MPMI Vol. 15, No. 12, 2002, pp. 1253–1267. Publication no. M-2002-1015-01R. © 2002 The American Phytopathological Society

Regulation of the *MPG1* Hydrophobin Gene in the Rice Blast Fungus *Magnaporthe grisea*

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Submitted 4 March 2002. Accepted 4 September 2002.

The hydrophobin-encoding gene *MPG1* of the rice blast fungus *Magnaporthe grisea* is highly expressed during the initial stages of host plant infection and targeted deletion of the gene results in a mutant strain that is reduced in virulence, conidiation, and appressorium formation. The green fluorescent protein-encoding allele *sGFP* was used as a reporter to investigate regulatory genes that control *MPG1* expression. The MAP kinase-encoding gene *PMK1* and the wide domain regulators of nitrogen source utilization, *NPR1* and *NUT1*, were required for full expression of *MPG1* in response to starvation stress. The *CPKA* gene, encoding the catalytic subunit of protein kinase A, was required for repression of *MPG1* during growth in rich nutrient conditions. During appressorium morphogenesis, high-level *MPG1* expression was found to require the *CPKA* and *NPR1* genes. Expression of a destabilized *GFP* allele indicated that de novo *MPG1* expression occurs during appressorium formation. Three regions of the *MPG1* promoter were identified which are required for high-level expression of *MPG1* during appressorium formation and are necessary for the biological activity of the MPG1 hydrophobin during spore formation and plant infection.

The filamentous ascomycete *Magnaporthe grisea* is the causal agent of rice blast, the most serious disease of cultivated rice (Ou 1985). The rice blast fungus brings about infection of rice leaves by elaborating specialized infection cells called appressoria which allow the fungus to rupture the leaf cuticle and gain entry to plant tissue. The fungus is genetically tractable and can form appressoria on artificial hydrophobic surfaces, and these features have enabled the identification and characterization of a number of genes controlling appressorium differentiation and maturation in *M. grisea* (Tucker and Talbot 2001). *MPG1* is a gene that is highly expressed during the early stages of plant infection and during disease symptom development (Talbot et al. 1993). *MPG1* encodes a small secreted, hydrophobic protein with the characteristics of a class I fungal hydrophobin (Talbot et al. 1996). Fungal hydrophobins are secreted proteins which react to interfaces between fungal cell walls and the air, or between fungal cell walls and solid surfaces, undergoing self-assembly into insoluble polymerized amphipathic monolayers (Wösten 2001). Targeted deletion of *MPG1* produced mutants which sporulate very poorly, make substantially fewer appressoria than wild-type *M. grisea* strains, and, consequently, are reduced in their virulence on rice. Significantly, *Δmpg1* mutants also exhibit an "easily wettable" phenotype due to the absence of the *MPG1*-encoded rodlet layer on the spore surface (Talbot et al. 1996). Therefore, the *MPG1* hydrophobin plays at least two distinct functions in *M. grisea*: formation of the hydrophobic spore coat during conidiogenesis and the efficient production of appressoria on the leaf surface.

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In this article, we describe the results of a series of experiments designed to determine how the *MPG1* gene is regulated. We were particularly interested in determining how *MPG1* expression is affected by mutations in the known regulatory genes that control appressorium formation in *M. grisea.* Appressorium development in *M. grisea* is regulated by at least two signal transduction pathways, a cyclic AMP response pathway which is required for appressorium formation on hydrophobic surfaces, and a MAP kinase signaling pathway which is required for appressorium formation and subsequent pathogenic development (Tucker and Talbot 2001). Here, we have taken advantage of the availability of developmental mutants in the cAMP/MAPK pathways involved in appressorium formation by *M. grisea* to explore the regulation of *MPG1*. We also have investigated the role of two further gene loci, *NPR1* and *NPR2,* which are involved in regulation of nitrogen metabolism and required for pathogenicity by *M. grisea. NPR1* and *NPR2* have been shown to be involved in *MPG1* regulation, based on RNA gel blot experiments (Lau and Hamer 1996). We have used a green fluorescent protein-encoding reporter gene to investigate temporal and spatial analysis of *MPG1* expression and to quantify *MPG1* expression during both appressorium formation and nutrient starvation of mycelium in different genetic backgrounds. We report that both positive and negative regulation of *MPG1* occurs in *M. grisea* during different stages of development and is influenced by the *PMK1* MAP kinase pathway, the cAMP-response pathway, and the wide domain regulators of nitrogen source utilization.

RESULTS

Our aim in this study was to provide a rigorous quantitative analysis of *MPG1* expression during infection-related development of *M. grisea* using the green fluorescent protein (GFP)-encoding gene as a reporter. We first carried out two experiments to investigate the nature of *MPG1* gene expression in more detail and the effect of DNA-mediated transformation on reporter gene expression. Previous use of a GFP reporter had indicated that *MPG1* is highly expressed in appressoria (Kershaw et al. 1998), but we were concerned that this observation might be due to expression of *MPG1* in conidia and the relative stability of

GFP, which has been reported to have a half-life of more than 24 h (Sheen et al. 1995). Therefore, we expressed an allele encoding a destabilized form of GFP (*d2EGFP*) under control of the *MPG1* promoter in *M. grisea*. The *d2EGFP* allele of GFP has a half-life of 2 h due to a PEST sequence at the C terminus of the protein, which targets it for intracellular proteasome degradation (Li et al. 1998). Transformants showing single copy integrations of the *MPG1(p)::d2EGFP* construct were selected and GFP fluorescence was observed during appressorium development (Fig. 1A). The pattern of GFP fluorescence was identical to that previously reported (Kershaw et al. 1998), indicating that de novo *MPG1* expression occurs during infection cell formation in *M. grisea*.

We also tested the effect of copy number and positional effects on reporter gene expression in *M. grisea.* Rates of homologous recombination in *M. grisea* are not sufficiently high to allow integration of a reporter gene construct at the same locus; therefore, DNA-mediated transformation results in random integration of a reporter gene plasmid throughout the genome. Thus, the level of expression of *GFP* in each transformant may be affected by the position in the fungal genome where the plasmid integrated or by the number of copies of the reporter gene integrated into the genome. To test the effect of gene copy number on reporter gene expression, mycelium from 10 transformants of *M. grisea* strain 44-R-7 containing various numbers of integrated copies of a *MPG1(p)::sGFP*

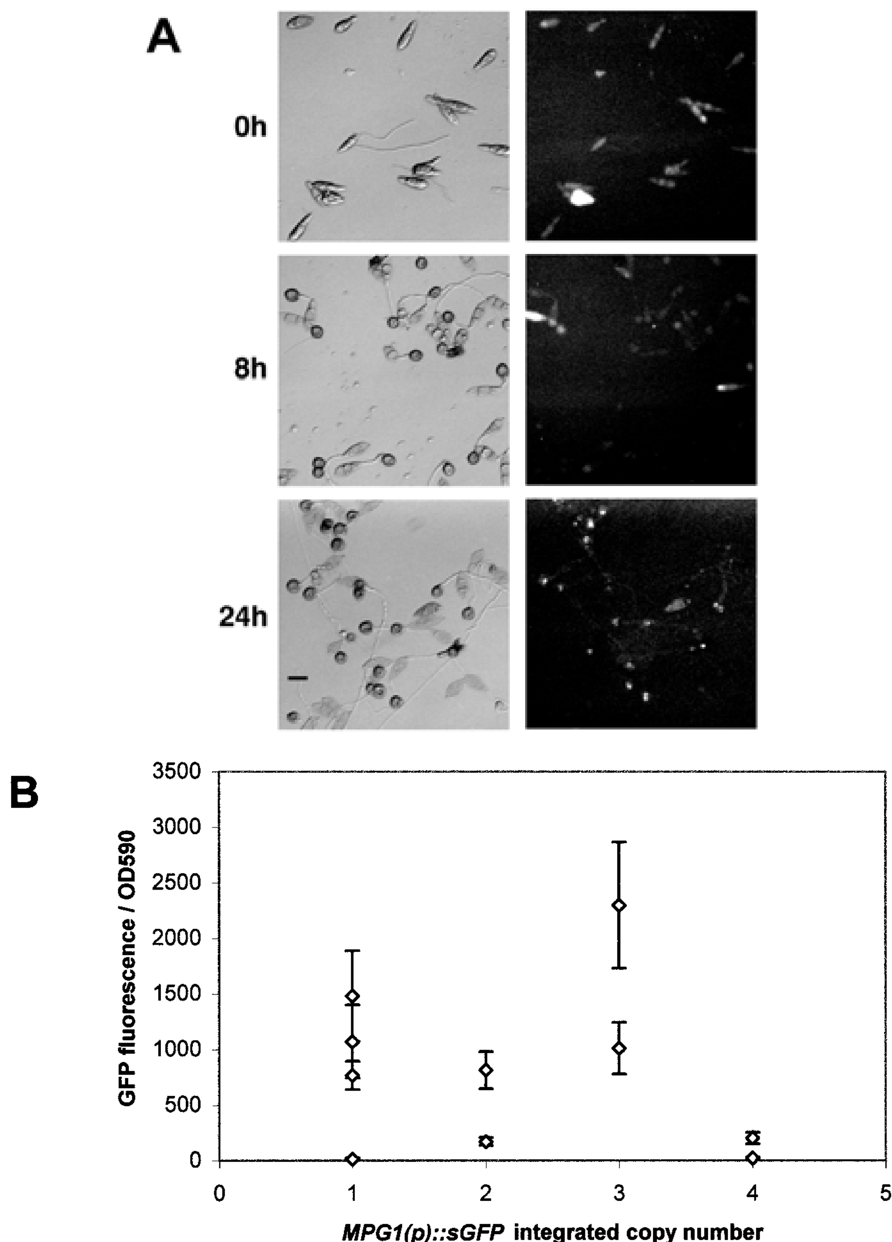


Fig. 1. A, Epifluorescence microscopy of appressorium development in *Magnaporthe grisea* showing *MPG1(p)::d2EGFP* expression. Conidia from *MPG1(p)::d2EGFP* transformant DSshl52 of wild-type strain Guy11 were harvested, and appressorium development was followed for 24 h on plastic surfaces. Micrographs were recorded under Hoffmann modulation optics or epifluorescence. Bar = 20 µm. The time after harvesting of conidia is given in hours. Similar patterns of green fluorescent protein (GFP) fluorescence were observed in independently generated transformants of identical genotype (data not shown). **B,** Scatter diagram showing the effect of integrated plasmid copy number on GFP fluorescence in *npr2* strains transformed with *MPG1(p)::sGFP*. The graph shows GFP fluorescence per optical density at 590 nm (OD_{590}) for 10 strains of 44-R-7 containing the stated number of copies of *MPG1(p)::sGFP* integrated into the fungal genome. Results shown are mean values calculated for mycelia grown for 48 h in complete medium (CM) at 25°C in 96-well microplates, after which fresh CM was added and the mycelia was incubated at 25°C for a further 24 h . The standard deviation was also calculated (n = 8).

construct was grown for 72 h in rich growth medium (complete medium; CM) and normalized GFP fluorescence was measured. A scattergram of integrated plasmid copy number against normalized GFP fluorescence showed no significant correlation between the two variables (Fig. 1B), which was confirmed by calculation of a correlation coefficient (–0.141). We can conclude that gene copy number has only a limited effect on the expression patterns of the *MPG1(p)::sGFP* reporter gene construct. Position effects, however, were very apparent. Among transformants carrying a single copy integration of the *MPG1(p)::sGFP* plasmid, for example, the most highly fluorescent strain had a fluorescence and optical density at 590 nm (OD_{590}) 150-fold greater than the least fluorescent strain. In view of this degree of variation, we decided to carry out all analysis reported in this study using up to four independent single-copy *M. grisea* transformants to verify expression patterns.

The effect of *CPKA* and *PMK1* on *MPG1* expression.

We first investigated whether the regulation of *MPG1* in response to starvation stress was influenced by either the *PMK1* MAP kinase gene or the *CPKA* protein kinase A gene which are required for appressorium morphogenesis and fungal virulence (Table 1). Two approaches were used to assay *MPG1* expression in mycelium from Δ*cpka* and Δ*pmk1* mutants. First, RNA gel blots were used to assay *MPG1* transcript abundance following starvation stress (Fig. 2A). Hyphal cultures from the two mutant *M. grisea* strains and the (isogenic) wild-type Guy11 were grown for 48 h in CM and then transferred to either CM or to minimal medium lacking a nitrogen source (MM-N) for a further 24 h before RNA extraction. In the wild-type *M. grisea* strain Guy11, there was a fivefold increase in *MPG1* transcript abundance during nitrogen starvation (Fig. 2A, lanes 1 and 4). In the Δ*pmk1* mutant, there was smaller increase in *MPG1* transcript abundance in response to starvation (Fig. 2A, lanes 3 and 6), suggesting that *PMK1* may be required for full induction of *MPG1* expression during nitrogen starvation. In the Δ*cpka* mutant, *MPG1* transcript abundance during nitrogen starvation was comparable to the wild-type strain but, in rich medium, Δ*cpka* showed a twofold greater level of *MPG1* expression than in Guy11 under identical conditions (Fig 2A, lane 2). This indicates that the *CPKA* gene is needed for repression of *MPG1* expression in nutrient-rich growth conditions.

To investigate these potential differences in *MPG1* expression in more detail, Δ*cpka* and Δ*pmk1* mutants were individually transformed with a *MPG1(p)::sGFP* reporter gene construct and transformants containing a single integrated copy of this construct were selected. The sGFP allele was used because of its optimized codon use for high-level expression in plants and fungi (Chiu et al. 1996). We then developed a method to assess *GFP* expression in a quantitative manner. Conidia from each of the mutant strains transformed with *MPG1(p)::sGFP*, and a wild-type Guy11 strain carrying the same plasmid, were used to inoculate CM in 96-well microplates. Mycelium was recovered after 30 h of growth by centrifugation and grown for a further 48 h in either CM, MM-N, or minimal medium lacking a carbon source (MM-C). GFP fluorescence was monitored over this time and then normalized to account for differences in mycelial growth by dividing the fluorescence value by an OD_{590} value which detects mycelial growth. All transformants routinely showed a fall in GFP fluorescence shortly after inoculation. This was due to inoculation being carried out with conidia which express *MPG1* at a high level, prior to germination. In each case, between two and four independent transformants carrying single copies of the *MPG1(p)::sGFP* reporter gene were analyzed for each *M. grisea* strain (Fig. 2C to F).

In the Guy11 *MPG1(p)::sGFP* transformant, GFP fluorescence increased during carbon and nitrogen starvation until, after 48 h , it was sevenfold and eightfold greater, respectively, than fluorescence observed in CM-grown mycelium (Fig. 2C). In a control experiment, a transformant carrying a single copy of *sGFP* under control of the glyceraldehyde-3-phosphate dehydrogenase (*GpdA*) promoter from *Aspergillus nidulans* (Punt et al. 1987) was used (Fig. 2D). This promoter gives constitutive high-level expression in *M. grisea*. After 48 h of growth, there was no significant difference in GFP fluorescence between mycelium grown in rich medium or exposed to starvation (t test, $P = 0.833$). The Δ*pmk1:MPG1(p)::sGFP* transformant (Fig. 2E) showed only a small increase in GFP fluorescence after 48 h , following nitrogen starvation or carbon starvation compared with GFP levels before starvation, but a greater increase in CM-grown mycelium which, after 48 h , showed significantly greater GFP fluorescence than the nitrogen-starved mycelium (t test, $P = 2.18 \times 10^{-5}$). In the Δ*cpka:MPG1(p)::sGFP* transformant, there was no increase in the level of GFP fluorescence in carbon-starved mycelium during the course of the experiment. In nitrogen-starved mycelium, there was a small increase in GFP fluorescence, consistent with the results of the RNA gel blot analysis (Fig. 2A). The largest increase in GFP fluorescence, however, was in nonstarved Δ*cpka:MPG1(p)::sGFP* mycelium, with a 12-fold increase during the course of the experiment. Three independent Δ*cpka:MPG1(p)::sGFP* transformants showed the same pattern of expression (Fig. 2F to H) .

Taken together, the results from RNA gel blot experiments and quantitative GFP expression analysis indicate that *CPKA* is required for full repression of *MPG1* during growth under high nutrient conditions and *PMK1* is required for full activation or derepression of *MPG1* during starvation stress.

The roles of *CPKA* and *PMK1* in regulating *MPG1* expression during conidial germination and appressorium formation also were investigated. Conidia were harvested from *MPG1(p)::sGFP* transformants and germinated on hydrophobic surfaces for a period of 24 h (Fig. 3). In *MPG1(p)::sGFP* transformants of the wild-type Guy11, high levels of GFP fluorescence were observed in conidia and appressoria (Fig. 3). Fluorescence was much greater than in Guy11 transformants expressing the *GpdA(p)::sGFP* gene (Guy11-c; Fig. 3), confirming that *MPG1* is highly expressed during appressorium formation. Although Δ*pmk1* strains do not produce appressoria, GFP expression in conidia of Δ*pmk1:MPG1(p)::sGFP* transformants was at the same level as in Guy11 *MPG1(p)::sGFP* transformants. In Δ*cpka: MPG1(p)::sGFP*

Table 1. Phenotype of mutant fungal strains used in this study

Genotype	Pathogenicity (compared with wild type)	Appressorium formation (compared with wild type)	Reference
Δpmk1	Reduced	No appressorium produced	Xu and Hamer 1996
Δcpka	Reduced	Delayed in appressorium formation	Mitchell and Dean 1995
npr1[a]	Reduced	Same as wild type	Lau and Hamer 1996
npr2[a]	Reduced	Same as wild type	Lau and Hamer 1996
nut1[a]	Same as wild type	Same as wild type	Froeliger and Carpenter 1996

[a] Defective in metabolism of complex sources of nitrogen.

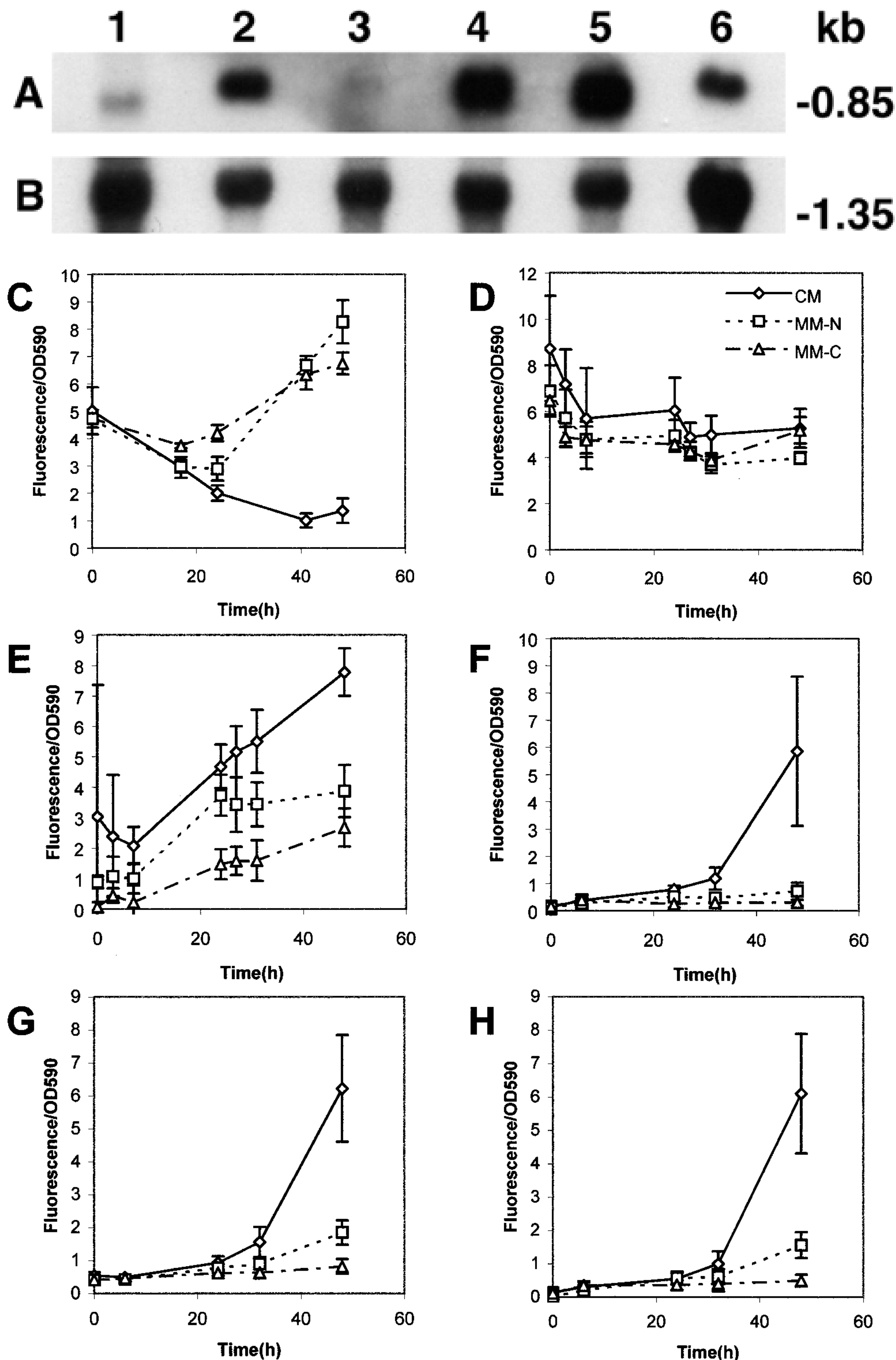


Fig. 2. Determining the effect of starvation stress on *MPG1* expression in developmental mutants of *Magnaporthe grisea* . **A,** Mycelium from a wild type strain Guy11 (lanes 1 and 4), (Δ*cpka* mutant DF51 (lanes 2 and 5), and a Δ*pmk1*mutant nn95 (lanes 3 and 6) was grown for 48 h in complete medium (CM), and transferred to either CM (lanes 1–3) or nitrogen starvation medium (MM-N, lanes 4 to 6) for 24 h . Total RNA was extracted and an RNA gel blot was probed with a 3.75-kb *Xba*I-*Hin*dIII genomic DNA fragment of the *MPG1* gene. **B,** Hybridization to the 18S rRNA gene from *M. grisea* as a loading control. **C** to **H**, Line graphs showing the results of microplate assay of green fluorescent protein (GFP) fluorescence in mycelium from *M. grisea* transformed with the *MPG1(p)::sGFP* construct and subjected to starvation stress. GFP fluorescence was normalized to account for fungal growth by dividing fluorescence value by the optical density at 590 nm (OD_{590}) and was plotted against time elapsed since mycelium was transferred to the growth media indicated. Similar patterns of GFP fluorescence were observed in independently generated transformants of identical genotypes (data not shown). Fluorescence/OD_{590} has been converted to arbitrary values. Each data point represents the mean from eight replicates. Error bar = standard deviation. **C,** *MPG1(p)::sGFP* transformant 72.3.4 of wild-type strain Guy11. **D**, *GPDA(p)::sGFP* transformant 70.2.1 of wild-type strain Guy11. **E,** Δ*pmk1:MPG1(p)::sGFP* transformant DS215. **F,** Δ*cpka:MPG1(p)::sGFP* transformant DS564. **G,** Δ*cpka:MPG1(p)::sGFP* transformant DS211. **H,** Δ*cpka:MPG1(p)::sGFP* transformant DS565.

transformants, GFP fluorescence was significantly lower than in the Guy11 transformant in both conidia and appressoria (*t* test, $P = 1.5 \times 10^{-7}$). Two independent Δ*cpka:MPG1(p)::sGFP* transformants showed the same expression pattern (data not shown). We conclude that *CPKA* is necessary for high levels of *MPG1* expression during appressorium formation.

Determining the effect of *NPR1*, *NPR2,* and *NUT1* on *MPG1* expression.

NPR1, NPR2, and *NUT1* encode wide-domain regulators of nitrogen metabolism in *M. grisea* (Lau and Hamer 1996). *NUT1* encodes a GATA transcription factor homologous to *areA* from *Aspergillus nidulans* and *nit2* from *Neurospora crassa* (Froeliger and Carpenter 1996). The *npr1*, *npr2,* and *nut1* mutants are all unable to utilize sources of nitrogen other than ammonia and urea, but *npr1* and *npr2* mutants are also nonpathogenic. It was previously reported that *NPR1*, *NPR2,* and *NUT1* are necessary for induction of *MPG1* expression during nutrient starvation of mycelium (Lau and Hamer 1996). To investigate this process in greater detail, the *MPG1(p)::sGFP* reporter plasmid was introduced into *M. grisea* strains 43-R-20 (*npr1*), 44-R-7 (*npr2*), and G11397 (*nut1*) and transformants containing single copies of the construct were selected. *MPG1* expression was assayed using RNA gel blots or by measurement of GFP fluorescence (Fig. 4). In RNA gel blot analysis, the isogenic wild-type *M. grisea* strain 35-R-24 showed a low level of *MPG1* expression in CM and under carbon starvation, with a threefold increase under nitrogen starvation (Fig. 4A, lanes 1 to 3). The *MPG1* transcript was undetectable in *npr1* mycelium grown in CM and only just detectable after nitrogen starvation, suggesting that the *NPR1* gene product is necessary for full induction of *MPG1* expression by nitrogen starvation and perhaps also to maintain the low basal level of *MPG1* expression in rich media (Fig. 4A, lanes 4 to 6). The *npr2* mutant showed a similar pattern of *MPG1* expression to the wild type under all conditions, with a small increase in *MPG1* transcript abundance following nitrogen starvation (Fig. 4A, lanes 7 to 9). This indicates that *NPR2* is not required for *MPG1* induction during nitrogen starvation, which is not consistent with previously published results (Lau and Hamer 1996), although the level of induction may be lower than in the wild-type strain. Analysis of GFP expression in mycelium of *MPG1(p)::sGFP* transformants grown in microplates provided results which were broadly consistent with RNA gel blots. The wild-type strain 35-R-24 showed elevation in normalized GFP fluorescence under nitrogen and carbon starvation stress (Fig. 4C), in contrast to a *nut1* mutant transformant (Fig. 4D). No significant increases in *MPG1(p)::sGFP* expression during nitrogen or carbon starvation of mycelium were observed in *npr1* or *npr2* mutant backgrounds (Fig. 4E to F).

MPG1 expression also was assayed during appressorium development using the *npr1*, *npr2,* and *nut1* strains transformed with a single copy of the *MPG1(p)::sGFP* construct (Fig. 3). Conidial germination and development of appressoria in *npr1*, *npr2,* and *nut1* transformants was identical to that observed in wild-type transformants (of either strain Guy11 or 35-R-24). The pattern of GFP expression observed was also similar. The intensity of GFP fluorescence in appressoria of the *nut1* transformant (DS174) was not significantly different from those of Guy11 transformants (*t* test, $P = 0.25$). In the *npr2* transformant (DS95), the intensity of GFP fluorescence in appressoria was not significantly different from that of Guy11 (*t* test, $P = 0.27$). The intensity of GFP fluorescence in conidia and appressoria from the *npr1* transformant (DS193) was significantly lower than in the Guy11 *MPG1(p)::sGFP* transformant (*t* test, $P < 0.0001$). Collectively, the results suggest that *NPR1*, *NPR2,* and *NUT1* are needed for induction of *MPG1* expression during nutrient starvation of mycelium, but only *NPR1* appears to play a significant role in regulating *MPG1* expression during appressorium formation (Table 2).

Identification of *cis*-acting elements of the *MPG1* promoter important in controlling *MPG1* expression.

In order to identify regions of the *MPG1* promoter necessary for control of *MPG1* expression, a 1.32-kb region of the *MPG1* promoter was sequenced (data not shown). This identified a series of putative binding sites for known transcription factors (Table 3). Based on the promoter sequence, four reporter gene constructs were created that contained *sGFP* under the control of 5′ truncations of the *MPG1* promoter (Fig. 5A). GFP fluorescence was visualized during appressorium development of Guy11 strains transformed with *MPG1(p)::sGFP* and the four *MPG1(pTr)::sGFP* constructs (Fig. 5B to G). Decreasing the length of the 1.3-kb *MPG1* promoter fragment caused a large reduction in gene expression, and all of the transformants carrying truncated promoter fragments showed significantly reduced GFP fluorescence in conidia and appressoria (*t* test, $P < 0.0001$). The initial truncation of the promoter fragment from –1,321 bp to –1,051 bp had a very pronounced effect on GFP expression in conidia and appressoria, which was lower than in transformants expressing either the full 1.3-kb promoter fragment or the shorter *MPG1(pTr2)* construct (Fig 5D to E). Similarly, shortening of the promoter between –266 bp and –95 bp had a similarly pronounced effect on gene expression (Fig. 5F to G). The results suggest that these two regions of *MPG1* promoter are important in controlling *MPG1* expression during appressorium development.

In order to determine the role of the *MPG1* promoter during starvation stress of hyphae, GFP fluorescence was assayed in microplate-cultured *M. grisea* mycelium from each of MPG1 truncated promoter transformants subjected to starvation stress (Fig. 6). In contrast to transformants expressing *sGFP* under control of the 1.3-kb *MPG1* promoter fragment, none of the transformants expressing *MPG1* promoter truncations showed induction of GFP expression in response to nitrogen or carbon source starvation (Fig. 6). This suggests that the region from 1,321 to 1,051 bp upstream from the *MPG1* locus is necessary for induction of *MPG1* expression during nutrient starvation. Wild-type strains transformed with *MPG1(pTr2)*, *MPG1(pTr3),* and *MPG1(pTr4)* also showed an increase in GFP fluorescence during growth in CM, indicating that the region from 1,051 to 811 bp upstream from the *MPG1* locus is necessary for repression of *MPG1* during growth in rich media (Table 4).

Determining the significance of the *MPG1* promoter in controlling the biological activity of *MPG1*.

All of the truncations of the *MPG1* promoter that we constructed caused a significant reduction in expression of the *sGFP* reporter. This suggested that, if *MPG1* was expressed under control of these promoter fragments, there would be a reduction in MPG1 hydrophobin production. Therefore, we decided to investigate the potential of the *MPG1* promoter fragments to control the biological activity of the MPG1 hydrophobin. A Δ*mpg1* strain of *M. grisea*, TM400-5, was selected for complementation analysis. This strain is reduced in pathogenicity, conidiation, and appressorium formation and lacks a hydrophobic rodlet layer on the surface of its conidia (Talbot et al. 1996). Four vectors were created carrying *MPG1* under control of each truncated fragment of the *MPG1* promoter (Fig. 5A) and introduced into the Δ*mpg1* strain TM400-5 of *M. grisea*. Four single-copy transformants of each strain were selected and analyzed. Conidia were harvested from each

strain and their surface was visualized by freeze-fracture and transmission electron microscopy of a carbon-platinum replica (Fig. 7A to D). The surface of the wild-type Guy11 shows characteristic 5-nm-diameter rodlets, which are absent from the Δ*mpg1* strain (Fig. 7A and B). No rodlets were observed on the surface of conidia from any of the Δ*mpg1* strains expressing *MPG1* under control of the *MPG1* promoter truncations. Two examples of the appearance of conidial surface replicas from these transformants are shown (Fig. 7C and D).

To assess other Mpg1-associated phenotypes, conidiogenesis and virulence on rice cultivars was assayed. The concentration of conidia produced by 10-day-old cultures of each *MPG1* promoter transformant was measured and compared with the wild-type Guy11 (Fig. 7E). In every case, the number of conidia produced by transformants was significantly lower than the number produced by the wild-type strain (*t* test, $P < 0.001$) and not significantly higher than the number produced by the Δ*mpg1* strain. In a few cases, the number of conidia produced

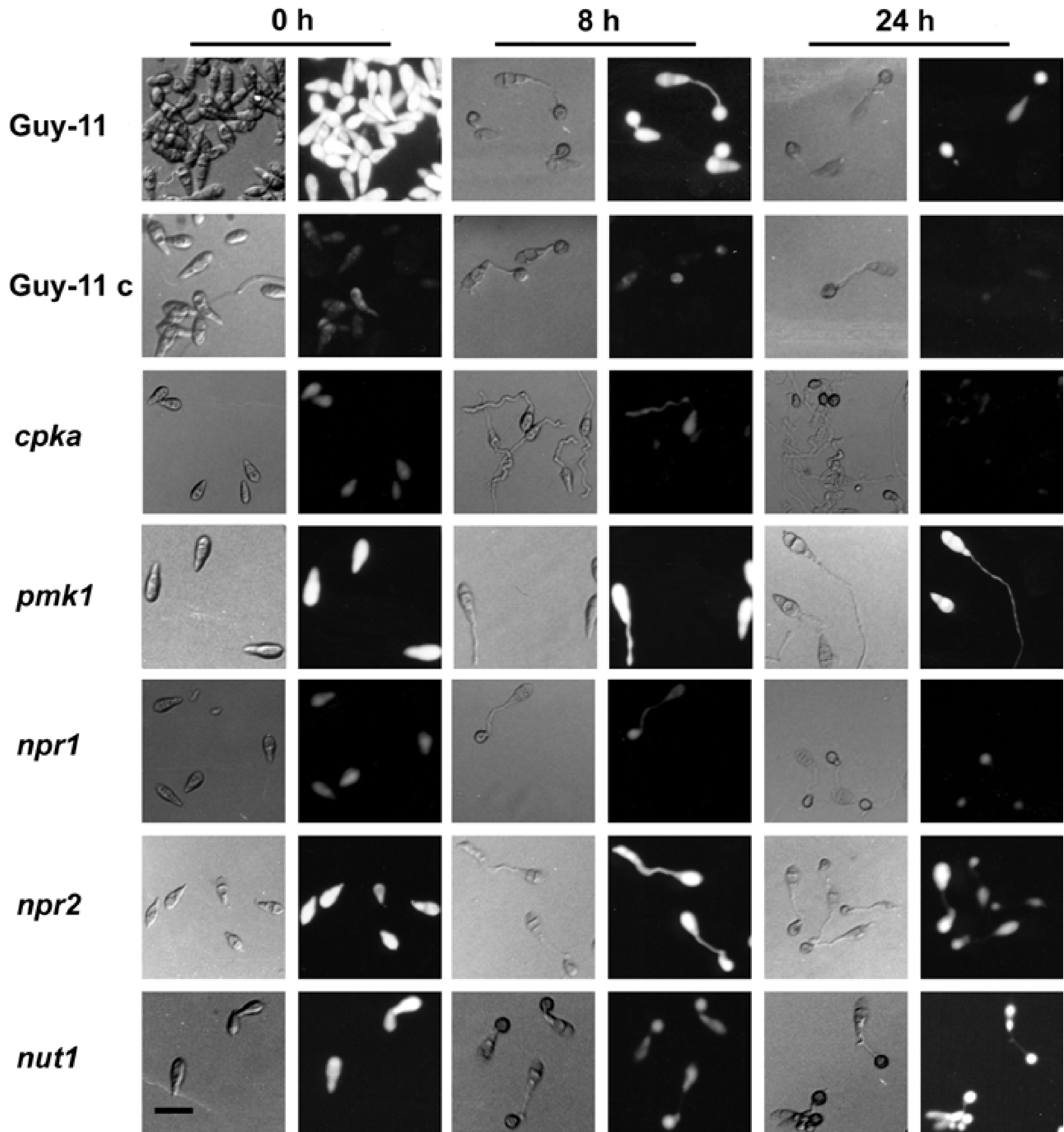


Fig. 3. Epifluorescence microscopy of appressorium development in *Magnaporthe grisea* showing *MPG1(p)::sGFP* expression. Conidia were harvested and allowed to undergo appressorium development for 24 h . Bright field images were recorded using Hoffmann modulation optics (Nikon). Individual *MPG1(p)::sGFP* transformants of the mutant strains indicated were observed. Guy11c is *GPDA(p)::sGFP* transformant 70.2.1 of Guy11 which was used as a control strain showing constitutive expression of green fluorescent protein (GFP). Similar patterns of GFP fluorescence were observed in independently generated transformants of identical genotypes (data not shown). Bar = 20 µm.

by the transformed strains was significantly less than that produced by the Δ*mpg1* strain, suggesting integration of the plasmid at a site that had a detrimental effect on conidial formation. Conidial suspensions of equal concentration from each transformant were used to infect rice plants, and the number of resulting disease lesions was counted after 4 days and compared with the wild-type *M. grisea* strain (Fig. 7F). In all cases, the number of lesions produced was significantly lower than for the wild-type strain. Taken together, the results suggest that complementation of the *mpg1* mutant phenotype is not possible when *MPG1* is expressed under control of an upstream sequence of less than 1,321 bp.

DISCUSSION

The *MPG1* hydrophobin gene plays diverse roles in the developmental biology of *M. grisea* (Talbot et al. 1993, 1996), requiring its expression to be regulated in response to different morphogenetic and environmental signals. The likely complexity of *MPG1* gene regulation prompted us to investigate its genetic control using the *GFP* reporter gene. Studying gene expression in *M. grisea* is not straightforward because, ideally, a reporter gene fusion should be integrated into the genome at the native gene locus (in this case, *MPG1*), as in studies of more genetically tractable fungi such as *Neurospora crassa*

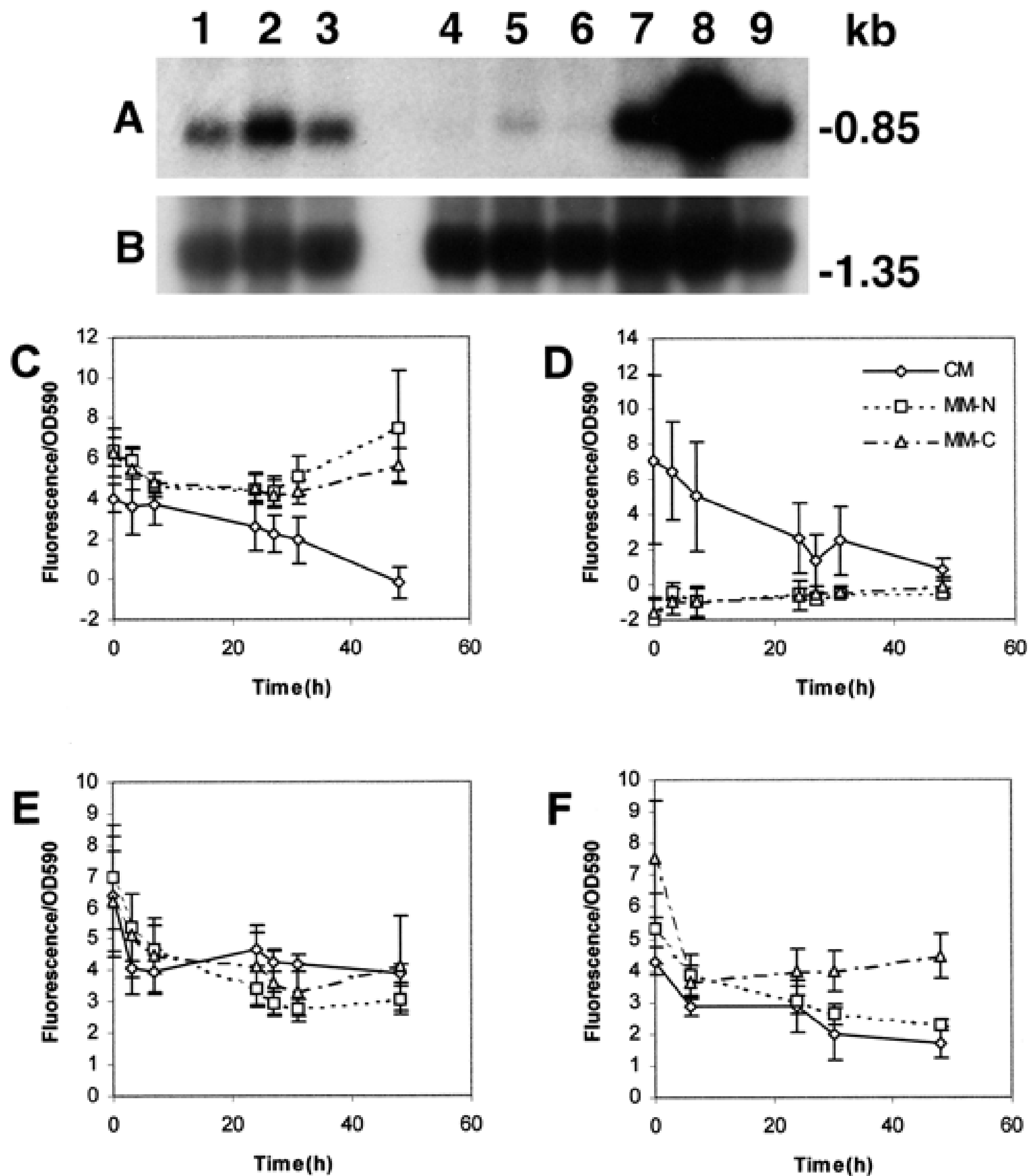


Fig. 4. Determining the effect of starvation stress on *MPG1* expression in wide domain regulatory mutants involved in nitrogen source utilization. **A,** Mycelium from 35-R-24 (a barley pathogenic wild-type strain of *Magnaporthe grisea*, lanes 1 to 3), 43-R-20 (an isogenic *npr1* mutant, lanes 4 to 6), and 44-R-7 (an isogenic *npr2* mutant, lanes 7 to 9) was grown for 48 h in complete medium (CM), then transferred to either CM (lanes 1, 4, and 7), nitrogen starvation medium (MM-N, lanes 2, 5, and 8), or carbon starvation medium (MM-C, lanes 3, 6, and 9) for 24 h . Total RNA and an RNA gel blot were probed with a 3.75-kb *Xba*I-*Hin*dIII genomic DNA fragment of the *MPG1* gene. **B,** Hybridization to the 18S rRNA gene from *M. grisea* as a loading control. **C** to **F,** Microplate assay of green fluorescent protein (GFP) fluorescence in mycelium from 35-R-24, *nut1*, *npr1,* and *npr2* strains of *M. grisea* expressing *MPG1(p)::sGFP* . GFP fluorescence was normalized to account for fungal growth by dividing fluorescence value by the optical density at 590 nm (OD_{590}) and was plotted against time elapsed since mycelium was transferred to the growth media indicated. Similar patterns of GFP fluorescence were observed in independently generated transformants of identical genotypes (data not shown). Fluorescence/OD_{590} has been converted to arbitrary values. Each data point represents the mean from eight replicates. Error bar = standard deviation. **C,** *MPG1(p)::sGFP* transformant DS63 of wild-type strain 35-R-24. **D,** *MPG1(p)::sGFP* transformant DS174 of mutant strain *nut1*. **E,** *MPG1(p)::sGFP* transformant DS193 of mutant strain *npr1*. **F,** *MPG1(p)::sGFP* transformant DS95 of mutant strain *npr2* (*MPG1(p)::sGFP*).

Table 2. Summary of the effect of regulatory mutants on the level of *MPG1* expression

Regulatory mutant	Effect on *MPG1* expression (compared with wild type)		
	Growth in rich medium	Nitrogen starvation	Appressorium formation
Δpmk1	No effect	Reduced	N/A[a]
Δcpka	Increased	No effect	Reduced
npr1	No effect	Reduced	Reduced
npr2	No effect	Reduced	No effect
nut1	No effect	Reduced	No effect

[a] The Δ*pmk1* strain does not produce appressoria.

Table 3. Putative *cis*-acting sites present in the *MPG1* promoter

Motif	Number of sites	Consensus sequence (5′–3′)[a]
Bristle response element[b]	8	MRAGGGR
GATA motif[c]	6	TATCTM
Heat shock element[d]	2	GAANN[e]
AbaA binding site[f]	3	CATTCY
PacC binding site[g]	4	GCCARG
Stress response elements[h]	4	TMAGGGG

[a] R = G/A, M = C/A, Y = T/C, N = A/C/G/T.
[b] Putative binding site for BrlA-like regulator of sporulation (Chang and Timberlake 1993).
[c] Putative binding site for AreA / Nit-2-like regulator of nitrogen metabolism (Fu and Marzluf 1990).
[d] Putative binding site for heat shock factor (Fernandes et al. 1994).
[e] Triple repeat of this 5-mer in either orientation.
[f] Putative binding site for AbaA-like regulator of sporulation (Andrianopoulos and Timberlake 1994).
[g] Putative binding site for PacC-like regulator of pH response (Tilburn et al. 1995).
[h] Putative binding site for stress response factor (Schüller et al. 1994).

(Bell-Pedersen et al. 1996) or *Ustilago maydis* (Basse et al. 2000). Unfortunately, rates of homologous recombination in *M. grisea* are very low, ranging from 3 to 11% (Talbot and Foster 2001), precluding homologous recombination as a viable strategy for production of sufficient reporter gene transformants. Instead, in this investigation, reporter gene constructs were integrated at random sites in the fungal genome. To minimize possible effects on reporter gene expression, we selected transformants where only a single copy of the plasmid had integrated into the genome and two or more transformants were routinely examined. This allowed an internal control for all subsequent measurements to account for possible position effects on expression. We also developed a robust method to measure GFP expression in a manner that could be quantified statistically.

The green fluorescent protein-encoding allele *sGFP* (Chiu et al. 1996) has been used as a reporter in several fungi (Bowyer et al. 2000; Lorang et al. 2001; Maor et al. 1998; Rohel et al. 2001; Spellig et al. 1996), but a potential problem in using *sGFP* as a reporter is the stability of the protein, which means that it may not faithfully report decreases in gene expression (Cubitt et al. 1995). For this reason, we initially expressed a destabilized version of GFP (*d2EGFP*) to ensure that GFP patterns reflected actual gene expression (Li et al. 1998).

Regulatory genes controlling *MPG1* expression.

RNA gel blots and GFP reporter gene assays indicated that *CPKA,* which encodes the catalytic subunit of protein kinase A, is required for full expression of *MPG1* during appressorium formation, consistent with its function as a regulator of appressorium morphogenesis (Mitchell and Dean 1995; Xu et al. 1997). *MPG1* may, therefore, be activated throughout appressorium development and not just at its initiation. *CPKA* is also necessary for repression of *MPG1* during growth in rich medium. Cyclic AMP is a component of the glucose repression pathway in both budding and fission yeasts which operates via activation of *CPKA* (Thevelein 1994). The fructose-1,6-bisphosphatase-encoding gene *fbp1* from *Schizosaccharomyces pombe,* for example, is repressed by this mechanism (Hoffman and Winston 1991). Repression of *MPG1* expression in hyphae is consistent with regulation of hydrophobin production by the glucose repression pathway.

The *PMK1* MAPK gene is essential for pathogenicity in *M. grisea*, acting as a regulator of appressorium formation and subsequent plant infection (Xu and Hamer 1996). *PMK1* is necessary for full induction of *MPG1* expression in response to nitrogen starvation, implicating *PMK1* in the response to starvation stress, and offering a potential reason why Δ*pmk1* mutants are unable to grow invasively in planta (Xu and Hamer 1996). In *Saccharomyces cerevisiae,* pseudohyphal invasive growth, for example, is stimulated by nitrogen starvation (Gimeno et al. 1992), a response regulated by the *KSS1* MAPK, a functional homolog of *PMK1* (Herskowitz 1995; Lengeler et al. 2000).

NPR1 and *NPR2* are wide-domain regulators of nitrogen metabolism required for pathogenicity and induction of *MPG1* expression during nutrient starvation (Lau and Hamer 1996). Although sharing similar mutant phenotypes with *areA* or *nit2* mutants of *A. nidulans* or *N. crassa* with respect to nitrogen source utilization, both represent distinct gene loci. *NUT1* encodes the *areA/nit2* homolog in *M. grisea* and is dispensable for virulence (Froeliger and Carpenter 1996). It is not clear whether *NPR1* and *NPR2* have homologs in *A. nidulans* or *N. crassa* because neither gene has yet been cloned, but they may represent pathogen-specific components of a starvation-induced response pathway for virulence (Snoeijers et al. 2000; Talbot et al. 1997). Results presented here suggest that *NPR1* and *NUT1* are necessary for induction of *MPG1* expression during starvation, but only *NPR1* is necessary for *MPG1* expression during appressorium development. Nitrogen starvation may, therefore, be a significant signal for expression of *MPG1* in hyphae, perhaps prior to symptom development and conidiogenesis, but is less significant in regulating appressorium morphogenesis. The role of *NPR1* in both regulatory processes indicates that it may be a wide domain regulator not exclusively involved in nitrogen repression. Consistent with this, we have recently observed that the virulence of *npr1* mutants can be partially restored by application of exogenous cAMP prior to plant infection (P. V. Balhadère and N. J. Talbot, *unpublished data*).

***Cis*-acting regions of the *MPG1* promoter.**

Sequence analysis of the *MPG1* promoter showed the presence of a number of putative *cis*-acting regulatory elements (Table 3). Despite the occurrence of a variety of putative enhancer sequences for stress response factors, there was no evidence that *MPG1* expression increased in response to pH, osmotic, or oxidative stress (data not shown). Only nitrogen and carbon starvation produced an obvious induction of *MPG1* expression. Three areas of the promoter appear important in regulation of *MPG1* expression. The region from –1,321 to –1,051 bp is necessary

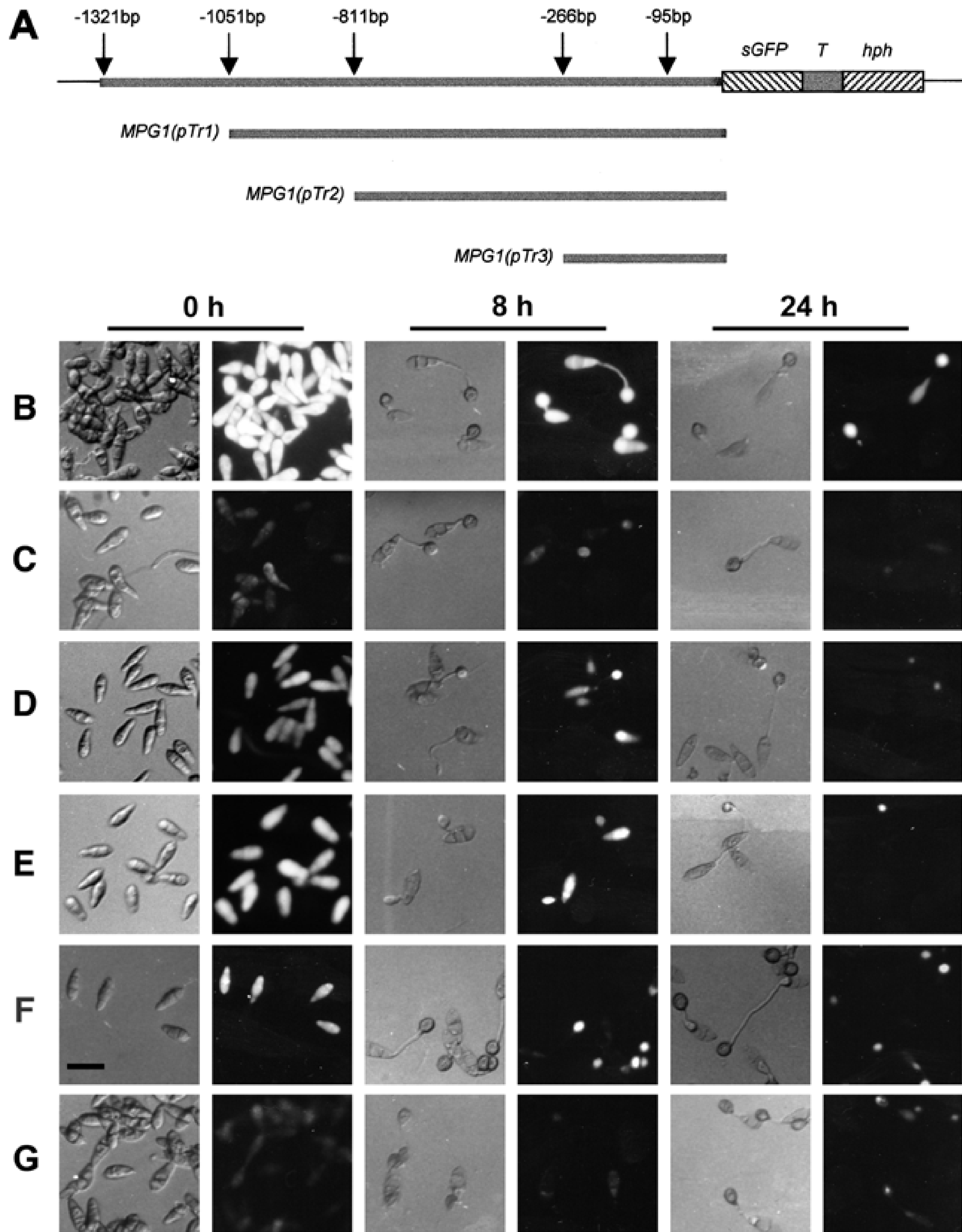


Fig. 5. *MPG1* promoter deletion analysis. **A,** *MPG1* promoter 5′ deletions used in this study in relation to the full length *MPG1* promoter. The length of the promoter in each truncation is indicated (arrows). Also shown are the green fluorescent protein encoding allele (*sGFP*), the *trpC* terminator (*T*), and the hygromycin B phosphotransferase selectable marker gene (*hph*). **B** to **G,** Epifluorescence microscopy of appressorium development in *Magnaporthe grisea* showing *MPG1(pTr)::sGFP* expression. Conidia from transformants of Guy11 expressing the following constructs were harvested and appressorium development followed for 24 h . **B,** *MPG1(p)::sGFP*. **C,** *GPDA(p)::sGFP*. **D,** *MPG1(pTr1)::sGFP*. **E,** *MPG1(pTr2)::sGFP*. **F,** *MPG1(pTr3)::sGFP*. **G**, *MPG1(pTr4)::sGFP*. Similar patterns of green fluorescent protein (GFP) fluorescence were observed in independently generated transformants of identical genotypes (data not shown). Bar = 20 µm.

for full expression of *MPG1* in conidia and appressoria, and induction of *MPG1* expression during starvation. The sequence from –1,051 to –811 bp is necessary for repression of *MPG1* expression in rich medium, and the region from –266 to –95 bp is important for expression of *MPG1* in conidia and appressoria.

The presence of six putative GATA elements in the *MPG1* promoter may represent putative binding sites for the NUT1 transcription factor and could be required for starvation control of *MPG1* and repression during growth of the fungus in rich medium, based on their location and studies in other fungi (Basse et al. 2000; Perez-Garcia et al. 2001). Studies of promoters of AreA-controlled genes from *A. nidulans* indicate, however, that not all the putative GATA elements are involved in AreA-mediated gene induction. For example, of 10 GATA sites in the intergenic promoter controlling expression of *niiA* and *niiD*, a central cluster of 4 elements is responsible for 80% of the transcriptional activity (Punt et al. 1995). Eleven GATA transcription factor–encoding genes have been identified in the *S. cerevisiae* genome (Lowry and Atchley 2000), so it is likely that several GATA elements in the *MPG1* promoter are binding sites for as yet undiscovered transcription factors. It also seems probable that the brlA response elements (BREs) are important motifs in the control of *MPG1* expression in conidia. The difference between having five BREs in the *MPG1p(Tr3)::sGFP* construct and two BREs in the *MPG1p(Tr4)::sGFP* construct, for example, may account for

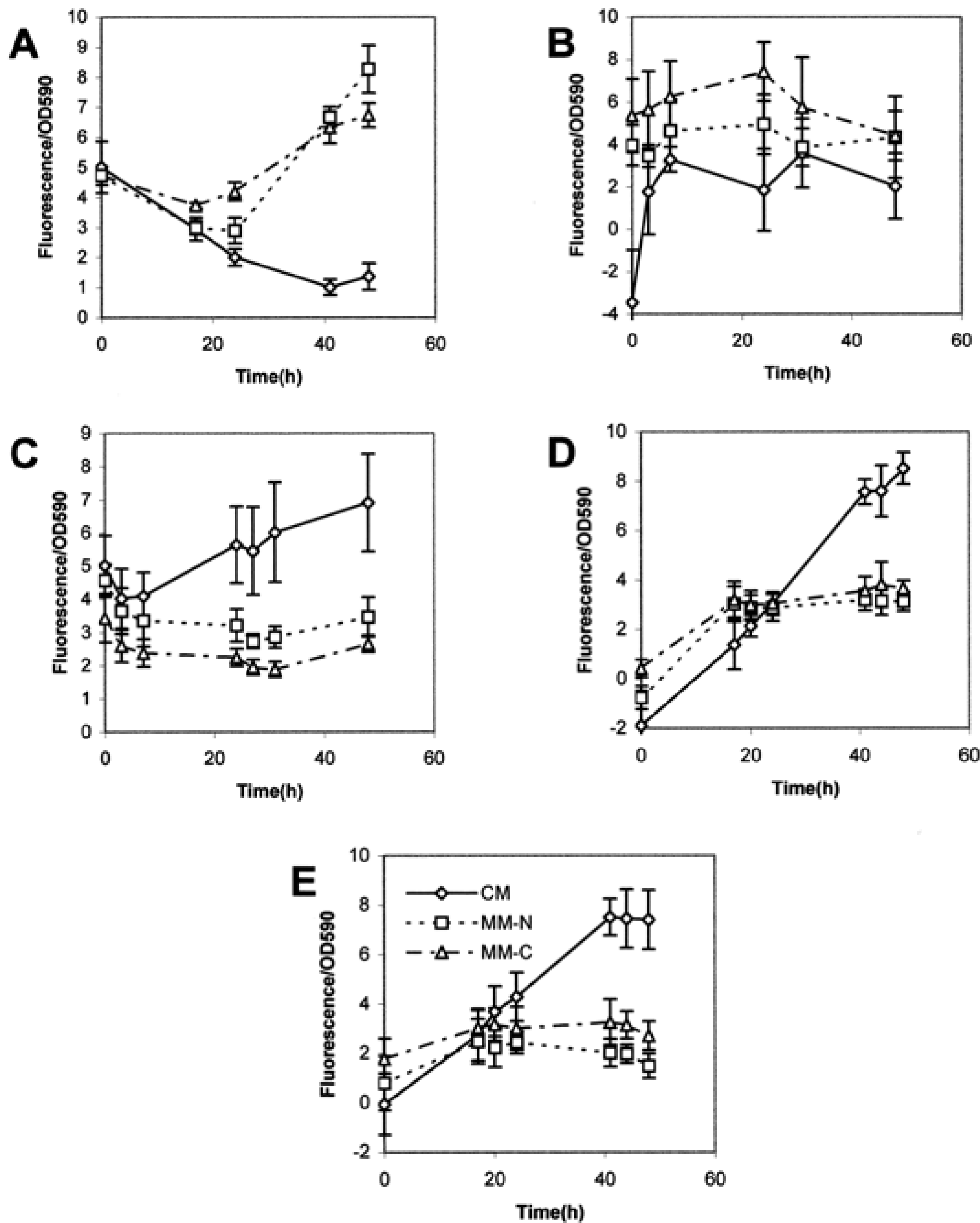


Fig. 6. Determining the effect of starvation stress on *sGFP* expression under control of *MPG1* promoter deletions. Mycelium was prepared in 96-well microplates and grown for 48 h in complete medium (CM) before being transferred to either CM, carbon starvation medium (MM-C), or nitrogen starvation medium (MM-N). Normalized green fluorescent protein (GFP) fluorescence was recorded at the time points indicated following transfer. All transformants were in the Guy11 wild-type strain background. **A,** *MPG1(p)::sGFP* transformant 72.3.4. **B,** *MPG1(pTr1)::sGFP* transformant DS649. **C,** *MPG1(pTr2)::sGFP* transformant DS627. **D,** *MPG1(pTr3)::sGFP* transformant DS666. **E,** *MPG1(pTr4)::sGFP* transformant DS449. Similar patterns of GFP fluorescence were observed in independently generated transformants of identical genotypes (data not shown). Fluorescence per optical density at 590 nm (OD_{590}) have been converted to arbitrary values. The results shown are mean data and standard deviations (n = 8).

the fourfold higher expression of GFP in conidia of *M. grisea MPG1p(Tr3)::sGFP* strains compared with those carrying the *MPG1p(Tr4)::sGFP* construct.

Complementation analysis emphasized the significance of the 5′ end of the *MPG1* promoter, which is absent in all promoter deletions tested. The absence of high level expression of *MPG1* did not allow production of the spore rodlet protein and was insufficient to restore virulence to a *Δmpg1* mutant. During conidial germination on rice leaves, *MPG1* is believed to self-assemble at the interface between the germ tube and the leaf, signaling the presence of a hydrophobic surface, which induces appressorium formation (Beckerman and Ebbole 1996; Talbot et al. 1996). This study has shown that high-level expression of *MPG1* during germ-tube extension is required for the hydrophobin to be able to fulfill this function and allow appressorium formation to proceed normally.

Production of a versatile morphogenetic protein such as the MPG1 hydrophobin, which plays roles in spore formation and plant infection, thus requires gene regulation in response to at least three signaling pathways (PMK1 MAPK, cAMP response, and nitrogen repression pathways), which operate through three large *cis*-acting domains upstream of the *MPG1* gene (Fig 8). Further analysis will be required to define enhancer motifs in detail and to identify specific transcription factors.

MATERIALS AND METHODS

Growth and maintenance of fungus stocks.

All isolates of *M. grisea* used in this study are stored at the University of Exeter. Fungal strains used were the wild-type strains Guy11 (Valent et al. 1991) and 35-R-24 (Lau and Hamer 1996) and the mutant strains 44-R-7, 43-R-20, G11397 (Lau and Hamer 1996), nn95 (Xu and Hamer 1996), DF51 (Xu et al. 1997), and TM400-5 (Talbot et al. 1993). The fungus routinely was grown in CM (Talbot et al. 1993) using standard procedures (Crawford et al. 1986).

Construction of reporter gene vectors.

The fungal transformation vector pDS1 was constructed by subcloning a 3-kb *Bst*XI/*Kpn*I fragment containing the *MPG1(p)::sGFP* construct from pMJK96 (Kershaw et al. 1998) into pCB1265, a vector conferring resistance to the herbicide bialophos (Sweigard et al. 1997).

Four fungal transformation vectors containing *sGFP* under the control of 5′ truncations of the *MPG1* promoter were constructed by amplification of the vector pMJK96 (Kershaw et al. 1998), which contains the *MPG1(p)::sGFP* construct, using four primers containing sequence identical to part of the *MPG1* promoter and the vector-specific T7 primer (Stratagene Inc., La Jolla, CA, U.S.A.). Four truncations were amplified using the following primers: Tr1 (5′-TATTAAGGCTTGCTATCTGCTACGTTGG-3′), Tr2 (5′- TATTAAGGCTTATGTTTGGGCTCTGCGG-3′), Tr3 (5′-TAAATAGGCTTCACACACACATACAGGG-3′), and Tr4 (5′-AATTAAGGCTTTGACTGAGTGACTGAGG-3′).

The sequences underlined introduced restriction sites for *Stu*I. Polymerase chain reaction (PCR) amplification from pMJK96 was performed in a Perkin-Elmer Gene Amp PCR 2400 with 25 cycles of amplification using *Pfu* proof-reading polymerase (Stratagene). Each cycle consisted of 45 sec of denaturation at 95°C, 45 sec of annealing at 55°C, and 2 min of extension at 72°C. A 5-min denaturation at 95°C preceded the first amplification cycle and a 10-min final extension at 72°C was performed after completion. The reaction volume of 50 µl contained supplied buffer, 50 ng of template, 20 pmol of each primer, 200 µM each dNTP, and 2.5 units of *Pfu* polymerase (added once reaction reached 95°C). PCR products were purified using Geneclean (BIO 101, Inc., Vista, CA, U.S.A.) and cloned into p-GEM-T (Promega Corp., Madison, WI, U.S.A.) using an A-tailing method (as per manufacturer's instructions). The truncations were recovered from the vector by digestion with *Apa*I/*Sac*I and subcloned into pCB1004 (Sweigard et al. 1997), which contains a gene conferring resistance to hygromycin B.

The fungal transformation vector pDS2 contained the destabilized allele of GFP (*d2EGFP*) (Clontech, Palo Alto, CA, U.S.A.) under control of the *MPG1* promoter. It was created by amplifying the *MPG1* promoter using the PCR conditions described above, and the primers 5′-AATTTAGGTACCAGACGCATGGTGGGTGGC-3′ and 5′-GAAGAAGGATCCGAGAAGAGACAGGAAAAG-3′. The underlined sequences introduced restriction sites for *Kpn*I and *Bam*HI, respectively. The *MPG1* promoter PCR product was digested with restriction enzymes *Kpn*I and *Bam*HI and ligated into pd2EGFP-1 (Clontech), immediately upstream of the *pd2EGFP* allele (Li et al. 1998). The *MPG1(p)::pd2EGFP* construct was recovered by digestion with *Kpn*I/*Not*I and ligated into pCB1004 (Sweigard et al. 1997).

Construction of the *MPG1* complementation vectors.

The vector pDS3 was created by subcloning a 3.75-kb *Kpn*I/*Xba*I genomic fragment containing the *MPG1* locus from pNT800 (Talbot et al. 1996) into pCB1265, which confers resistance to the herbicide bialophos (Sweigard et al. 1997). Four fungal transformation vectors containing *MPG1* under control of truncations of the *MPG1* promoter were created by PCR amplification of pDS3 using the conditions and four truncation primers described in the previous section (Tr1-4) and the primer 5′-AATATAGGCCTAACGAGTCCGCCCAGGG-3′. The underlined sequence introduces a *Stu*I restriction site. The resulting products were digested with *Stu*I and circularized by ligation.

Fungal transformations.

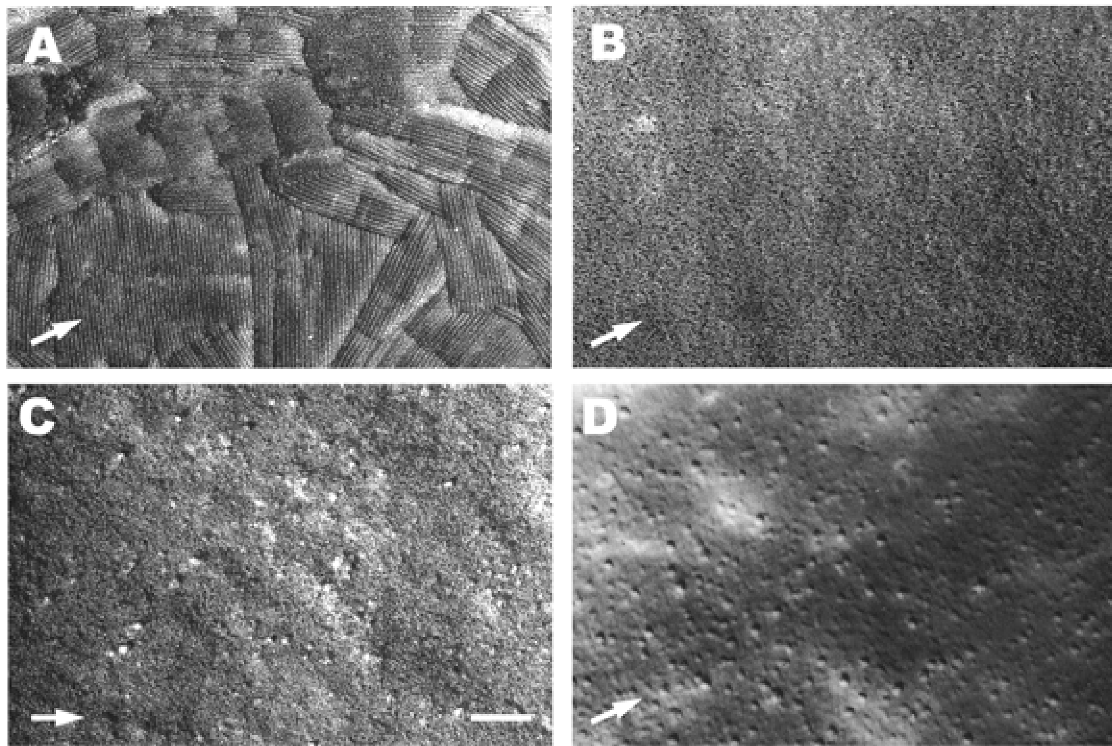
Protoplast preparation and transformations were performed as described previously (Talbot et al. 1993). Transformants were selected for hygromycin B resistance at 200 µg/ml or bialophos resistance at 30 µg/ml. Putative transformants were confirmed by Southern blot hybridization and those carrying single plasmid integrations were selected.

Nucleic acid isolations and analysis.

Genomic DNA was extracted from fungal mycelium using a hexadecyltrimethylammonium bromide (CTAB) procedure (Talbot et al. 1993). Gel electrophoresis, restriction enzyme digestion, and DNA gel blot hybridizations all were carried out

Table 4. Summary of the effect of truncations of the *MPG1* promoter on the level of *MPG1* expression

	Effect on *MPG1* expression (as compared to wild-type)		
Promoter truncation	**Growth in rich medium**	**Nitrogen starvation**	**Appressorium formation**
MPG1(pTr1)	Reduced	Reduced	Reduced
MPG1(pTr2)	Increased	Reduced	Reduced
MPG1(pTr3)	Increased	Reduced	Reduced
MPG1(pTr4)	Increased	Reduced	Greatly reduced



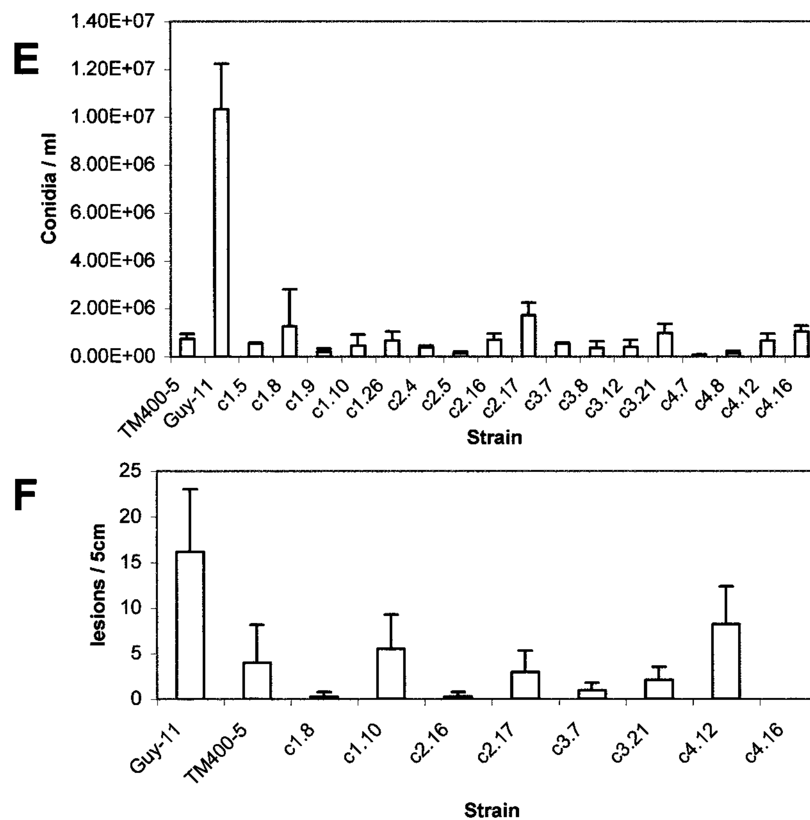


Fig. 7. Phenotypic analysis of *Δmpg1* transformants expressing *MPG1* under control of 5′ promoter deletions**. A–D,** Transmission electron micrographs of freeze-fractured conidium surfaces from four strains of *Magnaporthe grisea*. **A,** Guy11 (wild-type). **B,** TM400-5 (Δ*mpg1*). **C,** Δ*mpg1:MPG1(pTr1)::MPG1* transformant DS-c1.8. **D,** Δ*mpg1:MPG1(pTr2)::MPG1* transformant DS-c2.17. The arrows show the direction of shadowing. Bar = 100 nm. **E,** Bar chart showing the concentration of conidia produced by 10-day-old cultures of Δ*mpg1* strains of *M. grisea* transformed with *MPG1* under the control of *MPG1* promoter deletions. Individual transformant numbers are shown; the first number after the 'c' indicates the promoter deletion construct being expressed. Controls are TM400-5 (Δ*mpg1*) and Guy11. **F,** Bar chart showing the virulence of *M. grisea* transformants. Conidia from 10-day-old cultures of *M. grisea* strains were diluted to a concentration of 1×10^4 conidia per ml in 0.2% gelatin and used to inoculate 10-day-old rice seedlings. Disease lesions were counted from 5-cm sections of randomly selected leaves after 4 days. Strain numbers are shown; the first number after the 'c' indicates promoter deletion construct being expressed. Controls are TM400-5 (Δ*mpg1*) and Guy11.

using standard procedures (Sambrook et al. 1989). DNA hybridization probes were labeled using the random primer method (Feinberg and Vogelstein 1983) using the Stratagene Prime-It kit (Stratagene) and high-stringency washes were carried out as described previously (Talbot et al. 1993). RNA was isolated from hyphal cultures of *M. grisea* by the method of Timberlake (1980). Denaturing gel electrophoresis and RNA gel hybridizations were carried out using standard procedures (Sambrook et al. 1989).

Assay of GFP expression and fungal growth in 96-well microplates.

Ten-day-old cultures of fungal strains grown on CM agar plates (Talbot et al. 1993) were harvested by adding 5 ml of CM to each plate and removing mycelium with a sterile plastic spreader. The resulting suspension was filtered through sterile glass wool and diluted 1:10 in CM. The material was decanted in 100-µl aliquots into 96-well black microtiter plates with clear bottoms (Corning Costar, Cambridge, MA, U.S.A.) and incubated for 30 h at 25°C. Mycelium was recovered by centrifugation for 5 min at 3,000 × *g* in a Technospin R microtiter plate centrifuge (Sorvall Instruments, distributed by Kendro Laboratory Products Ltd., Newton, CT, U.S.A.), washed in sterile water, centrifuged again as before, and then flooded with 100 µl of either CM, MM-N (for nitrogen starvation), or MM-C (for carbon starvation) (Talbot et al., 1993) in each well. Microtiter plates routinely were incubated at 25°C for 48 h . Fungal growth was assayed by measuring the OD_{590} in a MR7000 microtiter plate reader (Dynatech Laboratories, Chantilly, VA, U.S.A.). GFP expression was assayed using a

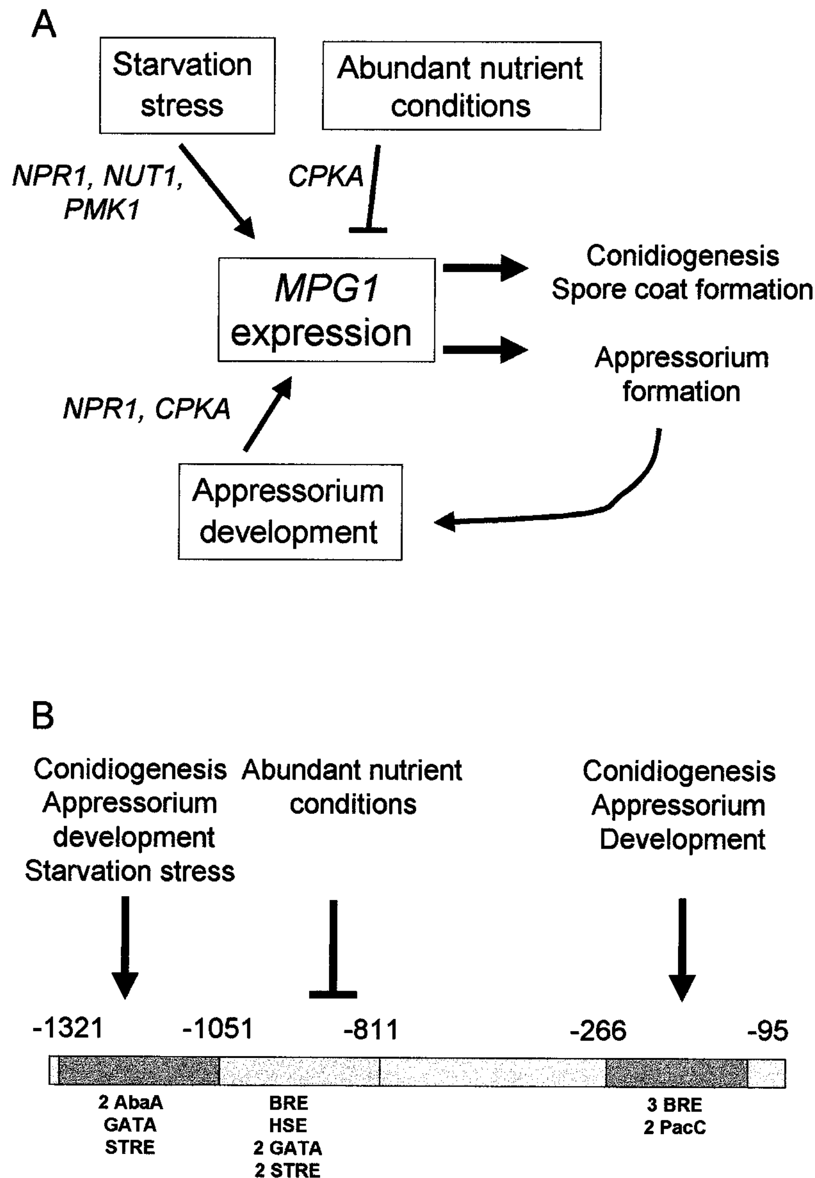


Fig. 8. Model showing genetic and environmental factors influencing *MPG1* hydrophobin gene expression. **A,** *MPG1* expression is induced in response to starvation stress and this response requires *NPR1*, *NUT1,* and the PMK1 MAPK gene. Repression of *MPG1* expression under rich growth conditions requires the cAMP-dependent protein kinase A catalytic subunit gene *CPKA*. During appressorium morphogenesis, *MPG1* expression is positively regulated by *NPR1* and *CPKA*. The MPG1 hydrophobin is required for efficient conidiogenesis and appressorium formation; therefore, its biological activity may positively feedback on its own expression through the cAMP response pathway and/or the action of *NPR1*. **B,** Regions of the *MPG1* promoter required for positive and negative regulation of *MPG1* hydrophobin expression. Values shown are base pairs upstream of the site of transcriptional initiation, determined previously by primer extension (Talbot et al. 1993). Abbreviations refer to putative *cis*-acting elements in each region. AbaA, Abacus conidiogenesis transcription factor binding site; GATA, binding site for GATA factor such a NUT1, BRE, BristleA conidogenesis factor response element; HSE, heat shock response element; STRE, stress response element; PacC, binding site for pH regulator transcription factor PacC.

Fluorolite 1000 microtiter plate-reading fluorometer (Dynatech Laboratories) or Victor Multilabel Counter (Wallac, distributed by Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, U.K.) with an excitation filter of 494 nm and an emission filter of 520 nm. Controls containing only media also were assayed for GFP fluorescence and OD_{590} and the results from these subtracted from raw data. Results were expressed as mean GFP fluorescence per OD_{590} from eight independent readings converted to arbitrary values between 1 and 10 (10 being the highest value measured for that particular strain) and standard deviations were calculated.

Visualization of GFP expression during appressorium development.

Germination and development of appressoria was monitored over time on plastic coverslips (PGC Scientific, Frederick, MD, USA) using a method adapted from that of Hamer and associates (1988). A conidial suspension in double-distilled water and 5×10^4 conidia/ml was prepared and 100 µl placed onto the surface of each coverslip. These were incubated in a moist chamber at 24°C. Developing conidia were observed using a Nikon Optiphot-2 compound microscope with Hoffmann Modulation Optics (Nikon, Inc., Melville, NY, U.S.A.). Photographs were taken with a Nikon HFX-DX camera and Nikon FX-35BX controller attached to this microscope on black-and-white 35-mm photographic film (TMAX 400, Kodak, Rochester, NY, U.S.A.) or 35-mm color photographic film (ASA 400, Kodak). Levels of GFP expression were observed by fluorescent excitation by a mercury vapor lamp (Nikon) using a B-2A filter (Nikon) (excitation 450 to 490 nm, emission 505 to 520 nm). GFP expression was quantified using NIH Image V1.62 (W. Rasband, National Institute of Health, USA).

Plant infection assays.

Rice infections were made using a dwarf Indica rice (*Oryza sativa*) cultivar, CO-39, which is very susceptible to rice blast (Valent et al. 1991). Ten-day-old cultures on CM agar were used for harvesting conidia in 3 ml of 0.2% gelatin (BDH). To assay for conidiation, the suspension was diluted to 1×10^4 conidia/ml and spray inoculated using an artists airbrush (Badger Airbrush, Franklin Park, IL, U.S.A.) onto 9-day-old (two- to three-leaf stage) plants. After spray inoculation, plants were watered well and incubated in polythene bags for 48 h and then for a further 2 to 3 days in a controlled environment chamber (Sanyo, Tokyo) at 24°C with a 12-h light and 12-h dark photophase and 90% relative humidity according to Valent and associates (1991). Lesion density was scored 96 h post inoculation from 10 randomly chosen leaves and means and standard deviations determined. Results from the pathogenicity assay were compared using the Student's *t* test (Sokal and Rohlf 1981).

Transmission electron microscopy.

The surfaces of conidia were viewed as replicas made after freeze etching (Kershaw et al. 1998). For freeze etching, conidia were fixed in 3% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.0) and washed three times in buffer. Samples were cryoprotected by sequential infiltration with 10% glycerol (1 h) and 20% glycerol (overnight). Conidia were frozen in Freon 22 (ICI, Runcorn, UK) and nitrogen slush. Freeze-fracturing was carried out in a Baltzer's BA301 (Baltzer Pfeiffer GmbH, Leichtenstein) and conidia were shadowed with carbon and platinum at 45°C. A backing layer of pure carbon was added at 90°C and the replicas floated onto distilled water. Replicas were cleaned overnight in 50% chromic acid and washed several times in distilled water before being picked up onto copper grids and viewed with a Jeol 100C transmission microscope (Jeol, Tokyo).

LITERATURE CITED

Andrianopoulos, A., and Timberlake, W. E. 1994. The *Aspergillus nidulans abaA* gene encodes a transcriptional activator that acts as a genetic switch to control development. Mol. Cell. Biol. 14:2503-2515.

Basse, C. W., Stumpferl, S., and Kahmann, R. 2000. Characterization of a *Ustilago maydis* gene specifically induced during the biotrophic phase: evidence for negative as well as positive regulation. Mol. Cell. Biol. 20:329-339.

Beckerman, J. L., and Ebbole, D. J. 1996. *MPG1*, encoding a fungal hydrophobin of *Magnaporthe grisea* is involved in surface recognition. Mol. Plant-Microbe Interact. 9:450-456.

Bell-Pedersen, D., Dunlap, J. C., and Loros, J. J. 1996. Distinct cis-acting elements mediate clock, light, and developmental regulation of the *Neurospora crassa* eas (ccg-2) gene. Mol. Cell. Biol. 16:513-521.

Bowyer, P., Mueller, E., and Lucas, J. 2000. Use of an isocitrate lyase promoter-GFP fusion to monitor carbon metabolism of the plant pathogen *Tapesia yallundae* during infection of wheat. Mol. Plant Pathol. 1:253-262.

Chang, Y. C., and Timberlake, W. E. 1993. Identification of *Aspergillus* brlA response elements (BREs) by genetic selection in yeast. Genetics 133:29-38.

Chiu, W., Niwa, Y., Zeng, E, Hirano, T., Kobayashi, H., and Sheen, J. 1996. Engineered GFP as a vital reporter in plants. Curr. Biol. 6:325-330.

Crawford, M. S., Chumley, F. G., Weaver, C. G., and Valent, B. 1986. Characterization of the herterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. Genetics 114:1111-1129.

Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. 1995. Understanding, improving and using green fluorescent proteins. Trends. Biochem. Sci. 20:448-55.

Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Fernandes, M., Xiao, H., and Lis, J. T. 1994. Fine structure analyses of the Drosophila and Saccharomyces heat shock factor—heat shock element interactions. Nucleic Acids Res. 22:167-173.

Froeliger, E. H., and Carpenter, B. E. 1996. *NUT1*, a major nitrogen regulatory gene in *Magnaporthe grisea*, is dispensable for pathogenicity. Mol. Gen. Genet. 251:647-656.

Fu, Y. H., and Marzluf, G. A. 1990. nit-2, the major positive-acting nitrogen regulatory gene of *Neurospora crassa,* encodes a sequence-specific DNA-binding protein. Proc. Natl. Acad. Sci. USA 87:5331-5335.

Gimeno, C. J., Ljungdahl, P. O., Styles, C. A., and Fink, G. R. 1992. Unipolar cell divisions in the yeast S. cerevisiae lead to filamentous growth: regulation by starvation and RAS. Cell 68:1077-1090.

Hamer, J. E., Howard, R. J., Chumley, F. G., and Valent, B. 1988. A mechanism for surface attachment in spores of a plant pathogenic fungus. Science 239:228-230.

Herskowitz, I. 1995. MAP kinase pathways in yeast: for mating and more. Cell 80:187-197.

Hoffman, C. S., and Winston, F. 1991. Glucose repression of transcription of the *Schizosaccharomyces pombe fbp1* gene occurs by a cAMP signaling pathway. Genes Dev. 5:561-571.

Kershaw, M. J., Wakley, G., and Talbot, N. J. 1998. Complementation of the *Mpg1* mutant phenotype in *Magnaporthe grisea* reveals functional relationships between fungal hydrophobins. (Eur. Mol. Biol. Organ.) J. 17:3838-3849.

Lau, G., and Hamer, J. E. 1996. Regulatory Genes Controlling *MPG1* Expression and Pathogenicity in the Rice Blast Fungus *Magnaporthe grisea*. Plant Cell 8: 771-781.

Lengeler, K. B., Davidson, R. C., D'souza, C., Harashima, T., Shen, W. C., Wang, P., Pan, X., Waugh, M., and Heitman, J. 2000. Signal transduction cascades regulating fungal development and virulence. Microbiol. Mol. Biol. Rev. 64:746-785.

Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C. C., and Kain, S. R. 1998. Generation of destabilized green fluorescent protein as a transcription reporter. J. Biol. Chem. 273:34970-34975.

Lorang, J. M., Tuori, R. P., Martinez, J. P., Sawyer, T. L., Redman, R. S., Rollins, J. A., Wolpert, T. J., Johnson, K. B., Rodriguez, R. J., Dickman, M. B., and Ciuffetti, L. M. 2001. Green fluorescent protein is lighting up fungal biology. Appl. Environ. Microbiol. 67:1987-1994.

Lowry, J. A., and Atchley, W. R. 2000. Molecular evolution of the GATA family of transcription factors: conservation within the DNA-binding domain. J. Mol. Evol. 50:103-115.

Maor, R., Puyesky, M., Horwitz, B. A., and Sharon, A. 1998. Use of green fluorescent protein (GFP) for studying development and fungal-plant interaction in Cochliobolus heterostrophus. Mycol. Res. 102:491-496.

Mitchell, T. K., and Dean, R. A. 1995. The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and

pathogenesis by the rice blast fungus Magnaporthe grisea. Plant Cell 7:1869-1878.
Ou, S. H. 1985. Rice Diseases. 2nd ed. Commonwealth Mycological Institute Kew, UK.
Perez-Garcia, A., Snoeijers, S. S., Joosten, M. H., Goosen, T., and De Wit, P. J. 2001. Expression of the avirulence gene Avr9 of the fungal tomato pathogen *Cladosporium fulvum* is regulated by the global nitrogen response factor NRF1. Mol. Plant-Microbe Interact. 14:316-325.
Punt, P. J., Oliver, R. P., Dingemanse, M. A., Pouwels, P. H., and van den Hondel, C. A. 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56:117-124.
Punt, P. J., Strauss, J., Smit, R., Kinghorn, J. R., van den Hondel, C. A., and Scazzocchio, C. 1995. The intergenic region between the divergently transcribed niiA and niaD genes of *Aspergillus nidulans* contains multiple NirA binding sites which act bidirectionally. Mol. Cell. Biol. 15:5688-5699.
Rohel, E. A., Payne, A. C., Fraaije, B. A., and Hollomon, D. W. 2001. Exploring infection of wheat and carbohydrate metabolism in *Mycosphaerella graminicola* transformants with differentially regulated green fluorescent protein expression. Mol. Plant-Microbe Interact. 14:156-163.
Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
Schuller, C., Brewster, J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. 1994.. The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae CTT1* gene. (Eur. Mol. Biol. Organ.) J. 13:4382-4389.
Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H., and Galbraith, D. W. 1995. Green-fluorescent protein as a new vital marker in plant cells. Plant J. 8:777-784.
Sokal, R. R., and Rohlf, F. J. 1981. Biometry. W. H. Freeman and Co., San Francisco, USA.
Snoeijers, S. S., Perez-Garcia, A., Joosten, M. H., and De Wit, P. J. 2000. The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. Eur. J. Plant Pathol. 106:493-506.
Spellig, T., Bottin, A., and Kahmann, R. 1996. Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. Mol. Gen. Genet. 252:503-509.
Sweigard, J. A., Chumley, F. G., Carroll, A., Farrall, L., and Valent, B. 1997. A series of vectors for fungal transformation. Fungal Genet. Newsl. 44:52-53.
Talbot, N. J. Ebbole, D. J., and Hamer, J. E 1993. 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., and Foster, A. J. 2001. Genetics and genomics of the rice blast fungus *Magnaporthe grisea*: Developing an experimental model for understanding fungal diseases of cereals. Adv. Bot. Res. 34:287-311.
Talbot, N. J., Kershaw, M. J., Wakley, G. E., DeVries, O. M. H., Wessels, 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., McCafferty, H. M., Ma, M., Moore, K., and Hamer, J. E. 1997. Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression. Physiol. Mol. Plant Pathol. 50:179-195.
Thevelein, J. M. 1994. Signal transduction in yeast. Yeast 10:1753-1790.
Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., Penalva, M. A., and Arst, H. N., Jr. 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. (Eur. Mol. Biol. Organ.) J. 14:779-790.
Timberlake, W. E. 1980. Developmental gene regulation in *Aspergillus nidulans*. Dev. Biol. 78:497-510.
Tucker, S. L., and Talbot, N. J. 2001. Surface attachment and pre-penetration stage development by plant pathogenic fungi. Annu. Rev. Phytopathol. 39:385-417.
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.
Wosten H. A. 2001. Hydrophobins: multipurpose proteins. Annu. Rev. Microbiol. 55:625-646.
Xu, J.-R., and Hamer, J. E. 1996. MAP kinase and cAMP signaling regulate infection structure formation and growth in the rice blast fungus *Magnaporthe grisea.* Genes Dev. 10:2696-2706.
Xu, J.-R., Urban, M., Sweigard, J., and Hamer, J. E. 1997 The *CPKA* gene of *Magnaporthe grisea* is essential for appressorial penetration. Mol. Plant-Microbe Interact. 10:187-194.