

# Identification of Pathogenicity Mutants of the Rice Blast Fungus *Magnaporthe grisea* by Insertional Mutagenesis

Pascale V. Balhadère, Andrew J. Foster, and Nicholas J. Talbot

Department of Biological Sciences, University of Exeter, Washington Singer Laboratories, Perry Road, Exeter EX4 4QG, U.K.

Accepted 13 October 1998.

**Restriction enzyme-mediated DNA integration (REMI) mutagenesis was used to identify mutants of *Magnaporthe grisea* impaired in pathogenicity. Three REMI protocols were evaluated and the frequency of REMIs determined. An REMI library of 3,527 *M. grisea* transformants was generated in three genetic backgrounds, and 1,150 transformants were screened for defects in pathogenicity with a barley cut leaf assay. Five mutants were identified and characterized. Two mutants (2029 and 2050) were impaired in appressorium function. Two other mutants, 125 and 130, were altered in conidial morphology, conidogenesis, and appressorium function. Mutant 130 was also a methionine auxotroph and methionine auxotrophy co-segregated with the reduction in pathogenicity. An additional mutant, 80, showed reduced pathogenicity on blast-susceptible rice cultivars but was fully pathogenic on barley. The reduction of pathogenicity in mutant 80 was associated with a delay in conidial germination and appressorium development. Genetic analysis suggested single-gene segregation for each mutant, but only two of the mutations co-segregated with the hygromycin resistance marker. The genetic loci in mutants 2029, 2050, 125, 130, and 80 were termed *PDE1*, *PDE2*, *IGD1*, *MET1*, and *GDE1*, respectively. *pde1* and *pde2* were non-allelic to *cpkA*, a mutation in the catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase A with a very similar phenotype. The results indicate the utility of REMI for studying fungal pathogenicity, but also highlight the requirement for rigorous genetic and phenotypic analysis.**

---

*Magnaporthe grisea* is a heterothallic ascomycete fungus that causes leaf-spot diseases on a range of grass species. It is best known for causing rice blast, one of the most important diseases of cultivated rice (for review see Talbot 1995) and a continuing threat to rice production (Baker et al. 1997). For this reason the biology of *M. grisea* has been investigated closely (for reviews see Howard and Valent 1996; Dean 1997), facilitated by the genetic and molecular tractability of the fungus and its amenability to DNA-mediated transformation (Parsons et al. 1987; Leung et al. 1990).

Rice blast disease starts when three-celled conidia are released from conidiophores by splashing water and transported onto neighboring uninfected host leaves. After release of spore tip mucilage (Hamer et al. 1988), which helps to anchor the conidium to the leaf cuticle, germination proceeds by extension of a germ tube from the basal and/or the apical cell. The germ tube undergoes hooking and swelling at its tip and then differentiates into an appressorium (Bourett and Howard 1990). The appressorium is a melanized, dome-shaped cell that penetrates the leaf via protrusion of a penetration peg through the cuticle. This process is largely mechanical, brought about by generation of high turgor (Howard et al. 1991) produced by accumulation of glycerol within the appressorium (de Jong et al. 1997). Melanin in the appressorium wall acts as a semi-permeable layer allowing free movement of water but retention of intracellular glycerol (de Jong et al. 1997; Money 1997), causing very high cellular turgor to be generated (Chumley and Valent 1990; Money and Howard 1996). Appressorial turgor is translated into mechanical force, causing a penetration peg to rupture the cuticle. Experiments carried out with the *CUTI* gene, encoding cutinase, suggest that extracellular enzymes may only have an accessory role in the penetration process (Sweigard et al. 1992), probably assisting and accelerating infection (Howard et al. 1991).

A number of signals have been implicated as triggers for induction of appressorium development in *M. grisea*, including cutin monomers (Gilbert et al. 1996), starvation stress (Jelitto et al. 1994), surface hardness (Xiao et al. 1994), and hydrophobicity of the leaf cuticle (Lee and Dean 1994). Surface hydrophobicity may be perceived by *M. grisea* due to the MPG1 hydrophobin, a secreted protein, which takes part in a hydrophobic surface interaction and is required for efficient appressorium formation (Beckerman and Ebbole 1996; Talbot et al. 1996). Secondary messengers such as cyclic AMP (cAMP) and diacyl glycerols have also been shown to induce appressorium formation on hydrophilic and hydrophobic surfaces, respectively, when applied to germinating conidia (Lee and Dean 1993; Thines et al. 1997a, 1997b) and have implicated both cAMP-dependent protein kinase A (Lee and Dean 1993; Mitchell and Dean 1995) and protein kinase C (Thines et al. 1997b) in appressorium induction. This is consistent with identification of several genes associated with infection in *M. grisea* that are essential for either conidium attachment (Liu and Dean 1997), appressorium formation (Xu and Hamer

1996; Choi and Dean 1997; Liu and Dean 1997), appressorium-mediated penetration (Mitchell and Dean 1995; Xu et al. 1997; Choi and Dean 1997), or invasive growth inside host tissues (Xu and Hamer 1996). These genes have quite specific roles in regulating pathogenicity functions (Hamer and Holden 1997) and encode components of general signaling pathways such as the G-protein-adenylate cyclase-protein kinase A pathway (reviewed by Kronstad 1997) and/or the pheromone/invasive response mitogen-activated protein (MAP) kinase cascade (reviewed by Herskowitz 1995). However, in spite of rapid progress in identifying genes involved in appressorium formation in *M. grisea*, the molecular basis of signal recognition and processing during appressorium formation and subsequent plant infection is still very much unknown, and the regulation of and interplay between the multiple signal transduction pathways deserve further investigation.

Genes involved in pathogenicity have been identified in *M. grisea* based on their expression pattern (Talbot et al. 1993a), by targeted deletion of genes encoding a known physiological function (Mitchell and Dean 1995; Xu and Hamer 1996; Choi and Dean 1997; Xu et al. 1997), or by isolation of pathogenicity mutants (Zhu et al. 1997; Sweigard et al. 1998). Of these approaches, identifying pathogenicity mutants clearly has the greatest potential for finding novel genes that encode determinants of pathogenicity. Insertional mutagenesis offers a mechanism not only for identifying mutants of interest but also for cloning the corresponding genes. Restriction enzyme-mediated DNA integration (REMI) is a procedure that results in direct gene tagging by plasmid DNA after inclusion of a restriction enzyme during transformation (Bölker et al. 1995). Since its first description in *Saccharomyces cerevisiae* (Schiestl and Petes 1991), and its application to the tagging of developmental mutations in *Dictyostelium discoideum* (Kuspa and Loomis 1992), REMI has been successfully applied to several fungal species, including *Cochliobolus heterostrophus* (Lu et al. 1994), *Neurospora crassa* (Garnand and Nelson 1995), *Ustilago maydis* (Bölker et al. 1995), *Alternaria alternata* (Akamatsu et al. 1997), *Coprinus cinereus* (Granado et al. 1997), and *M. grisea* (Shi et al. 1995; Sweigard et al. 1998).

In this report, we describe the use of REMI to generate a library of hygromycin-resistant transformants of *M. grisea*, which we have screened for pathogenicity defects. More than 1,000 transformants have been screened to date. Five new pathogenicity mutants have been identified and these have been characterized at the phenotypic, genetic, and molecular levels.

## RESULTS

### Generation and screening of the REMI library.

Before generation of an REMI mutant collection we decided to evaluate transformation procedures for *M. grisea* in order to maximize efficiency of the REMI mutant hunt. Plasmid insertion during *M. grisea* transformation has been reported to occur preferentially at regions of the genome showing short sequences of DNA homology (J. Sweigard, A. Carroll, L. Farrall, and B. Valent, *personal communication*), thus introducing a slight bias to perfectly random integration. To reduce this bias in REMI mutagenesis, we chose to use two different restriction enzymes, *Bam*HI and *Hind*III, that had previously been shown to be effective for REMI in *M. grisea*

(Shi et al. 1995). Plasmid pCB1003, which has been engineered to eliminate most common restriction sites in the hygromycin phosphotransferase gene (Carroll et al. 1994), was selected as the transforming plasmid to facilitate plasmid recovery from the *M. grisea* genome.

Choice of the recipient *M. grisea* strain was made based on the need for rapid pathogenicity screening with barley as a test host and for fertility in genetic crosses. The weeping lovegrass pathogenic strain 4091-5-8 (also pathogenic on barley) was initially selected (Valent et al. 1986) along with the highly pathogenic and fertile barley pathogen 35-R-24 (Lau and Hamer 1996). Subsequently, the rice pathogenic *M. grisea* strain Guy 11 (Leung et al. 1988) was also subjected to REMI transformation because of its more homogeneous response in pathogenicity and appressorium formation assays. Details of all *M. grisea* strains used in this study are shown in Table 1.

Three REMI transformation protocols were compared that, apart from minor variations such as the composition of polyethylene glycol (PEG) buffers and the successive temperatures of incubation, differed only in the conditions for protoplast regeneration. Full details of each procedure are given in Materials and Methods. The first REMI protocol (protocol 1) was adapted from a standard *M. grisea* transformation procedure used in our laboratory (Talbot et al. 1993a) while the second and third procedures tested (protocols 2 and 3) are published procedures from Sweigard et al. (1998) and Shi et al. (1995), respectively. By these methods, a library of 3,527 *M. grisea*

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

Strain	Characteristics	Source
Wild type		
Guy 11	Field strain, <i>MAT1-2</i> rice pathogen	Leung et al. 1988
TH3	Field strain, <i>MAT1-1</i> rice pathogen	J. L. Notteghem, unpublished
4091-5-8	Laboratory strain, <i>MAT1-2</i> weeping lovegrass and goosegrass pathogen	Valent et al. 1986
35-R-24	Laboratory strain, <i>MAT1-2</i> barley pathogen	Lau and Hamer 1996
35-R-56	Laboratory strain, <i>MAT1-1</i> barley pathogen	Lau and Hamer 1996
Mutants		
43-R-12	<i>npr1</i> mutant of 35-R-24 (F <sub>1</sub> progeny)	Lau and Hamer 1996
44-R-7	<i>npr2</i> of 35-R-24 (F <sub>1</sub> progeny)	Lau and Hamer 1996
DF51	<i>cpkA</i> mutant of 4091-5-8, <i>MAT1-2</i> , <i>hyg</i> <sup>r y</sup>	Xu et al. 1997
nn95	<i>pmk1</i> mutant of Guy 11, <i>MAT1-2</i> , <i>hyg</i> <sup>r</sup>	Xu and Hamer 1996
53-R-39	<i>mpg1</i> (F <sub>1</sub> progeny), <i>hyg</i> <sup>r</sup>	Talbot et al. 1996
2029	<i>pde1</i> mutant of 35-R-24 <sup>z</sup> , <i>MAT1-2</i> , <i>hyg</i> <sup>r</sup>	This study
2029-R-13	<i>pde1</i> (F <sub>1</sub> progeny), <i>MAT1-1</i> , <i>hyg</i> <sup>r</sup>	This study
2050	<i>pde2</i> mutant of 35-R-24 <sup>z</sup> , <i>MAT1-2</i> , <i>hyg</i> <sup>r</sup>	This study
2050-R-8	<i>pde2</i> (F <sub>1</sub> progeny), <i>MAT1-1</i>	This study
80	<i>gde1</i> mutant of Guy 11 <sup>z</sup> , <i>MAT1-2</i> , <i>hyg</i> <sup>r</sup>	This study
125	<i>igd1</i> mutant of Guy 11 <sup>z</sup> , <i>MAT1-2</i> , <i>hyg</i> <sup>r</sup>	This study
130	<i>met1</i> mutant of Guy 11 <sup>z</sup> , <i>MAT1-2</i> , <i>hyg</i> <sup>r</sup>	This study

<sup>y</sup> Hygromycin B resistant transformant.

<sup>z</sup> *pde*, *igd*, *me*, and *gde* = penetration defect, invasive growth defect, methionine auxotrophy, and germination delay, respectively.

transformants was produced in the three strain backgrounds 4091-5-8, 35-R-24, and Guy 11 with *Bam*HI and *Hind*III (92 and 8% of transformants, respectively). A comparison of each REMI protocol was carried out by Southern blot analysis of a random selection of transformants generated in a single strain background, 35-R-24 (Table 2). Transformants for each protocol were generated from more than one experiment. Despite the differences between the three REMI protocols, the transformation efficiencies obtained with 35 U ml<sup>-1</sup> of *Bam*HI were very similar (between 108 and 166 transformants µg<sup>-1</sup> DNA). Differences were observed, however, in the frequency with which REMI-mediated insertion of plasmid DNA occurred in the *M. grisea* genome (Table 2). REMI events were detected based on Southern blot analysis of genomic DNA from transformants in which the presence of a hybridizing band with a size similar to that of the plasmid could be detected. This corresponds to insertions where restriction sites at both ends of the plasmid are maintained, as occurs in REMI (Schiestl and Petes 1991; Shi et al. 1995). Integrations that did not preserve one or both restriction sites resulted in a hybridizing band of a larger size. Protocol 3 (Shi et al. 1995) was most effective in inducing REMI insertions and was significantly more efficient in doing so than protocols 1 ( $\chi^2 = 4.76$ ;  $P < 0.05$ ) or 2 ( $\chi^2 = 6.86$ ;  $P < 0.01$ ).

The REMI library was screened for mutants showing defects in pathogenicity and here we report the results from the 1,150 transformants so far screened. Pathogenicity was initially assessed with a barley cut leaf assay that we developed with the objective of quickly defining the relative pathogenicity of each transformant. In this assay a cut leaf section is suspended on agar and a drop of conidial suspension placed on the surface. Three replicate assays were carried out for each transformant analyzed and the disease allowed to progress for 4 days. All experiments were based on inoculation of a uniform concentration of conidia. Control experiments were carried out with a set of known pathogenicity mutants and corresponding isogenic wild-type strains (Fig. 1A). The non-

pathogenic *M. grisea* mutants *npr1*, *npr2*, and *pmk1* (Lau and Hamer 1996; Xu and Hamer 1996) produced no symptoms and were designated scale 0. In contrast, wild-type *M. grisea* strains showed intense necrosis of leaf sections, defined as scale 3 or 4 symptoms (Fig. 1A). Intermediate symptoms were found after inoculation with the appressorium-deficient *mpg1* mutant 53-R-39 (Talbot et al. 1996). After the first round of screening, between 8 and 9% of REMI transformants in each genetic background tested (i.e., 86 35-R-24 mutants and 12 Guy 11 mutants) scored 0 on this scale in all three replicate assays.

Two additional rounds of screening were carried out on a subset of the mutants with the cut leaf assay. This led to the identification of four mutants with consistently reduced pathogenicity (mutants 2029 and 2050 in the 35-R-24 genetic background, and mutants 80 and 130 in Guy 11). Another Guy 11 mutant (125) was also studied in detail. This mutant was selected based on defects in conidiospore morphology.

### Phenotypic characterization of pathogenicity mutants.

Pathogenicity assays on whole plants were carried out by spraying 14-day-old barley seedlings of cv. Golden Promise (Fig. 1B) and/or 21-day-old rice seedlings of cv. CO-39 (Fig. 1C). Mutants 2029 and 2050 showed clearly reduced disease symptoms on barley compared with 35-R-24 (Fig. 1B, leaves 2 and 3). Mean lesion densities per 5 cm of leaf were very reduced for 2029 ( $0.9 \pm 1.2$ ) and 2050 ( $0.4 \pm 1.1$ ) compared with 35-R-24 ( $6.3 \pm 2$ ). Among the Guy 11 mutants, 125 showed clearly reduced symptoms on barley (Fig. 1B, leaf 6) and rice (Fig. 1C, leaf 3). Similarly, mutant 130 showed reduced disease symptoms on barley (Fig. 1B, leaf 7) and rice (Fig. 1C, leaf 4). Interestingly, mutant 80 showed reduced pathogenicity in the barley cut leaf assay but appeared to be fully pathogenic when spray inoculated onto whole barley seedlings (Fig. 1B, leaf 5). This mutant was, however, markedly reduced in pathogenicity on rice (Fig. 1C, leaf 2), showing lesion densities of  $7.0 \pm 2.6$  per 5 cm of rice leaf surface, compared with Guy 11 ( $19.0 \pm 5.3$ ). The Guy 11 mutants that showed impaired pathogenicity on rice cv. CO-39 also showed comparable reductions in symptoms on a second rice cultivar, Maratelli (data not shown).

The development and function of appressoria in *M. grisea* has previously been shown to involve cAMP-dependent signaling (Lee and Dean 1993; Mitchell and Dean 1995; Choi and Dean 1997; Xu et al. 1997). In order to determine whether any of the pathogenicity mutants were impaired in cAMP signaling, we carried out infections with each mutant in the presence of 10 mM cAMP. This treatment caused a response only in mutant 130, in which pathogenicity toward CO-39 was partially restored. Mean lesion densities per 5 cm of rice leaf increased from  $6.1 \pm 2.2$  to  $13.5 \pm 4.2$  when conidia were inoculated in the presence of 10 mM cAMP. This compared with a lesion density of  $16.9 \pm 5.9$  when Guy 11 was inoculated under the same conditions.

### The effect of pathogenicity gene mutations on growth and development.

Having identified five pathogenicity mutants, we decided to examine the effects of each mutation on vegetative growth and asexual development. Growth of each mutant on complete medium showed small but significant reductions (2029, 2050,

**Table 2.** Effect of REMI (restriction enzyme-mediated DNA integration) protocol on transformation efficiency and the nature of integration events in *Magnaporthe grisea* strain 35-R-24

Transformation protocol <sup>u</sup>	Total (%)	Transformants analyzed (no.) <sup>v</sup>	Transformants showing single copy integration <sup>w</sup>	Transformants generated by true REMI <sup>x</sup>
1	61.8	22	9/22	5/9
2	13.6	20	10/20	4/10
3	24.6	21	17/21 <sup>y</sup>	9/17 <sup>z</sup>

<sup>u</sup> Protocol 1: modification of Talbot et al. 1993a, this study; Protocol 2: Sweigard et al. 1998 and J. Sweigard, A. Carroll, L. Farrall, F. G. Chumley, and B. Valent, *personal communication*; Protocol 3: Shi et al. 1995.

<sup>v</sup> Transformants selected for Southern analysis were generated with pCB1003 and *Bam*HI.

<sup>w</sup> Transformants that had undergone a single integration event as determined by Southern blot analysis.

<sup>x</sup> Single copy transformants in which pCB1003 has inserted at a *Bam*HI site, leaving restriction site intact. Characterized by hybridizing fragment corresponding in size exactly to introduced linearized plasmid.

<sup>y</sup> Frequency of single copy integration significantly different from protocol 1 ( $\chi^2 = 13.89$ ;  $P < 0.001$ ) or protocol 2 ( $\chi^2 = 8.05$ ;  $P < 0.01$ ).

<sup>z</sup> Frequency of REMI significantly different from protocol 1 ( $\chi^2 = 4.76$ ;  $P < 0.05$ ) or protocol 2 ( $\chi^2 = 6.86$ ;  $P < 0.01$ ).

125), a slight increase (80), or no significant difference (130) from corresponding wild-type strains (Table 3). Conidiogenesis was also similar to the parental wild-type strain 35-R-24 in mutants 2029 and 2050, while all Guy 11 mutants showed significant reductions in conidiogenesis compared with the wild type (Table 3). In addition to reduced numbers of conidia, both mutants 125 and 130 produced a proportion of conidia with aberrant morphology, as shown in Figure 2. Abnormalities included the presence of single- and two-celled spores, as well as various alterations in the shape of two- and three-celled spores (with or without conservation of bilateral symmetry about the longitudinal axis). Often, germ-tube-like projections were observed from the apical or basal cell of misshapen spores, but they never grew out from the spore and were therefore probably not signs of premature germination. Spore shapes were distinct from previously identified conidiation mutants (Hamer et al. 1989; Shi and Leung 1995). None of the alterations in spore morphology affected spore viability (Table 3), although conidial germination was delayed significantly in mutants 80, 125, and 130.

The growth of each mutant on minimal medium appeared similar to that observed in rich growth media, with the exception of mutant 130, which was unable to grow on minimal medium or on oatmeal agar. This was found to be due to an auxotrophic requirement for methionine. Growth of 130 could be supported by addition of cystathionine, homocysteine or methionine to minimal medium, but not by addition of homoserine. All remaining mutants were prototrophic and were not affected in nitrogen metabolism, unlike the nonpathogenic mutants *npr1* and *npr2* described by Lau and Hamer (1996). None of the mutants showed an easily wettable phenotype, suggesting that each mutant supports wild-type production of the MPG1 hydrophobin (Talbot et al. 1993a).

### Effect of mutations on appressorium-mediated plant infection.

Appressorium formation was studied by incubating conidia on artificial hydrophobic surfaces or epidermal strips. Development of appressoria on plastic surfaces occurred over a similar period of time in both genetic backgrounds, Guy 11 and 35-R-24. However, the frequency of appressorium differentiation was far more variable in 35-R-24 than in Guy 11 (Table 3). We have not been able to determine the external parameters affecting this (surface characteristics, light, and temperature of incubation appeared to have limited effects), although we found that the frequency of appressorium formation could be partially enhanced by addition of 10 mM cAMP (data not shown). Appressorium induction in both *M. grisea* strains was far more uniform on plant strips such as onion epidermis or cereal leaves, without any additional chemical induction (Table 3). Appressorium development was impaired significantly only in mutant 125, which showed an approximately 75% mean reduction compared with Guy 11, when assayed on coverslips (Table 3). The difference was much less pronounced on cereal leaves, however (Table 3). Mutant 125 produced an abundance of swollen, short, hyphal branches during appressorium development, compared with the wild type, perhaps representing premature abortion of appressorium formation (Fig. 2D and E).

Appressorium function was evaluated by measurement of internal turgor pressure and by assaying appressorium-

mediated penetration of epidermal cell layers (Chida and Sisler 1987). Glycerol has recently been found to be the major compatible solute that accumulates during appressorium turgor generation in *M. grisea* (de Jong et al. 1997). Cytorrhysis assays were therefore performed in order to estimate the intracellular concentration of glycerol. The resulting osmotic potential was extrapolated based on the regression equation between glycerol molarity and psychrometric measurement of osmotic potential (de Jong et al. 1997). With this analysis, mean internal appressorium turgor was estimated to be 4.1 MPa for Guy 11 and 4.4 MPa for 35-R-24 after 24 h of development (data not shown). All mutants generated similar appressorium turgor (3.8 to 4.4 MPa). In the absence of any detectable difference in turgor generation, the frequency of successful plant penetration was determined by both an onion epidermis assay (Chida and Sisler 1987) and a barley and rice leaf penetration test. Most appressoria (around 80%) produced by 35-R-24 and Guy 11 wild-type strains were able to invade onion epidermis (Fig. 3A and B) after 32 h. Penetration of onion, rice, and barley epidermis was of the same order for appressoria of Guy 11, but 35-R-24 appressoria were less efficient in penetration of barley epidermis (Fig. 3A). Appressoria of mutants 2029 and 2050 were significantly reduced in their ability to penetrate both onion and barley epidermis after 24 or 48 h of incubation (Fig. 3A). In contrast, the ability of appressoria of mutants 80 and 130 to penetrate onion epidermis was similar to that of the parental strain Guy 11, with mutant 125 showing a small reduction in appressorium penetration (Fig. 3B). On rice epidermis, however, appressoria from mutants 80, 125, and 130 were considerably reduced in their frequency of penetration, compared with Guy 11, particularly in the case of 125 and 130. Significantly, mutant 80—which is pathogenic on barley but reduced in symptom development on rice—was able to penetrate barley epidermis more quickly than rice epidermis (Fig. 3B).

Once inside epidermal cells, invasive growth of *M. grisea* proceeded as described by Heath et al. (1990), via intracellular ramification of secondary bulbous hyphae (Fig. 4A, B, and D) and colonization of adjacent cells with long, unbranched, and unbeaded hyphae. A comparative study of the penetration of barley and rice leaves by Guy 11 showed that epidermal colonization was more rapid in barley infections (data not shown), consistent with epidermal penetration assays (Fig. 3). Successful penetration mostly occurred in epidermal cells adjacent to veins in both grasses and necrosis of infected tissues appeared later in barley, between 48 and 72 h, compared with less than 48 h in rice. Unlike secondary hyphae, primary infection hyphae grew mainly in a direction perpendicular to the cuticle surface (Heath et al. 1990). Fungal colonization of host tissues was impaired in mutants 2029 and 2050, compared with 35-R-24. In mutant 2050, host colonization did not usually proceed farther than the appressorium, or a stunted club-shaped primary hypha originating from the penetration peg (arrowed in Figure 4C and F). In mutant 2029, some rare, secondary, branched hyphae were seen inside epidermal cells, although colonization generally was arrested at the primary infection stage.

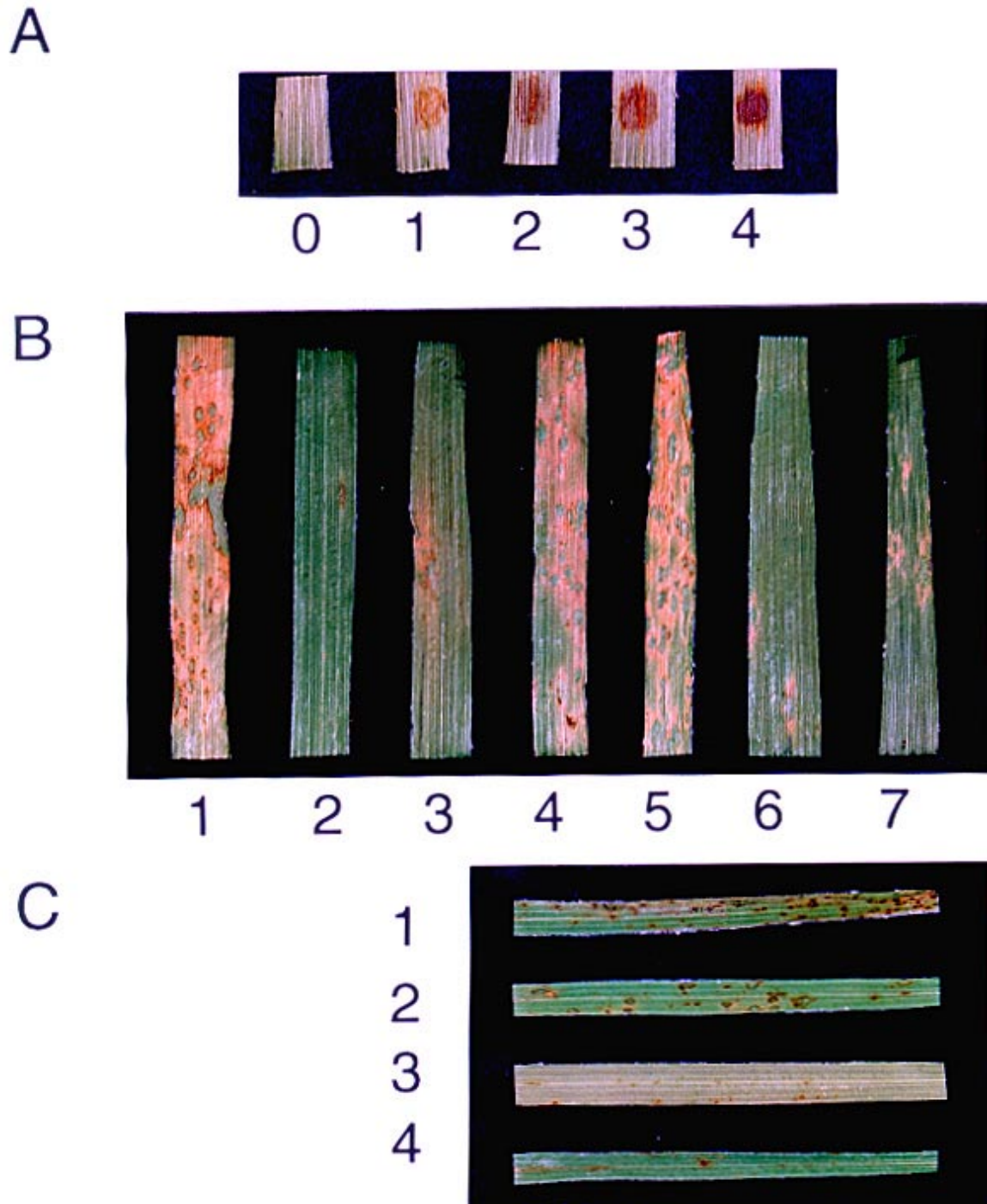
### Genetic and molecular analysis of the mutants.

Genomic DNA of the five pathogenicity mutants was digested by *Bam*HI and subjected to Southern blot analysis with

pCB1003 as a probe. In all cases the pCB1003 probe hybridized to a single genomic restriction fragment (data not shown) that was greater in size than the linearized plasmid (4.09 kb). This suggests that each transformant was the result of a single

plasmid integration into the *M. grisea* genome, with destruction of one or possibly both flanking *Bam*HI sites.

For each of the five mutants, the inheritance of related mutant phenotypes was then determined by random ascospore



**Fig. 1.** Pathogenicity assays of *Magnaporthe grisea* REMI (restriction enzyme-mediated DNA integration) mutants. **A**, Barley cut leaf assay. Droplets of a conidial suspension of  $10^4$  conidia  $\text{ml}^{-1}$  in water (25  $\mu\text{l}$ ) were applied to the surface of barley leaf segments and incubated on distilled water agar for 96 h. Mutants were scored on a 0 to 4 scale based on appearance of lesions as follows: 0, no symptoms (e.g., nn95, a *pmk1* mutant); 1 or 2, intermediate symptoms (e.g., a subset of REMI mutants); 3, necrotic reaction (e.g., 53-R-39, an isolate of the appressorium-deficient mutant *mpg1*); 4, intense necrotic reaction (e.g., Guy 11). **B**, Leaves from whole plant infection assays on barley cv. Golden Promise. Fourteen-day-old seedlings were spray inoculated with uniform conidial suspensions of the wild-type *M. grisea* strain 35-R-24 (leaf 1), mutants 2029 (leaf 2) and 2050 (leaf 3), wild-type strain Guy 11 (leaf 4), mutants 80 (leaf 5), 125 (leaf 6), and 130 (leaf 7). **C**, Leaves from whole plant infection assays on rice cv. CO39. Twenty-one-day-old seedlings were spray inoculated with wild-type Guy 11 (leaf 1), mutant 80 (leaf 2), mutant 125 (leaf 3), and mutant 130 (leaf 4).

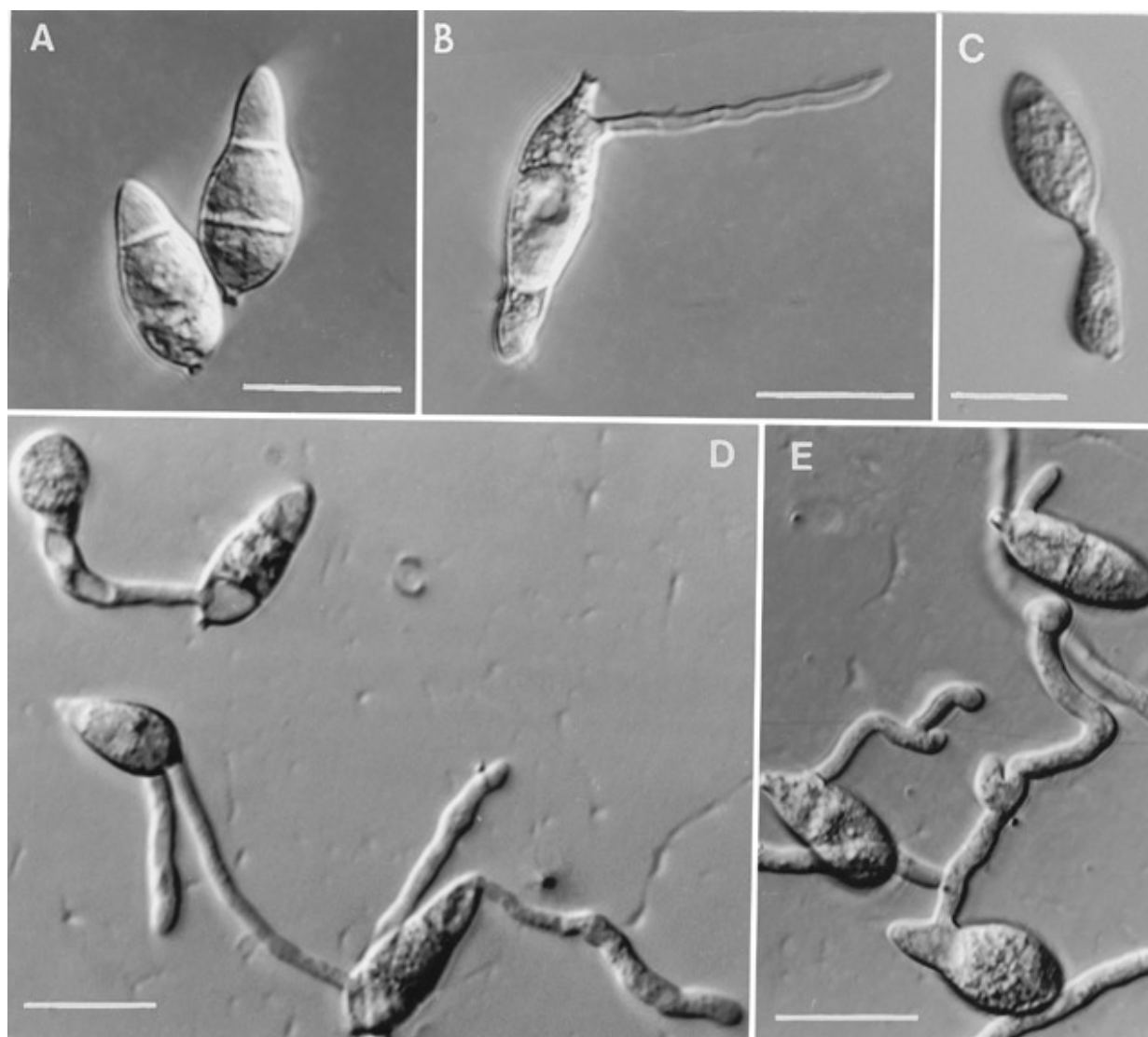
**Table 3.** Assessment of defects in growth and development of *Magnaporthe grisea* REMI (restriction enzyme-mediated DNA integration) mutants

Strain	Growth <sup>x</sup>	Conidia (no.) <sup>y</sup>	Conidial viability (%)	Appressorium development (%) <sup>z</sup>	
				(on coverslips)	(on leaf)
35-R-24	4.6 B	2.2 A	87.5 ± 2.2 A	30.7 ± 30.3 A	90.3 ± 3.5 A
2029	4.2 A	2.5 A	91.7 ± 1.5 A	29.4 ± 42.1 A	77.7 ± 14.6 A
2050	4.2 A	3.2 A	96.3 ± 2.6 A	35.4 ± 40.8 A	90.3 ± 4.0 A
Guy 11	3.6 b	19.3 a	96.9 ± 2.5 a	90.9 ± 11.5 b	77.0 ± 6.6 a
80	4.2 c	0.2 b	92.8 ± 7.2 a	75.3 ± 3.2 b	77.3 ± 10.1 a
125	2.6 a	1.7 c	94.7 ± 1.2 a	20.9 ± 8.1 a	85.8 ± 3.7 a
130	3.5 b	0.9 d	96.0 ± 2.7 a	63.4 ± 11.2 ab	87.7 ± 1.1 a

<sup>x</sup> Mean values followed by the same letter are not significantly different at  $P = 0.05$ . Comparisons only made between mutants from same genetic background. Capital letters are for 35-R-24 mutants. Colony diameter (cm) was measured after 7 days of growth on CM (complete medium) agar. Mean values are from 10 replicates.

<sup>y</sup> Conidiogenesis assessed in 10 replicate experiments. Means are expressed as numbers of conidia  $\times 10^4$  ml<sup>-1</sup> of conidial suspension cm<sup>-2</sup> of culture. Conidial viability is expressed as the mean percentage of conidia that had germinated after 24 h  $\pm$  SD.

<sup>z</sup> Appressorium development on plastic coverslips during 24 h. Three replicates of 200 conidia were counted for each observation. Appressorium formation also examined on barley leaves for 35-R-24, 2029, and 2050, and rice leaves for Guy 11, 80, 125, and 130 with 3 replicates of 100 conidia. Mean percent appressorium formation at 24 h  $\pm$  SD. SDs often exceeded mean values for 35-R-24-derived strains, which showed extreme variability in appressorium assays on artificial surfaces (see text).

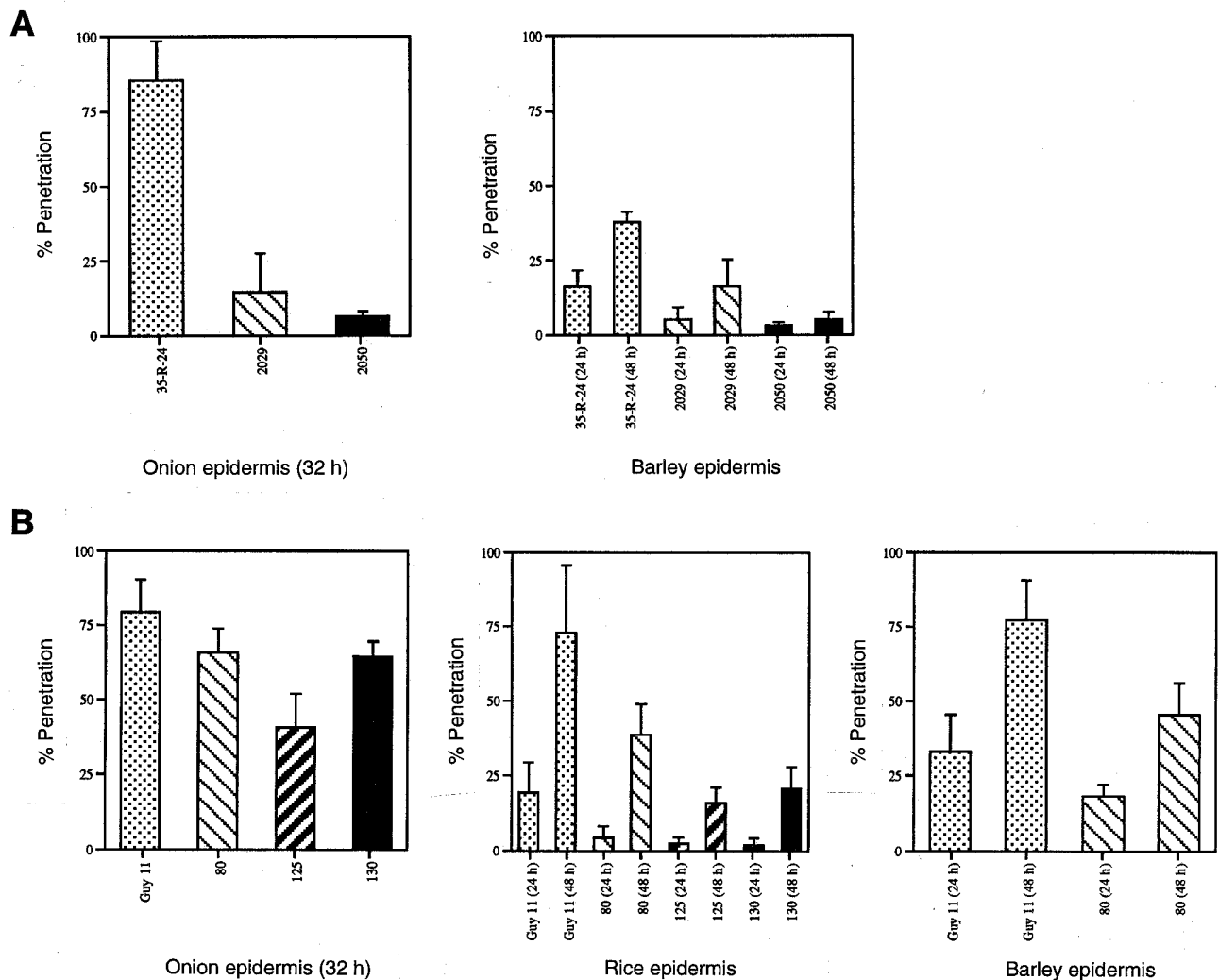


**Fig. 2.** Conidial morphology of REMI (restriction enzyme-mediated DNA integration) mutants 125 and 130. Conidia were harvested from plate cultures of Guy 11, 125, and 130 and viewed immediately or after germination on plastic coverslips. Micrographs are of conidia viewed with Hoffman modulation contrast with a Nikon Optiphot microscope. **A**, Pyriform-shaped conidia produced by the wild-type Guy 11. **B**, Elongated, three-celled conidium produced by mutant 125. The apical cell is extended through a germ-tube-like projection. **C**, Elongated, two-celled conidium produced in mutant 130. **D**, Germ tube hooking in wild-type conidia after 6 h. **E**, Germ tube hooking in 125 conidia after 6 h. Scale bar = 5  $\mu$ m.

analysis. Co-segregation of each phenotype with the hygromycin B resistance (*Hph*) marker was also determined. Comparisons of  $\chi^2$  distribution to the expected 1:1 ratio predicted single gene inheritance for all phenotypes studied (Table 4). Only in the cross between 130 and TH3 did the calculated  $\chi^2$  deviate from the expected 1:1 ratio, showing a significant deficit in both hygromycin-resistant and methionine-auxotrophic offspring. Segregation of hygromycin B resistance was supported in all cases by Southern blot data (data not shown). For mutants 2029 and 80, hygromycin B resistance co-segregated with all mutant phenotypes, confirming that insertion of plasmid DNA had caused the mutation (Table 4). However, mutant phenotypes exhibited by 2050, 125, and 130 showed no co-segregation with *Hph*, suggesting that the mutations responsible for these various phenotypes arose independently of plasmid insertion. In the case of mutant 130 the reduction in pathogenicity co-segregated with methionine auxotrophy, which may facilitate gene cloning by comple-

mentation. In mutant 125, however, conidial morphology and pathogenicity segregated as single un-tagged mutations, which complicates analysis of this mutant. The loci corresponding to each mutant identified were named *pde1* (2029) and *pde2* (2050) for penetration defective, *gde1* (80) for germination delayed, *igd1* (125) for invasive growth defect, and *met1* for methionine auxotrophy.

The pathogenicity phenotypes of mutants 2029 and 2050 were similar to the mutant phenotype reported after targeted deletion of the *CPKA* gene, which encodes a catalytic subunit of cAMP-dependent protein kinase A (Mitchell and Dean 1995; Xu et al. 1997). Allelism tests of the single tagged mutation in 2029 (*pde1*) and the untagged mutation in 2050 (*pde2*) with the  $\Delta cpkA$  mutation were therefore carried out. Mutant strains 2029 (*pde1*) and DF51 ( $\Delta cpkA$ ) (Xu et al. 1997) were both crossed with a hygromycin-sensitive F<sub>1</sub> progeny strain, 2050-R-8. In parallel, the allelism of mutation *pde1* was also checked to *cpkA* by crossing the hy-



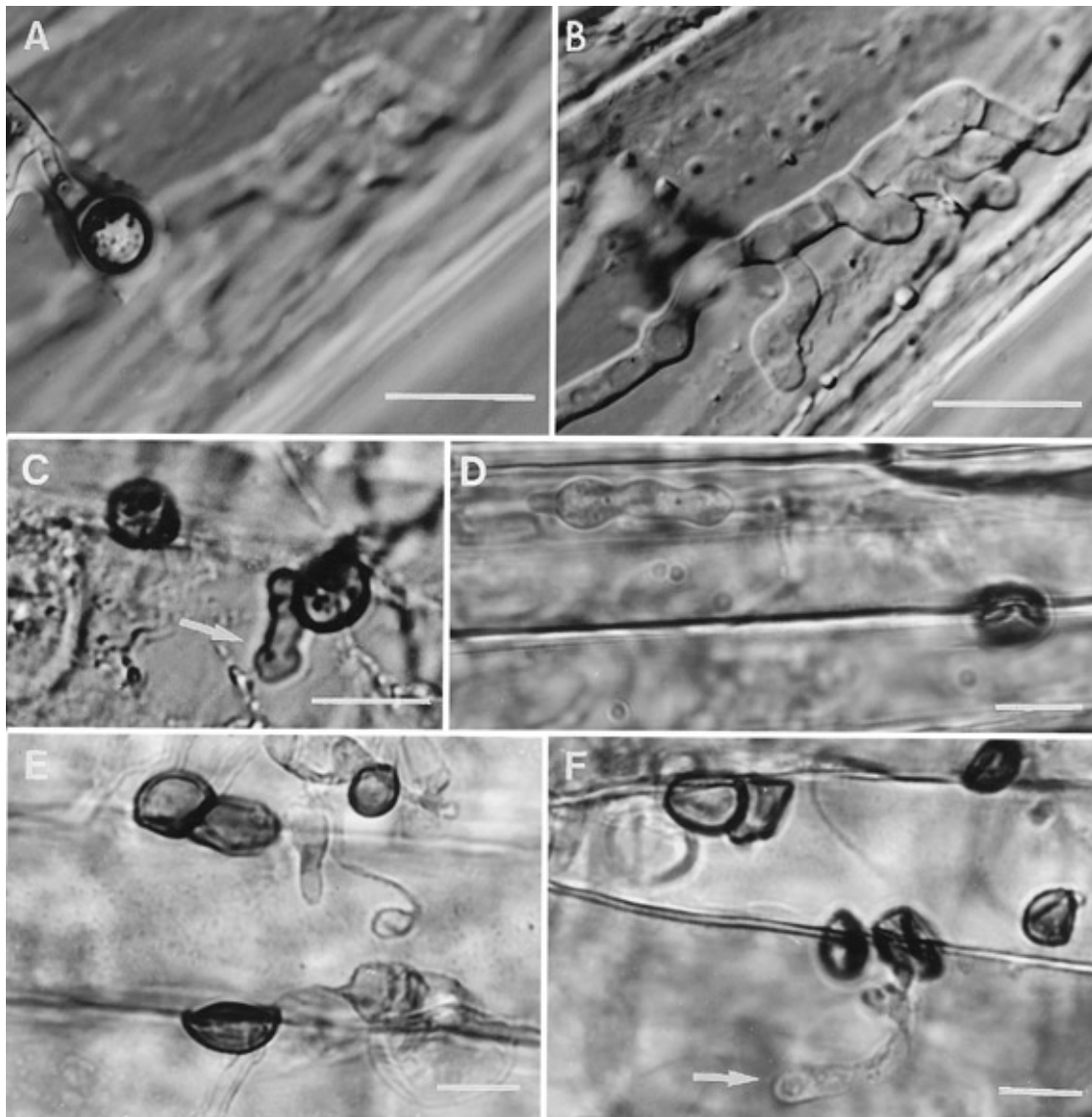
**Fig. 3.** Appressorium-mediated penetration of *Magnaporthe grisea* wild-type strains and REMI (restriction enzyme-mediated DNA integration) mutants. Conidial suspensions were incubated on sterilized onion epidermis (Chida and Sisler 1987) and intact barley or rice epidermis. Percentage of appressoria that had penetrated the epidermis, by elaboration of a penetration peg, was recorded at 32 h after inoculation for onion epidermis, 24 h for barley epidermis, and 48 h for rice epidermis. **A**, Appressorium-mediated penetration assays of 35-R-24 mutants 2029 and 2050. **B**, Appressorium-mediated penetration assays of Guy 11 mutants 80, 125, and 130. Error bar = standard deviation.

gromycin resistant  $F_1$  progeny strain 2029-R-13 with DF51. For each allelism test, the involvement of two separate genes was confirmed by the presence of wild-type recombinant offspring in the  $F_1$  generation (Table 5). Consistent with *PDE1* and *PDE2* being loci distinct from *CPKA*, appressorium formation was not delayed in 2029 and 2050, whereas a substantial delay occurs in *cpkA* strains (Xu et al. 1997). Distribution of the three *Hph* segregations and two of the pathogenicity segregations deviated from the expected ratios with an excess of wild-type progeny, possibly suggesting lethality of double mutants. So far this hypothesis can only be checked among progeny of the cross between 2029-13 and DF51, as *pde2* is not tagged.

## DISCUSSION

### Assessment of REMI mutagenesis for identification of pathogenicity mutants.

REMI has previously been reviewed as a method for gene tagging in filamentous fungi (Sweigard 1996). Although the random nature of plasmid integration has been questioned (Sweigard et al. 1998), REMI has already proved useful in recovering novel mutations in plant-pathogenic fungi (Lu et al. 1994; Bölker et al. 1995; Akamatsu et al. 1997). In *M. grisea*, REMI has allowed more than 30 genes involved in conidiation (Shi et al. 1995) and pathogenicity (Sweigard et al. 1998) to be tagged. Using this technique, we have tagged



**Fig. 4.** Detail of appressorium-mediated penetration by *Magnaporthe grisea* mutant 2050. **A and B**, Onion penetration assay by wild-type *M. grisea* strain 35-R-24 after 32 h. Appressorium formed on surface of the onion strip (**A**) has penetrated the underlying epidermal cell and formed beaded hyphae (**B**). **C**, Defective penetration of sterile onion epidermis by mutant 2050 after 32 h. Two appressoria are shown but only one has penetrated the cuticle where growth was arrested with a club-shaped infection hypha. **D**, Penetration of intact barley leaf by wild-type *M. grisea* strain 35-R-24 after 48 h. One appressorium is shown on the surface stained with acid fuchsin (slightly out of focus). In the underlying cell, a bulbous secondary hypha (in focus and unstained) grows away from initial infection site. **E and F**, Penetration of intact barley leaf by mutant 2050 after 48 h. Several appressoria are shown together with collapsed conidia from which they originated. Only one appressorium (arrowed in **F**) has successfully penetrated the underlying cell and has only formed a stunted infection hypha. Scale bar = 5 μm.

two additional genes related to pathogenicity and/or conidiation processes and characterized three un-tagged mutations involved in pathogenicity functions.

The five new pathogenicity mutants reported here were identified after screening 1,150 *M. grisea* transformants, corresponding to a frequency of 0.4% (close to the 0.5% value recently reported by Sweigard et al. 1998). The percentage of tagged mutations involved in pathogenicity was also similar (0.2% compared with 0.33%; Sweigard et al. 1998). Taken together, these values suggest that REMI mutant screens in *M. grisea* are likely to be reproducible in their efficiency of mutant isolation. The low percentage of tagged mutations also probably emphasizes the potential mutagenicity of adding restriction endonucleases to protoplasts during transformation, previously reported in other fungi (Winegar et al. 1992; Granado et al. 1997).

The molecular basis of REMI in filamentous fungi may involve activation of different recombinogenic-repair processes, explaining the varied insertion of plasmid DNA, which includes true REMI, single insertion with elimination of flanking restriction sites, ectopic integration with no correspondence to restriction site, or more elaborate genomic rearrangements (Asch et al. 1992; Schiestl et al. 1994; Sweigard et al. 1998). Our comparison of three different REMI treatments in a single strain of *M. grisea* with a single restriction endonuclease (*Bam*HI) shows that, at least in *M. grisea*, the conditions for transformation may influence the way plasmid integration proceeds (Table 2). The main difference between the transformation protocols we compared was in the method of protoplast regeneration. In protocols 2 and 3 (Sweigard et al. 1998; Shi et al. 1995), PEG, added to protoplasts to bring about DNA-mediated transformation, was removed by centrifugation. In protocol 3 (Shi et al. 1995), which promoted the highest frequency of REMI, protoplast regeneration also involved a prolonged incubation in osmotically stabilized liquid medium subsequent to removal of PEG. Removal of PEG buffer in protocols 2 and 3 may limit the uptake of transforming DNA and restriction endonuclease into protoplasts, preventing a high frequency of chromosomal breaks triggered by prolonged exposure to restriction endonuclease, and thereby favoring single-plasmid insertion events at endonuclease-cleaved sites.

**Table 4.** Segregation analysis of random ascospore F<sub>1</sub> progeny from crosses between REMI (restriction enzyme-mediated DNA integration) mutants and wild-type strains of *Magnaporthe grisea*

Cross	Hygromycin sensitivity <sup>v</sup>		$\chi^2$	Pathogenicity <sup>w</sup>		$\chi^2$	F	Conidiation <sup>x</sup>		$\chi^2$	F	Co-segregation of phenotypes <sup>y</sup>
	hyg <sup>r</sup>	hyg <sup>s</sup>		Mutant	Wild type			Mutant	Wild type			
35-R-56 × 2029	16	27	2.8	16 (0.6)	27 (9.9)	2.8	148.1 <sup>z</sup>					1-2
35-R-56 × 2050	25	25	0	26 (0.8)	24 (11.1)	0.1	126.1 <sup>z</sup>					...
TH3 × 80	24	31	0.9	24 (18.6)	31 (34.3)	0.9	19.4 <sup>z</sup>	24	31	0.9	51.8 <sup>z</sup>	1-2-3
TH3 × 125	17	29	3.1	17 (0.8)	29 (13.2)	3.1	106.6 <sup>z</sup>	19	27	1.4	110.8 <sup>z</sup>	...
TH3 × 130	9	38	17.9 <sup>z</sup>	9 (7.4)	38 (22.3)	17.9 <sup>e</sup>	51.3 <sup>z</sup>	9	38	17.9 <sup>z</sup>	2.6	2-3

<sup>v</sup> hyg<sup>r</sup> and hyg<sup>s</sup> stand for hygromycin B resistant and hygromycin B sensitive, respectively

<sup>w</sup> Pathogenicity of progeny was assessed on barley cv. Golden Promise except for progeny of the third cross, which were tested on rice cv. CO39. Mean lesion densities (per 5 cm) are given in brackets. F = the value of the Fisher-Snedecor test obtained by one-way analysis of variance.

<sup>x</sup> Conidiation was determined by flooding plate cultures with 3 ml of water. The mean number of conidia for wild-type progeny was  $9.1 \times 10^5 \text{ ml}^{-1}$  ( $\pm 4.2 \times 10^5 \text{ ml}^{-1}$ ). The mean number of conidia from mutant progeny was  $1.5 \times 10^5 \text{ ml}^{-1}$  ( $\pm 1.4 \times 10^5 \text{ ml}^{-1}$ ).

<sup>y</sup> Phenotypes 1, 2, and 3 correspond to hygromycin B resistance, pathogenicity phenotype, and conidiation phenotype, respectively. - indicates no co-segregation.

<sup>z</sup> Significant at  $P = 0.001$ .

## Analysis of pathogenicity mutants.

Development of a simple, detached, leaf assay for pathogenicity was essential in screening large numbers of *M. grisea* transformants. In this way we were able to quickly discriminate pathogenicity mutants from other transformants. This was aided greatly by being able to carry out control inoculations with known pathogenicity mutants, which alleviated variation between experiments. Once identified, we re-tested all mutants by spray inoculating whole seedlings, which allowed further selection of mutants showing reduced blast symptoms.

To determine the likely cause of impaired pathogenicity in each mutant we carried out a comprehensive examination of mutant phenotypes. Only one of the mutants, 125 (*igd1*), was defective in appressorium development although this phenotype was observed only in assays on artificial substrates (appressoria formed efficiently on leaf surfaces). This is in contrast to previously identified mutants such as *mpg1*, *pmk1*, *mac1*, *app1*, and *magB* (Talbot et al. 1993a; Xu and Hamer 1996; Zhu et al. 1996; Choi and Dean 1997; Liu and Dean 1997), which are all defective in elaboration of infection structures and pathogenicity. Two of the mutants, 2029 (*pde1*) and 2050 (*pde2*), were, however, severely impaired in appressorium-mediated penetration. This was observed in penetration assays of both onion and barley epidermis (Fig. 3) and was similar to the phenotype exhibited by *cpkA* mutants (Xu et al. 1997). However, both *pde* mutations were found to affect two separate loci distinct from *CPKA*. Neither of the *pde*

**Table 5.** Genetic relationships between *pde1*, *pde2*, and *cpkA* mutations in *Magnaporthe grisea*

Cross	Random ascospore progeny				
	Hygromycin sensitivity <sup>w</sup>			Pathogenicity <sup>x</sup>	
	hyg <sup>r</sup>	hyg <sup>s</sup>	$\chi^2$	Wild type	$\chi^2$
2050-R-8 × 2029	15	32	6.2 <sup>y</sup>	16	2.1
2050-R-8 × DF51	13	39	13 <sup>z</sup>	27	20.1 <sup>z</sup>
2029-R-13 × DF51	9	33	64.3 <sup>z</sup>	33	64.3 <sup>z</sup>

<sup>w</sup> hyg<sup>r</sup> and hyg<sup>s</sup> stand for hygromycin B resistance and sensitivity phenotype, respectively. The  $\chi^2$  values tested a 1:1 ratio for hyg<sup>r</sup>/hyg<sup>s</sup> segregation in the first two crosses and a 3:1 ratio in the third cross.

<sup>x</sup> Pathogenicity of progeny was assessed on barley cv. Golden Promise. For pathogenicity segregation  $\chi^2$  values tested a 3:1 ratio.

<sup>y</sup> Significant at  $P = 0.05$ .

<sup>z</sup> Significant at  $P = 0.001$ .

mutants was affected in appressorium turgor, indicating that the penetration process is blocked subsequent to inflation of the appressorium. Where Pde mutants were able to penetrate cuticle layers they often formed stunted, club-shaped, penetration pegs (Fig. 4) consistent with a block in sustaining cell polarity necessary for extension of the penetration peg. The possible lethality of *pde1cpkA* and *pde2cpkA* double mutants (yet to be confirmed) may indicate that *pde* and *cpkA* mutations affect genes involved in alternate transduction pathways required for penetration peg extension. Preliminary observations indicate that, like *cpkA* mutants (Xu et al. 1997), both *pde1* and *pde2* mutants are able to grow inside tissues and produce lesions if conidial suspensions are injected through the sheath of barley leaves, consistent with a defect in cuticle penetration.

The three mutants isolated in the Guy 11 genetic background all displayed a range of conidiation-related phenotypes. These phenotypes all segregated as a single genetic character for mutants 80 (*gde1*) and 130 (*met1*), illustrating the frequent link between conidiogenesis and pathogenicity-related mutant phenotypes (Hamer and Givan 1990; Shi and Leung 1995). All three Guy 11 mutants were able to penetrate onion epidermis, although mutant 125 showed reduced frequency, compared with wild type. All the mutants were, however, reduced in rice leaf penetration to varying extents (Fig. 3). This feature may reflect a defect in appressorium function—although turgor appeared similar to wild-type values (data not shown)—or in formation of the penetration pegs and infectious hyphae.

The *gde1* mutant was very reduced in disease symptom development on rice cultivars and yet was fully pathogenic on barley (Fig. 1). This may be due to the defect in appressorium-mediated penetration because when *gde1* appressoria developed on the two plant surfaces (barley and rice) that differ in surface hardness, appressoria penetrated at different rates. The reduced and delayed penetration of rice, concomitant with an earlier host response, may therefore be sufficient to account for the reduced pathogenicity of *gde1* on this host. Once growth was established within plant tissues, for example, no difference could be detected in the rates of growth of *gde1* compared with the wild type based on microscopy. The *gde1* mutation also caused a reduction in conidiogenesis (Table 3) and conidium attachment, and a delay in the onset of spore germination. The defect might therefore be associated either with polarity establishment during germination and appressorium-mediated infection, or in mobilization of storage compounds for active growth (for review of these germination-associated processes see d'Enfert 1997). Mutants 125 (*igd1*) and 130 (*met1*) were distinct from both the *pde* mutants and *gde1* because the reduction in plant infection in these mutants was also associated with reduced colonization of rice epidermal cells, leading to fewer and often smaller lesions.

In mutant 130 (*met1*), methionine auxotrophy segregated with reduced pathogenicity and abnormal conidial morphology. Growth on minimal medium showed the auxotrophic requirement could be fulfilled by addition of methionine, homocysteine, and cystathionine, but not of homoserine. This is consistent with a mutation in the gene encoding either cystathionine  $\gamma$ -synthase or homocysteine synthase. Free amino acid determination from mycelial extracts of 130 was consistent with either of these possible mutations (M. Droux,

P. V. Balhadère, M.-H. Lebrun, and N. J. Talbot, unpublished results). It is possible, however, that a mutation in a gene encoding a general transcription activator such as *CYS3* of *N. crassa* or *MET4/MET28* of *S. cerevisiae* could also cause methionine auxotrophy (Marzluf 1997). In *S. cerevisiae*, methionine auxotrophy has, for example, been associated with various blocks directly or indirectly involved in sulfur assimilation, including structural genes for transport and metabolism and corresponding regulatory genes (Slekar et al. 1996). Definite confirmation will await the results of cloning the target gene by complementation of the methionine auxotrophy. The conidiogenesis phenotype associated with *met1* (Fig. 2) may be due to inefficient translocation of any available methionine into aerial hyphae and conidiophores. This is currently being investigated by adding increasing doses of methionine to the growth medium and studying complementation of the conidial phenotypes. The underlying causes for reduction of pathogenicity in the methionine auxotrophic mutant are likely to be due to the defect in cuticle penetration and colonization of host tissues. As conidia are probably limited in methionine storage, this could in turn represent a rate-limiting factor for appressorium-mediated penetration, due to a constraint on protein synthesis. Consistent with a defect in appressorium-mediated penetration, the *met1* mutant showed partially restored pathogenicity after exogenous application of cAMP. cAMP signaling has been shown to be required for development of functional appressoria (Lee and Dean 1993; Xu et al. 1997) and addition of cAMP is known to re-mediate other reduced pathogenicity mutants, including *mpg1* and *magB* strains (Beckerman and Ebbole 1996; Talbot et al. 1996; Liu and Dean 1997). The addition of cAMP might therefore allow a higher percentage of appressoria to successfully penetrate rice leaves. The relatively poor growth subsequent to infection by 130 (*met1*) might, however, also reflect limited availability of free methionine from plant tissues requiring amino acid synthesis, perhaps from free sulfate. Inhibition of methionine biosynthesis has recently been proposed to be the mode of action of the anilinopyrimidine fungicides, consistent with the potential importance of this pathway for pathogenesis (Fritz et al. 1997). Interestingly, one of the pathogenicity mutants identified in a recent *M. grisea* REMI screen, *pth3*, was a histidine auxotroph with an insertion in a gene putatively encoding imidazole glycerol phosphate dehydratase (Sweigard et al. 1998). Taken together, these results show the potential importance of genes involved in basic metabolism for production of disease symptoms by fungal pathogens and the potential of REMI for identifying targets for disease control agents.

REMI had allowed us to characterize five mutations displaying an array of phenotypes in relation to appressorium function, invasive growth, conidiogenesis, and conidium germination. These mutations probably affect new pathogenicity genes based on the novel phenotypes observed, compared with previous mutant screens (Shi et al. 1995; Sweigard et al. 1998) and limited allelism tests so far performed. This study further demonstrates the use of REMI in the discovery of genes involved in fungal pathogenicity. However, the fact that only two out of five mutations were tagged reinforces the critical need for genetic analysis if this method is to be widely used as an alternative to conventional mutagenic approaches (Shi et al. 1995; Sweigard et al. 1998). Plasmid rescue, phe-

notypic complementation-based cloning, and positional cloning should allow us to identify the genes of interest in the near future.

## MATERIALS AND METHODS

### Fungal strains, media, and genetic crosses.

Characteristics of the strains used in this study are summarized in Table 1. Laboratory strains 35-R-24, 35-R-56, DF51 (*cpkA*), nn95 (*pmk1*), 43-R-12 (*npr1*), and 44-R-7 (*npr2*) were kindly provided by J. E. Hamer (Purdue University, West Lafayette, IN). The field strain TH3 was a generous gift from J.-L. Notteghem (CIRAD, Montpellier, France).

Standard procedures for cultivation and preservation of *M. grisea* were as previously described (Crawford et al. 1986; Talbot et al. 1993a, 1996; Valent et al. 1991). For genetic analysis, perithecia were removed onto 4% (wt/vol) double-distilled water agar plates, crushed open, and dissected with a metal needle. Individual asci were picked and transferred to germinate on oatmeal agar plates. A monoconidial isolate derived from a single ascospore was subsequently obtained from each of these cultures. Guy 11 and 35-R-24 pathogenicity mutants were crossed with the non-isogenic TH3 wild-type strain and the near-isogenic 35-R-56 wild-type strain (Lau and Hamer 1996), respectively. Allelism tests were conducted on *cpkA*, *pde1* and *pde2* mutations.

### Fungal transformation.

Protoplast isolation was carried out as described by Talbot et al. (1993a), but with replacement of sorbitol by sucrose as an osmoprotectant, to avoid a slight cytotoxic effect of sorbitol that had been previously observed (N. J. Talbot, *unpublished*). Preparations were made from the three wild-type strains Guy 11, 4091-5-8, and 35-R-24. Protoplasts were finally resuspended in STC buffer (0.6 M sucrose, 10 mM Tris-HCl pH 7.5, 10 mM CaCl<sub>2</sub>) at a concentration of  $3.3 \times 10^7$  ml<sup>-1</sup> and slowly frozen at -70°C for long-term storage.

A 150- $\mu$ l aliquot of protoplast suspension was used in each transformation experiment. Two different plasmids conferring resistance to hygromycin B were used as mutagenic tags: pAN7-1 (Punt et al. 1987) and pCB1003 (Carroll et al. 1994). Plasmids were linearized with *Bam*HI or *Hind*III restriction endonucleases, followed by heat inactivation at 70°C for 20 min, and then phenol-chloroform extraction and ethanol precipitation of the DNA. Each transformation used 2  $\mu$ g of DNA and 6 U of restriction enzyme. Three different protocols were evaluated for generation of the REMI library.

Protocol 1 was a modification of the conventional transformation protocol of Talbot et al. (1993a). Protoplasts were incubated for 20 min on ice in the presence of both linearized plasmid and restriction endonuclease, prior to addition of 1 ml of PTC buffer (60% wt/vol PEG 4000, 10 mM Tris-HCl pH 7.5, 10 mM CaCl<sub>2</sub>). After a further 20 min of incubation at room temperature, the suspension was thoroughly mixed with 150 ml of TB3 molten agar (0.3% wt/vol yeast extract, 0.3% wt/vol Casamino Acids, 20% wt/vol sucrose, 2% wt/vol agar; pH 6.5) and poured into 8.5-cm plates. Protoplasts were regenerated overnight at 24°C in the dark and each plate was then overlaid with 15 ml of 2 YEG agar (0.2% wt/vol yeast extract, 1% wt/vol glucose, 1.8% agar; pH 6.5) containing 267  $\mu$ g ml<sup>-1</sup> hygromycin B (Calbiochem, La Jolla, CA).

Protocol 2 was developed by Sweigard et al. (1998). Protoplasts were incubated for 15 min at room temperature with plasmid DNA before addition of restriction enzyme and PEG solution (40% wt/vol PEG 8000, 50 mM Tris-HCl pH 8, 20% wt/vol sucrose, 50 mM CaCl<sub>2</sub>, 50 mM NaCl, 50 mM KCl, 10 mM MgCl<sub>2</sub>) and additional incubation for 20 min. After incubation for 1 h on an orbital shaker with addition of 3 ml of TB3, PEG solution was removed by centrifugation at  $3,750 \times g$  for 10 min and the protoplasts re-suspended in 100  $\mu$ l of STC buffer. The rest of the procedure was identical to protocol 1, apart from substitution of TB3 agar for 2 YEG agar in the regeneration overlay.

Protocol 3 corresponded to the procedure published by Shi et al. (1995). Protoplasts were mixed with plasmid DNA and restriction endonuclease and incubated for 20 min on ice, before addition of 2 ml of PEG solution (60% wt/vol PEG 4000, 25 mM Tris-HCl pH 7.5, 25 mM CaCl<sub>2</sub>) and further incubation on ice for 20 min. The protoplast suspension was then centrifuged at  $3,750 \times g$  for 12 min in the presence of 30 ml of ice-cold STC buffer. Protoplasts were re-suspended in 1 ml of liquid complete medium (CM; Talbot et al. 1993a) supplemented with 20% sucrose, and left overnight at room temperature. Subsequent steps were identical to the previous protocols with regeneration in TB3 agar. In all treatments, stable, hygromycin-resistant transformants were recovered after 7 days of incubation in the dark at 24°C and subcultured to CM agar plates supplemented with 100  $\mu$ g ml<sup>-1</sup> hygromycin B. Transformants were stored at -20°C as desiccated, sterile, filter paper disks in 96-well plates (Corning Glassworks, NY).

### DNA manipulations.

General procedures for nucleic acid analysis followed standard protocols (Sambrook et al. 1989). For Southern blot analysis, genomic DNA was extracted as described by Talbot et al. (1993b). Restriction digestions were routinely carried out with 2  $\mu$ g of genomic DNA according to manufacturer's instructions (Promega, Madison, WI). Digested DNA was size fractionated in 0.8% (wt/vol) agarose gels and blotted to Hybond-N membranes (Amersham Life Science, Bucks, UK), following the manufacturer's instructions. Radiolabeled probes were generated through random priming (Feinberg and Vogelstein 1983), from the linear plasmids used for transformation. Hybridization and washes were conducted under high stringency as previously described (Talbot et al. 1993a).

### Pathogenicity screening tests.

Pathogenicity of primary transformants was assessed with a barley cut leaf assay. Leaf segments were excised from the second leaf of a 14-day-old barley seedling approximately 1 cm from its base. Barley cv. Golden Promise was used due to its high susceptibility toward rice- and barley-pathogenic strains of *M. grisea*, and its ease of cultivation (Lau and Hamer 1996). Conidial suspensions were prepared from 7-day-old fungus cultures grown on 5.5-cm CM plates, adjusted to  $10^5$  ml<sup>-1</sup>, and 25- $\mu$ l droplets deposited onto the upper side of nine 0.5-cm leaf segments maintained on 4% (wt/vol) distilled water agar plates. Three leaf segments originating from three separate barley seedlings were used per transformant examined. Disease lesions were scored after 4 days of incubation at 24°C with a 12-h photophase, and compared with wild-type and mutant strains from the laboratory (Fig. 1). An average

representative score was thus deduced. Only strains with a score of 0 or 1 were selected for further analysis. Under these conditions, 48 transformants could be processed in a single day together with three control strains (the parental wild-type pathogenic strain, Guy 11 [4], 4091-5-8 [3], or 35-R-24 [4] and two pathogenicity mutants, nn95 [*pmk1*; Xu and Hamer 1997], which scored 0, and 53-R-39 [*mpg1*; Talbot et al. 1996], which scored 3). Before extensive analysis of the selected transformants, the test was repeated on single-spore transformants at the same conidial concentration and a concentration twice as high.

Pathogenicity of 35-R-24 and Guy 11 wild-type and mutant strains was tested by spraying trays containing 20 14-day-old seedlings of the barley cv. Golden Promise with conidial suspensions (see below). Guy 11 mutants were inoculated in the same way onto rice cvs. CO39 and Maratelli, by spraying trays containing 100 21-day-old seedlings. For  $F_1$  pathogenicity analysis, pots of eight 8-day-old seedlings from barley cv. Golden Promise or 21-day-old seedlings of rice cv. CO39 were used. Conidia were harvested in 0.2% (wt/vol) gelatin from 10-day-old CM plates. Plants were sprayed with an artist's airbrush (Badger, Franklin Park, IL). For barley, and rice cv. Maratelli, 5 ml of a  $1 \times 10^5$  conidia  $\text{ml}^{-1}$  suspension was used, whereas for rice cv. CO39, 3 ml of a  $5 \times 10^4$  conidia  $\text{ml}^{-1}$  suspension was used. cAMP responsiveness of pathogenicity mutant defects was also examined by spraying plants with conidial suspensions containing 10 mM cAMP (Sigma, St. Louis, MO). Symptoms were recorded after 4 days of incubation in a controlled growth chamber at 24°C, with a 12-h photophase. Lesions were scored on 10 of the most infected leaves and the mean lesion density per 5 cm of leaf length was deduced.

#### Analysis of fungal growth and sporulation.

Fungal growth (assessed by radial growth assays), conidiation (expressed as the number of conidia produced per ml of a plate culture flooded with 3 ml of water, or number of conidia produced per  $\text{cm}^2$  of a plate culture), and conidial morphology were assessed with cultures that had been incubated on 5.5-cm CM plates kept for 7 days at 24°C with a 12-h photophase. At least 10 replicates were observed for each strain and each light treatment. Mutants were also tested for nitrogen metabolism defects by comparing growth on minimal medium supplemented with 6 mM ammonium, glutamine, or nitrate. Controls included isogenic parental strains as well as *npr1* and *npr2* mutant strains (Lau and Hamer 1996). In the case of mutant 130, which was unable to grow on oatmeal agar, an auxotrophic profile was established following the protocol recommended for yeast (Rose et al. 1990). Additional assays involved checking for the easily wettable phenotype associated with defects in hydrophobin production, using the protocol described by Talbot et al. (1993a) and an *mpg1* control strain.

#### Analysis of appressorium development.

Development of infection structures was monitored over time on microscope plastic coverslips (PGC Scientifics, Frederick, MD), as previously described by Hamer et al. (1988). Droplets (100  $\mu\text{l}$ ) of double-distilled water containing  $10^4$  conidia each were deposited onto coverslips and kept at 24°C under humid conditions. For each observation time, three replicates of 200 conidia were examined and compared with

wild-type parental conidia. The responsiveness of appressorium formation to cAMP was studied following the protocol of Lee and Dean (1993).

Appressorium cytorrhysis assays were performed as described by Howard et al. (1991) and de Jong et al. (1997). Appressoria were allowed to form in water droplets on plastic coverslips for 20 h at 24°C. The mean percentage of appressorium collapse was calculated at four different glycerol molarities (1, 2, 3, and 4 M) from at least three replicates of 100 appressoria.

Appressorium penetration of onion epidermis strips was observed in a procedure adapted from Chida and Sisler (1987). Ten-microliter droplets of conidial suspensions ( $5 \times 10^4$  conidia  $\text{ml}^{-1}$ ) were deposited onto the hydrophobic side of the epidermis and kept in a humid chamber for 32 h at 24°C. Appressorium-mediated penetration by 35-R-24 and Guy 11 mutants was also examined on excised leaves of barley cv. Golden Promise and rice cv. CO39, respectively. Leaves were infected and incubated as described for the ex planta leaf assay, but 10- $\mu\text{l}$  instead of 100- $\mu\text{l}$  droplets were used. After 24 or 48 h of incubation, leaves were treated overnight in methanol at room temperature to remove chlorophyll and were subsequently stained with 0.1% (wt/vol) acid fuchsin in lactic acid (Anonymous 1968). This stained all fungal material attached to the plant cuticle bright red, while leaving all fungal invasive structures clear because of poor impregnation of the stain inside plant tissues. All micrographs were produced with a Nikon (Swindon, UK) Optiphot-2 microscope with Hoffman modulation contrast, except for barley penetration images generated with a Leitz (Reading, UK) Ortholux bright-field microscope.

#### Statistical analysis.

Distribution of frequencies ( $F_1$  segregations, penetration ability) were compared with a  $\chi^2$  test, whereas distribution of mean numbers (lesions, colony diameters, conidiation numbers) were studied by analysis of variance after transformation into  $\text{Log}_x$  or  $\text{Log}(x+1)$ . Distribution of proportions (conidial germination, appressorium formation) were also compared by analysis of variance after transformation into  $\text{Arcsin}\sqrt{x}$ . Means were further discriminated through a Student's *t* test.

#### ACKNOWLEDGMENTS

This work was supported by award of a BBSRC research grant (AO6404) to N. J. T. We thank Amy Taylor and Andrew Winkley for help generating and screening the REMI library, James Kingdom for growth of all plants used in this study, Roland Weber and Gavin Wakley for assistance with photographic work, and Nicholas Tongue for technical assistance. Work on rice blast in N. J. T.'s laboratory is authorized by the Plant Health division of the Ministry of Agriculture Fisheries and Food license no. PHF 43/2823/9/1998. N. J. T. is a Nuffield Foundation Science Research Fellow.

#### LITERATURE CITED

- Akamatsu, H., Itoh, Y., Kodama, M., Otani, H., and Kohmoto, K. 1997. AAL-toxin deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme mediated integration. *Phytopathology* 87:967-972.
- Anonymous. 1968. *Plant Pathologist's Pocketbook*. Commonwealth Mycological Institute, Kew, Surrey, U.K.
- Asch, D. K., Frederick, G., Kinsey, J. A., and Perkins, D. D. 1992. Analysis of junction sequences resulting from integration at nonho-

- mologous loci in *Neurospora crassa*. *Genetics* 130:737-748.
- Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S. P. 1997. Signalling in plant-microbe interactions. *Science* 276:726-733.
- Beckerman, J. L., and Ebbole, D. J. 1996. MPG1, a gene encoding a fungal hydrophobin of *Magnaporthe grisea*, is involved in surface recognition. *Mol. Plant-Microbe Interact.* 9:450-456.
- Bölker, M., Böhnert, H. U., Braun, K. H., Görl, J., and Kahmann, R. 1995. Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Genet.* 248:547-552.
- Bourett, T. M., and Howard, R. J. 1990. *In vitro* development of penetration structures in the rice blast fungus *Magnaporthe grisea*. *Can. J. Bot.* 68:329-342.
- Carroll, A. M., Sweigard, J. A., and Valent, B. 1994. Improved vectors for selecting resistance to hygromycin. *Fungal Genet. Newsl.* 41:22.
- Chida, T., and Sisler, H. D. 1987. Restoration of appressorial penetration ability by melanin precursors in *Pyricularia oryzae* treated with anti-penetrants and in melanin-deficient mutants. *J. Pestic. Sci.* 12:49-55.
- Choi, W., and Dean, R. A. 1997. The adenylate cyclase gene *MAC1* of *Magnaporthe grisea* controls appressorium formation and other aspects of growth and development. *Plant Cell* 9:1973-1983.
- Chumley, F. G., and Valent, B., 1990. Genetic analysis of melanin-deficient, nonpathogenic mutants of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 3:135-143.
- Crawford, M. S., Chumley, F. G., Weaver, C. G., and Valent, B. 1986. Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. *Genetics* 114:1111-1129.
- de Jong, J. C., McCormack, B. J., Smirnov, N., and Talbot, N. J. 1997. Glycerol generates turgor in rice blast. *Nature* 389:244-245.
- Dean, R. A. 1997. Signal pathways and appressorium morphogenesis. *Annu. Rev. Phytopathol.* 35:211-234.
- d'Enfert, C. 1997. Fungal spore germination: Insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. *Fungal Genet. Biol.* 21:163-172.
- Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132: 6-13.
- Fritz, R., Lanen, C., Colas, V., and Leroux, P. 1997. Inhibition of methionine biosynthesis in *Botrytis cinerea* by the anilinopyrimidine fungicide pyrimethanil. *Pestic. Sci.* 49:40-46
- Garnand, K., and Nelson, M. A. 1995. The effect of DNA structure and restriction enzymes on transformation efficiencies in *Neurospora crassa*. *Fungal Genet. Newsl.* 42:29-31.
- Gilbert, R. D., Johnson, A. M., and Dean, R. A. 1996. Chemical signals responsible for appressorium formation in the rice blast fungus. *Physiol. Mol. Plant Pathol.* 48:335-346.
- Granado, J. D., Kerstet-Chaloupková, K., Aebi, M., and Kües, U. 1997. Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. *Mol. Gen. Genet.* 256:28-36.
- Hamer, J. E., and Givan, S. 1990. Genetic mapping with dispersed repeated sequences in the rice blast fungus: Mapping the *SMO* locus. *Mol. Gen. Genet.* 223:487-495.
- Hamer, J. E., and Holden, D. W. 1997. Linking approaches in the study of fungal pathogenesis: A commentary. *Fungal Genet. Biol.* 21:11-16.
- Hamer, J. E., Howard, R. J., Chumley, F. G., and Valent, B. 1988. A mechanism for surface attachment of spores of a plant pathogenic fungus. *Science* 239:288-290.
- Hamer, J. E., Valent, B., and Chumley, F. G. 1989. Mutations at the *SMO* locus affect the shapes of diverse cell types in the rice blast fungus. *Genetics* 122:351-361.
- Heath, M. C., Valent, B., Howard, R. J., and Chumley, F. G. 1990. Interactions of two strains of *Magnaporthe grisea* with rice, goosegrass and weeping lovegrass. *Can. J. Bot.* 68:1627-1637.
- Herskowitz, I. 1995. MAP kinase pathways in yeast: For mating and more. *Cell* 80:187-197.
- Howard, R. J., Ferrari, M. A., Roach, D. H., and Money, N. P. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl. Acad. Sci. USA* 88:11281-11284.
- Howard, R. J., and Valent, B. 1996. Breaking and entering - host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annu. Rev. Microbiol.* 50:491-512.
- Jelitto, T. C., Page, H. A., and Read, N. D. 1994. Role of external signals in regulating the pre-penetration phase of infection by the rice blast fungus, *Magnaporthe grisea*. *Planta* 194:471-477.
- Kronstad, J. W. 1997. Virulence and cAMP in smuts, blights and blights. *TIPS* 2:193-199.
- Kuspa, A., and Loomis, W. F. 1992. Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. USA* 89:8803-8807.
- Lau, G. W., and Hamer, J. E. 1996. Regulatory genes controlling *MPG1* expression and pathogenicity in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 8:771-781.
- Lee, Y.-H., and Dean, R. A. 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *Plant Cell* 5:693-700.
- Lee, Y.-H., and Dean, R. A. 1994. Hydrophobicity of contact surface induces appressorium formation in *Magnaporthe grisea*. *FEMS Microbiol. Lett.* 115:71-75.
- Leung, H., Borromeo, E. S., Bernardo, M. A., and Notteghem, J. L. 1988. Genetic analysis of virulence in the rice blast fungus *Magnaporthe grisea*. *Phytopathology* 78:1227-1233.
- Leung, H., Lehtinen, U., Karjalainen, R., Skinner, D. Z., Tooley, P. W., Leong, S. A., and Ellingboe, A. H. 1990. Transformation of the rice blast fungus *Magnaporthe grisea* to hygromycin B resistance. *Curr. Genet.* 17:409-411.
- Liu, S., and Dean, R. A. 1997. G protein  $\alpha$  subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 10:1075-1086.
- Lu, S., Lyngholm, L., Yang, G., Bronson, C., Yoder, O. C., and Turgeon, B. G. 1994. Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. *Proc. Natl. Acad. Sci. USA* 91:12649-12653.
- Marzluf, G. A. 1997. Molecular genetics of sulfur assimilation in filamentous fungi and yeast. *Annu. Rev. Microbiol.* 51:73-96.
- Mitchell, T. K., and Dean, R. A. 1995. The cAMP-dependent protein kinase catalytic sub-unit is required for appressorium formation and pathogenesis by the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 7:1869-1878.
- Money, N. P. 1997. Mechanism linking cellular pigmentation and pathogenicity in rice blast disease. *Fungal Genet. Biol.* 22:151-152.
- Money, N. P., and Howard, R. J. 1996. Confirmation of a link between fungal pigmentation, turgor pressure, and pathogenicity using a new method of turgor measurement. *Fungal Genet. Biol.* 20:217-227.
- Parsons, K. A., Chumley F. G., and Valent, B. 1987. Genetic transformation of the fungal pathogen responsible for rice blast disease. *Proc. Natl. Acad. Sci. USA* 84:4161-4165.
- Punt, P. J., Oliver, R. P., Dingemans, M. A., Pouwels, P. H., and van den Hondel, C. A. M. J. J. 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* 56:117-124.
- Rose, M. D., Winston, F., and Hieter, P. 1990. *Methods in Yeast Genetics: A Laboratory Course Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Schiestl, R. H., and Petes, T. D. 1991. Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 88:7585-7589.
- Schiestl, R. H., Zhu, J., and Petes, T. D. 1994. Effect of mutations in genes affecting homologous recombination on restriction enzyme-mediated and illegitimate recombination in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 14:4493-4500.
- Shi, Z., Christian, D., and Leung, H. 1995. Enhanced transformation in *Magnaporthe grisea* by restriction enzyme mediated integration of plasmid DNA. *Phytopathology* 85:329-333.
- Shi, Z., and Leung, H. 1995. Genetic analysis of sporulation in *Magnaporthe grisea* by chemical and insertional mutagenesis. *Mol. Plant-Microbe Interact.* 8:949-959.
- Slekar, K. H., Kosman, D. J., and Culotta, V. C. 1996. The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection. *J. Biol. Chem.* 271: 28831-28836.
- Sweigard, J. 1996. A REMI primer for filamentous fungi. *IS-MPMI-Rep.* 5:3-5.
- Sweigard, J. A., Carroll, A. M., Farrall, L., Chumley, F. G., and Valent, B. 1998. *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Mol. Plant-Microbe Interact.* 11: 404-412.
- Sweigard, J. A., Chumley, F. G., and Valent, B. 1992. Disruption of a

- Magnaporthe grisea* cutinase gene. *Mol. Gen. Genet.* 232:183-190.
- Talbot, N. J. 1995. Having a blast: Exploring the pathogenicity of *Magnaporthe grisea*. *Trends Microbiol.* 3:9-16.
- Talbot, N. J., Ebbole, D. J., and Hamer, J. E. 1993a. Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 5:1575-1590.
- Talbot, N. J., Kershaw, M. J., Wakley, G. E., de Vries, O. M. H., Wesels, J. G. H., and Hamer, J. E. 1996. *MPG1* encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnaporthe grisea*. *Plant Cell* 8:985-999.
- Talbot, N. J., Salch, Y., Ma, M., and Hamer, J. E. 1993b. Karyotype variation within clonal lineages of the rice blast fungus, *Magnaporthe grisea*. *Appl. Environ. Microbiol.* 59:585-593.
- Thines, E., Eilbert, F., Sterner, O., and Anke, H. 1997a. Glisoprenin A, an inhibitor of the signal transduction pathway leading to appressorium formation in germinating conidia of *Magnaporthe grisea* on hydrophobic surfaces. *FEMS Microbiol. Lett.* 151:219-224.
- Thines, E., Eilbert, F., Sterner, O., and Anke, H. 1997b. Signal transduction leading to appressorium formation in germinating conidia of *Magnaporthe grisea*: Effects of second messengers diacylglycerol, ceramides and sphingomyelin. *FEMS Microbiol. Lett.* 156:91-94.
- Valent, B., Crawford, M. S., Weaver, C. G., and Chumley, F. G. 1986. Genetic studies of fertility and pathogenicity in *Magnaporthe grisea*. *Iowa State J. Res.* 60:569-594.
- Valent, B., Farrall, L., and Chumley, F. G. 1991. *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* 127:87-101.
- Winegar, R. A., Lutze, L. H., Rufer, J. T., and Morgan, W. F. 1992. Spectrum of mutations produced by specific types of restriction enzyme-mediated double strand breaks. *Mutagenesis* 7:439-445.
- Xiao, J.-Z., Watanabe, T., Kamakura, T., Ohshima, A., and Yamaguchi, I. 1994. Studies on cellular differentiation of *Magnaporthe grisea*. Physicochemical aspects of substratum surfaces in relation to appressorium formation. *Physiol. Mol. Plant Pathol.* 44:227-236.
- Xu, J.-R., and Hamer, J. E. 1996. MAP Kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.* 10:2696-2706.
- Xu, J.-R., Urban, M., Sweigard, J. A., and Hamer, J. E. 1997. The *CPKA* gene of *Magnaporthe grisea* is essential for appressorial penetration. *Mol. Plant-Microbe Interact.* 10:187-194.
- Zhu, H., Whitehead, D. S., Lee, Y.-H., and Dean, R. A. 1996. Genetic analysis of developmental mutants and rapid chromosome mapping of *APPI*, a gene required for appressorium formation in *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 9:767-774.