental mutants (Fig. 2). Most of the hybrid spores were elongated (similar to the ellipsoid conidia of the *con4* mutant) but with a single septum (characteristic of *con2*).

When pGT562A was introduced into Guy11-*con2*, a transformant, GR19-8, with new phenotypes was recovered. GR19-8 produced *con2* type spores but had colony morphology characteristic of the *con7* mutant (erect and fragile conidio-

Table 1. Strains of *Magnaporthe grisea* and plasmids used in this study

Strain or plasmid	Genotypes and relevant characteristics	Reference
<i>M. grisea</i>		
Guy11	<i>MAT-2</i>	Leung et al. 1988
2539	<i>MAT-1</i>	Leung et al. 1988
89-8-1	<i>MAT-2, con1-C</i>	Gift of A. H. Ellingboe, Univ. of Wisconsin-Madison
Guy11- <i>con2</i>	<i>MAT-2, con2</i>	Leung and Shi 1994
GT20	<i>MAT-2, con4</i>	Shi and Leung 1995
GT562	<i>MAT-2, con7</i>	Shi and Leung 1995
GR14-1	Guy11- <i>con2/CON4</i>	This study
GR14-2	Guy11- <i>con2/CON4</i>	This study
GR14-3	Guy11- <i>con2/con4</i>	This study
GR14-7	Guy11- <i>con2/con4</i>	This study
GR16-3	Guy11/ <i>CON7</i>	This study
GR16-4	Guy11/ <i>CON7</i>	This study
GR16-17	Guy11/ <i>con7</i>	This study
GR16-35	Guy11/ <i>con7</i>	This study
GR17-1	Guy11/ <i>con4</i>	This study
GR17-3	Guy11/ <i>CON4</i>	This study
GR17-4	Guy11/ <i>CON4</i>	This study
GR19-7	Guy11- <i>con2/CON7</i>	This study
GR19-8	Guy11- <i>con2/con7</i>	This study
GR28-9	<i>con1-C/CON4</i>	This study
GR28-4	<i>con1-C/con4</i> , no spore, reduced growth	This study
GR29-9	<i>con1-C/CON7</i>	This study
GR29-1	<i>con1-C/con7</i> , no spore, fragile conidiophore	This study
GR29-8	<i>con1-C/con7</i> , no spore, fragile conidiophore	This study
GR43-3	<i>con7</i> mutant produced by gene disruption with pCON7::bar	This study
GR44-1	<i>con4/con7</i>	This study
GR44-3	<i>con4/con7</i>	This study
GR44-10	<i>CON4/con7</i>	This study
Plasmid		
pAN7-2	9.2-kb cosmid vector containing <i>Escherichia coli</i> hygromycin B phosphotransferase gene	Punt et al. 1987
pBARGEM7-2	4.9-kb plasmid containing the <i>bar</i> gene, which confers phosphinothricin resistance	Pall and Brunelli 1993
pGT20A	Self-ligated plasmid containing pAN7-2 plus flanking DNA from the <i>con4</i> locus in GT20	This study
pGT562A	Self-ligated plasmid containing pAN7-2 plus flanking DNA from the <i>con7</i> locus in GT562	This study
pCON7::bar	<i>CON7</i> cDNA disrupted by insertion of the <i>bar</i> cassette at <i>EcoRV</i> site	This study

phores), though much reduced in sporulation capacity (similar to the *con2* mutant). Unlike Guy11-*con2*, conidia of GR19-8 germinated poorly and did not produce appressoria (Fig. 2).

Introduction of pGT20A into 89-8-1 (*con1-C* mutation) generated 24 transformants. One transformant, GR28-4, was aconidial and exhibited reduced radial growth. Two aconidial mutants (GR29-1 and GR29-8) were recovered from 89-8-1 transformed with pGT562A. These two transformants formed abundant conidiophore-like hyphae with little reduction in vegetative growth. The conidiophore-like hyphae appeared to be fragile (a characteristic of the *con7* mutant), as many conidiophore fragments were found in suspension of aerial mycelia.

Since both *con4* and *con7* are insertional mutants carrying hygromycin B resistance, a different approach involving an alternate selection marker was used to construct a *con4/con7* mutant. Cloning of the cDNA corresponding to the *CON7* gene allowed us to construct a gene disruption vector, pCON7::bar, in which the *CON7* open reading frame was disrupted by the insertion of the *bar* gene cassette from pBARGEM7-2. pCON7::bar was used to disrupt the *CON7* gene in Guy11 to generate *con7* mutants with phosphinothricin resistance (Z. Shi and H. Leung, unpublished results). A representative *con7* mutant (GR43-3) was used as the recipient for transformation with pGT20A. Eight out of 70 hygromycin B resistant transformants (11%) had spore characteristics intermediate between those of the *con4* and *con7* mutants (Fig. 2). All transformants with novel spore morphology and/or other phenotypes were considered putative gene

disruption mutants and were subjected to genetic and molecular analyses.

Genotypes of double mutants confirmed by genetic and Southern blot analyses.

To confirm that the transformants with novel phenotypes were caused by double mutations at the respective loci, progeny were obtained from crosses between the double mutants and wild-type strain 2539. While crosses between two single mutants were sterile (e.g., *con1* × *con7* or *con2* × *con4*), crosses between the putative double mutants and 2539 were sufficiently fertile to allow for segregation analysis. No genetic data were obtained from the putative *con1/con4* mutant GR28-4 as numerous attempts to cross the mutant to 2539 were not successful.

In crosses involving putative *con2/con4*, *con1/con7*, and *con4/con7* double mutants, all four expected genotypes (two parental and two recombinant types) were recovered in the progeny, confirming that the novel morphologies were the result of double mutations (Table 2). For cross 1559 (*con2/con4* × 2539), the segregation ratio of the progeny was in close agreement with the expected 1:1:1:1 ratio, indicating that the *con2* and *con4* loci are not genetically linked. In cross 1562 (*con1/con7* × 2539), there was an excess of *con1/con7* and *con1/CON7* progeny, which we cannot explain at this time. In crosses 1572 and 1573 (*con4/con7* × 2539), there was an excess of wild type, which may reflect a reduced viability of the mutant progeny.

Three phenotypic classes were detected among progeny from cross 1581 (GR19-8 × 2539; GR19-8 was derived from transformation of Guy11-*con2* with pGT562A): *con2*, *con7*, and wild type. The recovery of *con7* progeny confirmed that GR19-8 harbored a *con7* mutation. The most frequent category was *con2* progeny, which were further classified according to their sensitivity to hygromycin B. Approximately half of the *con2* progeny were resistant to hygromycin and the other half sensitive. The hygromycin-resistant *con2* progeny produced very few spores, a phenotype similar to the GR19-8 parent. It was inferred that these *con2* progeny have the *con2/con7* genotype. The segregation ratio of the four genotypes did not deviate significantly from the 1:1:1:1 ratio, which is consistent with the hypothesis that the *con2* mutation is epistatic to *con7* in defining spore morphology and that the two loci are genetically unlinked.

To further confirm the genotypes of these double mutants, genomic DNA of representative transformants and the original mutants were subjected to Southern analysis with pAN7-2 as the hybridization probe. For the *con2/con4* (GR14-3), *con1/con4* (GR28-4), and *con4/con7* (GR44-1) mutants generated by transformation with pGT20A, the hybridization patterns were identical to those of the original *con4* mutant (Fig. 1A, compare GR14-3 and GR28-4 with GT20; Fig. 1B, GR44-1 with GT20). Similarly, gene disruptions in the 89-8-1 and Guy11-*con2* mutants mediated by transformation with pGT562A gave a hybridization pattern identical to that of the original insertional mutant, GT562 (Fig. 1A, compare GR19-8 and GR29-1 with GT562). The detection of identical flanking DNA fragments indicated that gene replacement events were mediated by a double cross-over between homologous regions of the genome and the introduced plasmids. One exception in the Southern analysis is the pattern of GR44-3 (*con4/con7*

Fig. 1. Southern analysis of double mutants and ectopic integrants of *Magnaporthe grisea*. Genomic DNA was digested with *Bam*HI and electrophoretically separated on 0.7% agarose gel. Southern blot was probed with pAN7-2. Size in kb of linearized pAN7-2 is indicated on the left side. **A**, Representative single and double mutants and ectopic integrants generated by introducing plasmids pGT20A or pGT562A into strains Guy11, Guy11-*con2*, and 89-8-1. Gene replacement mutants (GR) show pattern identical to that of the original mutations: GR14-3 (*con2/con4*), GR17-2 (*con4*), GR28-4 (*con1/con4*) have the same hybridization pattern as GT20; GR19-8 (*con2/con7*), GR16-35 (*con7*), GR29-1 (*con1/con7*) have the same pattern as GT562. The other GR strains are ectopic integrants. **B**, *con4/con7* double mutants generated by insertion of pGT20A into *con7* mutant strain GR43-3. GT20 and GR44-1 share two common bands. Uppermost band represents homology between parental vectors pAN7-2 and pBARGEM7-2. Strain GR44-3 is also considered a gene replacement mutation; however, sequence changes may have occurred that disrupted one *Bam*HI restriction site. GR44-10 is an ectopic integrant.

double mutant, compare it with GT20 in Figure 1B). It is possible that GR44-3 is the result of gene disruption due to single cross-over. Alternately, the unexpected pattern may be due to sequence changes occurring at one of the *Bam*HI sites in a gene replacement event. Transformants that had phenotypes identical to parental recipient strains were also examined for their integration patterns. In all cases, ectopic integrations were observed (Fig. 1).

Thus, genetic analyses and Southern hybridization patterns together confirmed that all the morphological variants are double mutants caused by gene replacement or disruption events at the targeted loci. The fact that ectopic integrants had spore morphology identical to parental recipient strains indicates that the wild-type alleles of the *CON4* and *CON7* loci are dominant to the mutant alleles (*con4* and *con7*).

Interactions between morphogenetic loci in appressorium formation and pathogenesis.

Table 3 summarizes the radial growth and light response of representative double mutants generated by gene disruption or replacement. Ectopic transformants from the corresponding experiments were included as controls. Radial growth was similar in the single and double mutants except for the *con1/con4* mutant (GR28-4), which was significantly reduced in vegetative growth. The effect of the *con7* mutation on light response was evident in the *con4/con7* mutants (GR44-1 and

GR44-3), which produced similar amounts of spores with or without light. The light response of the *con2/con7* mutant was similar to that of the *con2* mutant, although the double mutant showed much reduced sporulation.

Appressorium formation of double mutants was evaluated on hydrophobic microscope slide surfaces and on detached leaf sheaths. The detached leaf assay was conducted to test whether signal(s) from plants may play a role in the induction of appressorium formation. As expected, the *con2/con4* mutant formed appressoria since both the parental *con2* and *con4* mutants can form appressoria, albeit at a reduced level (Shi and Leung 1995). No appressoria were formed by the *con4/con7* and *con2/con7* mutants, indicating that *con7* is epistatic to *con2* and *con4* in appressorium formation. In all cases, the patterns of appressorium formation on slides and leaf sheath were similar. Thus, there was no evidence of inductive factors from the plant that would compensate for the defects in these mutants.

Since cyclic AMP (cAMP) has been shown to induce appressorium formation in *M. grisea* (Lee and Dean 1993), we evaluated the responses of single and double mutants to exogenous cAMP (Fig. 3). Appressorium formation in the *con2* and *con4* mutants was partially responsive to cAMP such that exogenously added cAMP induced appressorium formation on a hydrophilic surface (ethanol-cleaned microscope coverslip). The concentration of cAMP required to induce appressorium

Fig. 2. Conidial morphology of selected single and double mutants of *Magnaporthe grisea*. Double mutants show hybrid spore morphology. *con2/con4* mutant shows a slight narrowing of the basal cell and has only a single septum. *con2/con7* mutant produces *con2*-type conidia that do not form appressoria. *con4/con7* mutant shows a narrowing of the basal cell and does not form appressoria. *con1/con4* and *con1/con7* mutants are aconidial (not shown).

formation in the *con4* mutant was greater than that needed for the wild type. For the *con2* mutant, approximately 50% of the germ tubes produced appressoria when treated with 50 mM cAMP. No response to cAMP was observed in the *con2/con4* double mutant even though *con2* and *con4* mutants were partially responsive to cAMP. This would suggest that the defects in response to cAMP were additive in the *con2/con4* double mutant. The *con1* and *con7* mutants did not form appressoria on a hydrophobic surface and were not responsive to cAMP within the range of concentrations tested. No appressoria were formed by the *con2/con7* and *con4/con7* double mutants in response to exogenous cAMP, indicating that *con7* is epistatic to *con2* and *con4* in its response to cAMP.

Pathogenicity of the gene replacement mutants and their corresponding ectopic integrants was tested on rice variety M103, which is highly susceptible to the parental strain Guy11 (Table 4). The gene-disrupted *con4* and *con7* mutants both exhibited pathogenic properties identical to the original mutants, GT20 and GT562, respectively. For example, GR17-1 (*con4*) caused only a few lesions at a high inoculum level; GR16-17 and GR16-35 (*con7*) were nonpathogenic. The *con2/con4* mutants (GR14-3 and GR14-7) were weakly pathogenic on M103 as assayed by spray inoculation. The number of lesions produced by the *con2/con4* mutants was less than that produced by either single-mutation parent (*con2* or *con4*). The *con2/con7* and *con4/con7* double mutants were nonpathogenic on rice as assayed by spray inoculation (Table 4). The lack of macroscopic symptoms suggests that few, if any, cells were invaded by the double mutants. Pathogenicity of mutants was also assayed by injecting spore suspension into the leaf sheaths of plants. The wound inoculation assay allowed us to test whether the mutants were capable of colonizing the plant tissue if the penetration

step was by-passed. The results of wound inoculation were consistent with those of spray inoculation. With the exception of the *con2/con4* mutant, no double mutants caused lesions on injected leaves (Table 4).

DISCUSSION

The conventional approach for investigating epistatic relationships among single-gene mutations is to determine the phenotypes of double mutants generated by genetic crosses. Double mutants, however, can be difficult to produce due to reduced fertility of the mutants. Furthermore, the double mutant recovered among segregating progeny has a genetic background different from either parental strain where isogenic strains are not available for genetic analysis, and hence the trait of interest may be masked by other segregating genes. We demonstrate in this report that these problems can be overcome by transformation-mediated gene disruption. This approach will be especially important for some fungi that lack a sexual stage for genetic analysis. We made use of three morphogenetic mutants, *con1-C* (spontaneous), *con2* (chemically induced), and *con7* (*CON7* gene disrupted in GR43-3), as recipients for transformation. Retrieved plasmids carrying an inactivated gene (*con4* or *con7*) and a selectable marker, hygromycin B phosphotransferase, were used to replace the corresponding wild-type genes. A combination of genetic and molecular analyses confirmed the genotypes of transformants with double mutations. Gene replacement mutants were recovered at a high frequency. All double mutants examined, with the exception of GR44-3 (*con4/con7*), are the result of gene replacement events, which occur in 4 to 40% of transformants.

Table 2. Segregation analysis of double mutants of *Magnaporthe grisea* generated by gene replacement

Cross no.	Parents	Parental genotype	Expected progeny genotype	Observed progeny (no.)	χ^2	P value
1559	GR14-3 × 2539	<i>con2/con4</i> × <i>CON2/CON4</i>	<i>con2/con4</i> <i>CON2/CON4</i> <i>con2/CON4</i> <i>CON2/con4</i>	20 27 26 26	1.24	0.75 > P > 0.5
1559B	GR14-7 × 2539	<i>con2/con4</i> × <i>CON2/CON4</i>	<i>con2/con4</i> <i>CON2/CON4</i> <i>con2/CON4</i> <i>CON2/con4</i>	7 11 6 10	1.99	0.75 > P > 0.5
1581	GR19-8 × 2539	<i>con2/con7</i> × <i>CON2/CON7</i>	<i>con2/con7</i> <i>CON2/CON7</i> <i>con2/CON7</i> <i>CON2/con7</i>	4 ^a 8 7 7 ^a	1.37	0.75 > P > 0.5
1562	GR29-8 × 2539	<i>con1/con7</i> × <i>CON1/CON7</i>	<i>con1/con7</i> <i>CON1/CON7</i> <i>con1/CON7</i> <i>CON1/con7</i>	17 4 14 7	14.9	0.01 > P
1572	GR44-1 × 2539	<i>con4/con7</i> × <i>CON4/CON7</i>	<i>con4/con7</i> <i>CON4/CON7</i> <i>con4/CON7</i> <i>CON4/con7</i>	2 9 1 6	9.1	0.05 > P > 0.01
1573	GR44-3 × 2539	<i>con4/con7</i> × <i>CON4/CON7</i>	<i>con4/con7</i> <i>CON4/CON7</i> <i>con4/CON7</i> <i>CON4/con7</i>	6 18 6 7	11.1	0.05 > P > 0.01

^a Hygromycin B resistant.

Our initial model assumed sporulation to be controlled by a linear pathway such that genes controlling earlier conidial development would be epistatic to those controlling later stages. We have shown by genetic analysis that *con2* is epistatic to *con1* (Shi and Leung 1995). Considering the underdeveloped conidial morphology of the *con2* mutant, we anticipated that *con2* would be epistatic to both *con4* and *con7*. Results from this study indicate that *con2* is epistatic to *con7* but not to *con4*, as evidenced by the formation of a hybrid spore type in the *con2/con4* double mutant. Novel morphogenetic phenotypes were observed in other gene interactions as well. Sporulation was completely suppressed in the *con1/con4* and *con1/con7* mutants and hybrid spores were produced in the *con2/con4* and *con4/con7* mutants. The most prominent phenotype of the *con2* mutation is the production of one- or two-celled conidia. Since the morphogenetic abnormality in the *con1* and *con7* mutants may only be manifested at the three-celled stage, *con2* becomes epistatic to *con1* and *con7* by blocking the development of a three-celled conidium. Results from this study, together with our previous work (Shi and Leung 1995) indicate the presence of a core pathway (*con5* > *con6* > *con2* > *con1* and *con7*) determining conidial development in *M. grisea*. However, spore morphology can be modified by other genes functioning independently of this core pathway (e.g., the *CON4* gene). The role of *CON4* in this sporulation pathway is consistent with the nature of the gene. Preliminary sequence analysis indicates that this gene encodes a membrane-associated glycoprotein (D. Fujimoto, Z. Shi, D. Christian, and H. Leung, unpublished results). It is important to point out that the epistatic relationships described here are entirely based upon on the morphogenetic phenotypes and do not necessarily imply a regulatory relationship as has been demonstrated in *A. nidulans* (Busby et al. 1996; Miller 1990; Timberlake 1993).

Figure 4A summarizes the different gene interactions occurring during spore development. In the spore morphogenesis pathway, *con2* acts upstream at a branch point before *con1* and *con7* in the determination of spore shape. Interaction between the *con1* and *con7* mutations results in the production of conidiophores only. Interestingly, this conidiophore-only phenotype is similar to that of an upstream mutation, *con6*, described previously (Shi and Leung 1995). It is possible that developmental blocks in both *con1* and *con7* are functionally

equivalent to a single block in an upstream gene. In this model, *con4* functions independently of the core pathway, but exerts a strong influence on spore morphogenesis.

Examination of traits other than spore morphogenesis, however, shows different epistatic relationships among the morphogenetic loci (Table 5). The *con7* mutation is completely epistatic to *con2* and *con4* in the formation of appressoria and responsiveness to cAMP. cAMP has been shown to play an important role in the regulation of fungal development. For instance, exogenously added cAMP restored the budding phenotype in a filamentous *Ustilago maydis* mutant with a defective adenylate cyclase that abolishes cAMP synthesis (Gold et al. 1994). In *M. grisea*, appressorium formation is induced by hydrophobicity and by exogenously added cAMP (Lee and Dean 1993). We found that appressorium formation can be induced by cAMP in the *con2* and *con4* mutants but not in the *con1* and *con7* mutants (Fig. 3). The *con2/con4* double mutant no longer responded to cAMP induction even though individ-

Fig. 3. Effect of exogenously added cyclic AMP on appressorium formation of single and double mutants of *Magnaporthe grisea*. Wild-type Guy11 shows increasing response throughout the range of concentrations tested (heavily dotted bar, 0.5 mM; lightly dotted bar, 5 mM; solid bar, 50 mM). Single mutants *con2* and *con4* show a slight response at concentrations of 5 and 0.5 mM, respectively. Single mutants of *con1* and *con7* and their respective double mutants are nonresponsive.

Table 3. Growth and sporulation of representative double mutants and ectopic transformants of *Magnaporthe grisea*^a

Recipient strain + plasmid	Plasmid integration	Strain	Mutation	Radial growth (mm)	Sporulation (×10 ⁴ /ml)	
					Light	Dark
GR43-3 + pGT20A	Replacement	GR44-1	<i>con4/con7</i>	37 ± 1.4 ^b	43.9 ± 8.0	46.8 ± 8.0
	Ectopic	GR44-10	<i>con7</i>	36.5 ± 1.3	278.1 ± 14.9	273.8 ± 39.1
Guy11-con2 + pGT20A	None	Guy11-con2	<i>con2</i>	42.8 ± 0.6	5.5 ± 1.3	0
	Replacement	GR14-3	<i>con2/con4</i>	41.9 ± 0.5	12.6 ± 5.4	0.3 ± 0.5
	Ectopic	GR14-1	<i>con2</i>	36 ± 0	14 ± 3.1	0.3 ± 0.5
Guy11 + pAN7-2	Insertion	GT20	<i>con4</i>	37.1 ± 0.4	158.2 ± 13.0	1.1 ± 0.4
Guy11-con2 + pGT562A	Replacement	GR19-8	<i>con2/con7</i>	38.3 ± 0.5	1.6 ± 0.7	0.13 ± 0.2
	Ectopic	GR19-7	<i>con2</i>	41.6 ± 0.7	21.3 ± 5.5	0.3 ± 0.6
89-8-1 + pGT20A	None	89-8-1	<i>con1-C</i>	41.2 ± 0.5	1.6 ± 0.4	0.8 ± 0.2
	Replacement	GR28-4	<i>con1-C/con4</i>	24.2 ± 0.5	0	0
	Ectopic	GR28-9	<i>con1-C</i>	42.0 ± 0.0	1.8 ± 0.5	0.4 ± 0.1
89-8-1 + pGT562A	Replacement	GR29-1	<i>con1-C/con7</i>	41.5 ± 1.0	0	0
	Ectopic	GR29-9	<i>con1-C</i>	41.1 ± 1.0	2.5 ± 0.7	0.3 ± 0.1

^a Vegetative growth was measured at ninth day before sporulation was assayed by washing cultures in a 6-cm petri plate with 1 ml of sterile water.

^b ± standard deviation.

ual parental mutants responded at a reduced level. These data suggest that *con2* and *con4* mutations may be influenced directly or indirectly by the cAMP signaling pathway, whereas *con7* and *con1* are not.

Pathogenicity tests of double mutants show additive as well as epistatic interactions between the mutations. The original *con2* and *con4* mutants are weakly pathogenic to rice (Shi and Leung 1995). The *con2/con4* mutants, however, produced only a few lesions with spray and wound inoculation even when a high inoculum intensity (5×10^6 spores) was used, suggesting that the accumulated developmental defects have led to a further reduction in pathogenicity in the *con2/con4* mutant. In contrast, the *con7* mutation is pathogenically epistatic to *con2* and *con4* (Fig. 4B). There was no sign of infection with the *con2/con7* and *con4/con7* mutants when plants were spray or wound inoculated with a high level of inoculum (10^5 to 10^6 spores). That epistatic relationship is dependent on the phenotypes assayed has been demonstrated in *A. nidulans* (Martinelli 1979). For instance, the *bristle* mutation is epistatic to the *medusa* mutation in conidiophore structure, yet the *medusa* mutations are epistatic to a leaky *bristle* mutation in pigmentation. The different phenotypic expressions of gene interactions observed in *M. grisea* suggest that each gene has multiple functions in different developmental processes and, consequently, their interactions lead to diverse phenotypic expression.

The epistatic relationships described in this paper are based on the assumption that the parental mutants (Guy11-*con2*, 89-8-1, and GR43-1) used as transformation recipients and the two retrieved plasmids used for gene disruption each contain a single mutated gene. Our genetic and molecular analyses conducted on these mutants, so far, support this assumption (Shi and Leung 1995; Z. Shi and H. Leung, *unpublished data*). The gene disruption experiments conducted with the retrieved *con4* and *con7* genes (pGT20A and pGT562A) further support this conclusion. It is possible, however, that one mutation effects two or more genes that are genetically inseparable. Fur-

thermore, the epistatic relationships described in this paper are based on the phenotypic characterization of double mutants, which reflects our current knowledge of the developmental processes of sporulation, appressorium formation, and pathogenesis. A molecular understanding of the interactions among these genes will require a full characterization of the genes and their gene products.

In conclusion, we have demonstrated that morphogenetic genes have multiple functions, and hence their interactions result in epistatic relationships unique to a specific develop-

Fig. 4. A, Model depicting epistatic and interactive relationships among morphogenetic loci in conidiogenesis in *Magnaporthe grisea*. Solid arrows indicate direction of epistasis; broken two-way arrows indicate interactions producing hybrid phenotypes. *con2* acts at a branch point before *con1* and *con7*. *con4* acts independently of the core pathway, but strongly influences spore morphogenesis. Disruption of *con1*, along with either *con4* or *con7*, suppresses the formation of conidia. **B,** The epistatic relationships change; however, when pathogenicity is considered, *con7* becomes epistatic to both *con2* and *con4*.

Table 4. Pathogenicity of representative double mutants, ectopic transformants, and recipient strains of *Magnaporthe grisea*

Strain	Integrated plasmid	Genotype	Lesions per leaf of M103 (no.) at two inoculum levels ^a		Wound inoculation ^b
			5×10^5	5×10^6	
GR17-1	pGT20A	<i>con4</i>	0	0.8 ± 1.3	+
GR17-3	pGT20A	wild type	6.4 ± 1.7	19.2 ± 2.5	+
GR17-4	pGT20A	wild type	3.3 ± 1.9	11.5 ± 0.9	+
GR14-3	pGT20A	<i>con2/con4</i>	0	0.4 ± 0.6	+
GR14-7	pGT20A	<i>con2/con4</i>	0	0.3 ± 0.7	+
GR16-17	pGT562A	<i>con7</i>	0	0	—
GR16-35	pGT562A	<i>con7</i>	0	0	—
GR16-3	pGT562A	Wild type	3.4 ± 0.5	15.0 ± 5.2	+
GR16-4	pGT562A	Wild type	9.2 ± 2.2	11.0 ± 1.7	+
GR19-7	pGT562A	<i>con2</i>	0.1 ± 0.2	4 ± 3.4	+
GR19-8	pGT562A	<i>con2/con7</i>	0	0	—
GR44-10	pGT20A	<i>con7</i>	0	0	—
GR44-1	pGT20A	<i>con4/con7</i>	0	0	—
GR44-3	pGT20A	<i>con4/con7</i>	0	0	—
Guy11- <i>con2</i>	None	<i>con2</i>	0.2 ± 0.4	1.6 ± 1.9	+
GT20	pAN7-2	<i>con4</i>	0.2 ± 0.4	0.3 ± 0.6	+
GT562	pAN7-2	<i>con7</i>	0	0	—
GR43-3	pCON7::bar	<i>con7</i>	0	0	—
Guy11	None	Wild type	21.5 ± 9.3	>30	+

^a Total number of spores delivered in 8 ml of spore suspension. Approximately 10 to 15 plants were inoculated with each strain. Inoculation was conducted at least twice. $\pm$ standard deviation.

^b + = lesions observed, — = no lesion.

mental process. There exists a core pathway determining the progression of conidial development yet other genes can exert strong effects on the morphogenetic process. Double mutations in different pathways can lead to changes in spore morphology, suppression of sporulation, and reduced pathogenicity. A practical objective in our investigation of sporulation in *M. grisea* is to identify genes or developmental processes as targets for intervention. This strategy requires a clear understanding of the degree of autonomy of a developmental pathway. The suppression of spore formation and pathogenesis shown in double mutants suggests that it may be possible to identify multiple sites for intervention.

MATERIALS AND METHODS

Strains and media.

Characteristics of the *M. grisea* strains and plasmids used and generated in this study are listed in Table 1. Transformants generated from gene disruption experiments were designated as the GR series. Cultures were maintained on oatmeal agar and colonized paper disks as described (Leung et al. 1988). *E. coli* strains DH5 α and DH10B were used for propagation of plasmid DNA. *E. coli* harboring plasmids were grown in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics (Sambrook et al. 1989).

Retrieval of the mutated *CON4* and *CON7* loci.

Genomic DNA of the GT20 (*con4*) and GT562 (*con7*) mutants was isolated by the CTAB method as described (Borromeo et al. 1993). Fungal genomic sequences flanking the inserted plasmid in GT20 and GT562 were obtained by plasmid rescue as described (Kuspa and Loomis 1992). Briefly, approximately 0.5 μ g of DNA was digested with *Apa*I, which does not cut within pAN7-2. The digested DNA was ethanol precipitated, dried, and redissolved in Tris-EDTA. The DNA was circularized in a 500- μ l ligation reaction containing 5 units of T4 DNA ligase, and incubated at 14°C overnight. Ligated DNA was precipitated and redissolved in sterile water to a final concentration of 0.1 μ g/ μ l. One microliter of the ligated DNA was used to transform *E. coli* DH10B cells by electroporation with a cell porator (Cell-Porator *E. coli* Electroporation System, Gibco BRL, Gaithersburg, MD). *E. coli* transformants were selected on LB agar plates containing ampicillin (50 μ g/ml) and the plasmids were recovered by the alkaline lysis method as described (Sambrook et al. 1989). The retrieved plasmids were analyzed by restriction mapping

to confirm that they contained the original pAN7-2 plus genomic sequences presumably representing the mutated genes in the mutants. The retrieved plasmids were used for gene disruption.

Transformation.

The plasmids containing the inactivated genes (*con4* or *con7*) were linearized with *Apa*I and introduced into Guy11 (wild type), Guy11-*con2* (*con2*), 89-8-1 (*con1-C*), and GR43-3 (*con7*) by the transformation procedure described by Leung et al. (1990) and Shi et al. (1995). Hygromycin B resistant transformants were transferred to oatmeal agar and incubated under direct illumination (140 μ E $\cdot$ s⁻¹ $\cdot$ m⁻²) for 5 days to induce sporulation. Conidial morphology and colony morphology were examined by light microscopy to identify gene-disrupted mutants. Putative gene-disruption mutants and ectopic transformants with recipient strain phenotypes were subjected to phenotypic, genetic, and molecular analyses.

Analysis of transformants.

Transformants with novel conidial and colony morphology were purified by single sporing to confirm the mutant phenotypes and then crossed to 2539 to determine their inheritance by standard crossing methods (Leung and Williams 1985). Segregation patterns and linkage relationship between two genes were tested by Chi-square goodness-of-fit test. Gene-disrupted mutants and representative recipient type transformants were analyzed by Southern hybridization to determine whether the mutant phenotypes were the result of gene replacement at the corresponding loci. DNA was digested with *Eco*RI or *Bam*HI, electrophoresed, and blotted onto nylon membrane (Hybond N-plus, Amersham, Chicago) according to manufacturer's instructions. pAN7-2 was labeled with ³²P-dATP (DECAprime DNA Labeling Kit, Ambion, Austin, TX) and used to probe the genomic blots. Hybridization signals were detected by standard autoradiography (Sambrook et al. 1989).

Confirmed gene-replaced mutants and recipient type transformants (ectopic integrants) were examined for conidial morphology by differential interference contrast microscopy. Appressorium formation, growth, and sporulation were assayed as described by Shi and Leung (1995). The response of the mutants to cAMP (Sigma Chemical, St. Louis, MO) was tested by a modified procedure described by Lee and Dean (1993). Microscope coverslips were soaked in 95% ethanol, then flamed, to make them hydro-

Table 5. Summary of epistatic relationships between gene pairs in *Magnaporthe grisea*^a

Mutant genotype	Morphology		Light response of sporulation	Appressorium formation	Cyclic AMP response	Pathogenicity
	spore	colony ^b				
<i>con1/con4</i>	NA ^c	Additive ^d	NA	NA	NA	NA
<i>con1/con7</i>	NA	Hybrid	NA	NA	NA	NA
<i>con2/con4</i>	Hybrid ^e	Hybrid	Hybrid	Additive	Additive	Additive
<i>con2/con7</i>	<i>con2>con7</i> ^f	<i>con7>con2</i>	<i>con2>con7</i>	<i>con7>con2</i>	<i>con7>con2</i>	<i>con7>con2</i>
<i>con4/con7</i>	Hybrid	<i>con7>con4</i>	<i>con7>con4</i>	<i>con7>con4</i>	<i>con7>con4</i>	<i>con7>con4</i>

^a Epistatic relationships are determined based on the phenotypes of double mutants in respective developmental processes.

^b Features that determine an overall colony appearance include vegetative growth, pigmentation, abundance of mycelia, and conidiophores.

^c Not applicable.

^d Additive effect of two mutated genes was observed in the double mutant. In all cases, the defect was more pronounced than in the single-gene mutants.

^e Hybrid phenotype.

^f > indicates direction of epistasis.

philic. A 20- μ l spore suspension (approximately 10^4 spores/ml) in different concentrations of cAMP (water control, 0.5 mM, 5 mM, and 50 mM) was placed on a sterile coverslip. The samples were incubated in a moist chamber for 24 h and then inverted (with the drop of spore suspension facing down) and placed on a microscope slide. The proportion of germ tubes forming appressoria was determined by counting approximately 100 germlings per slide. Appressorium formation was also observed on excised leaf sheaths, affixed to microscope slides with "double coated" tape, by spotting spore suspension (approximately 10^5 spores per ml) onto the tissue and incubating in a moist chamber for 24 h. Appressorium formation was observed microscopically. Experiments were conducted at least three times. Pathogenicity of transformants and the original mutants was tested on rice variety M103 (provided by David MacKill, University of California-Davis). Plants were inoculated by spraying and leaf-sheath injection and the number of lesions recorded as described (Shi and Leung 1994). Approximately 10 to 15 plants were inoculated with each strain per experiment and at least two independent inoculation tests were conducted.

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