MPMI Vol. 9, No. 6 1996, pp. 450-456. Publication no. M-1996-0621-02R.

MPG1, a Gene Encoding a Fungal Hydrophobin of *Magnaporthe grisea*, Is Involved in Surface Recognition

Janna L. Beckerman and Daniel J. Ebbole

Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843 U.S.A.
Received 9 February 1996. Accepted 5 April 1996.

Upon encountering a leaf surface, emergent germ tubes from conidia of the rice blast fungus, *Magnaporthe grisea*, form infection structures called appressoria that allow direct penetration of plant cells. The *MPG1* gene encodes a fungal hydrophobin of *M. grisea* that is expressed during development of aerial hyphae, conidia, and appressoria. Deletion of *MPG1* reduces the efficiency of appressorium formation. We found that yeast extract repressed *MPG1* expression in vitro and inhibited appressorium development of the rice pathogen, strain Guy11. Appressorium formation of *mpg1* mutants is rescued in *trans* by coinoculation with wild-type cells. *MPG1* is required for efficient induction of appressoria in response to a host surface or highly hydrophobic artificial substrates. However, we identified several artificial substrates that can support efficient appressorium formation of *mpg1* strains. This finding suggests that Mpg1p is not specifically required for appressorium formation, but is involved in the interaction with, and recognition of, the host surface. Additionally, a time window of competence to form appressoria was identified; the decision to form appressoria occurs approximately 6 to 8 h following conidial germination. After this critical time, cells are no longer able to form appressoria in response to inductive cues. These studies indicate that *MPG1* hydrophobin is required for host recognition and that it acts as a morphogenetic signal for cellular differentiation.

Fungal pathogens must form an intimate contact with their hosts in order for successful parasitism to occur. Following contact, signaling between host and parasite determines the outcome of the interaction. The signals that trigger development of appressoria in fungal pathogens are poorly understood. In the case of the bean rust fungus, *Uromyces appendiculatus,* germ tubes are able to sense the height of the stomatal guard cells as a signal to differentiate (Hoch et al. 1987; Wynn 1976). This thigmotropic response provides a means for positioning appressoria at optimal sites for host penetration. This mechanism can be contrasted to that of *Colletotrichum gloeosporioides*, which selectively responds to the surface wax of its host, avocado. Waxes from other plants fail to induce appressoria formation in *C. gloeosporioides* (Kolattukudy et al. 1995). In the case of the rice blast fungus, *Magnaporthe grisea* (anamorph = *Pyricularia grisea* Sacc.), surface hydrophobicity has been implicated as an important cue for appressoria development (Lee and Dean 1994). However, other workers have suggested that surface hardness and other characteristics are important in substrate recognition (Jelitto et al. 1994; Xiao et al. 1994b). In addition, treatments that interfere with adhesion of hyphae to substrate block development (Xiao et al. 1994a).

Corresponding author: D. J. Ebbole; E-mail: dje0282@zeus.tamu.edu

The infection cycle of *M. grisea* begins with adhesion of conidia through spore tip mucilage released from the periplasmic space at the apex of the conidium (Hamer et al. 1988). Conidia germinate on the hydrophobic host surface and appressoria develop at the tips of the nascent germ tubes. The appressorium is a complex biomechanical device that uses hydrostatic pressure to assist in penetration of the host cell (Howard et al. 1991). However, only certain surfaces readily support development. If conidia are deposited on a non-inductive substrate, germ tubes fail to develop appressoria. To gain a better understanding of the events that influence this key stage in the life cycle of the pathogen we investigated the role of the fungal hydrophobin encoded by *MPG1* (Talbot et al. 1993).

Fungal hydrophobins represent a class of cell wall proteins that influence the hydrophobic character of the cell surface and are involved in the emergence of aerial hyphae from liquid media (Wessels et al. 1991). Hydrophobins form the rodlet layer visible on the surface of conidia of many fungal species, and facilitate the dissemination of the conidia through air (Beever and Dempsey 1978). The assembly of rodlets is catalyzed at hydrophilic-hydrophobic interfaces (Wösten et al. 1994) and can contribute to adhesion of hyphae to hydrophobic surfaces (Wösten et al. 1994). As with other fungi, the hydrophobin mutant of *M. grisea* displays a "wettable" phenotype (water droplets bead on the surface of aerial hyphae in wild-type but wet the hyphae of hydrophobin-less mutants) (Talbot et al. 1993). *mpg1* strains inefficiently form appressoria (5 to 10% of wild-type), suggesting that *MPG1* assists in the initiation of development.

Lee and Dean (1993) demonstrated that the need for an inductive surface could be bypassed by addition of cAMP to germinating conidia. They suggested that the interaction of cell wall protein in the germ tube with the substrate results in the increased production of cAMP and initiation of the developmental program of appressorium formation. The low levels

of appressorium formation in the *MPG1* deletion mutants and in wild-type germlings inoculated onto noninductive hydrophilic substrates could be explained by a simple model in which the interaction of the germ tube with hydrophobin rodlets assembled at the hyphae-substrate interface signals the presence of a "host" surface. Alternatively, hydrophobin assembly may assist in adhesion of hyphae to certain substrates, as with Sc3 hydrophobin (Wösten et al. 1994). The degree of adhesion of the hypha to substrate may generate a signal to initiate development.

To characterize *MPG1* and its role in development, we examined the effect of nutrients on expression of *MPG1* and the effects of nutrients, cAMP, and substrate on appressorium formation. Previous work in our laboratory (JLB and DJE, unpublished) and by others (Lee and Dean 1994) failed to identify simple nutrients that could inhibit formation of appressoria. However, we found that nutrient sources that repressed *MPG1* expression prevented appressorium formation in vitro and that cAMP restored development. These findings allowed us to examine the time during which germinating conidia are competent to form appressoria. We also examined the response of the wild-type strain, Guy11, and isogenic *MPG1* deletion mutants to a variety of substrates and determined whether coinoculation with Guy11 could rescue appressorium formation of *MPG1* deletion mutants. Our results are consistent with the view that Mpg1p is important for host recognition and that it functions as a morphogenetic cue for infection structure differentiation during a narrow time window of developmental competence.

RESULTS

MPG1 expression and *M. grisea* development are regulated in response to nutrient source.

Limitation for carbon and nitrogen in a modified complete medium leads to derepression of *MPG1* (Talbot et al. 1993), suggesting that *MPG1* is regulated by carbon and nitrogen catabolite repression. Repression of *MPG1* was observed in complete medium containing yeast extract, Casamino Acids, and peptone (Talbot et al. 1993). However, we found that full repression of *MPG1* expression required a medium containing yeast extract. Repression occurred in media containing 2% yeast extract even in the absence of additional carbon or nitrogen sources (Fig. 1). Peptone, tryptone, and Casamino Acids were far less effective at repression of *MPG1* expression. *MPG1* was highly expressed in minimal medium containing 70 mM nitrate and 1% glucose (Fig. 1A). Starvation for carbon or nitrogen did not strongly stimulate *MPG1* expression over the already substantial level observed in minimal medium (Fig. 1B). Individual testing of several abundant carbon sources present in yeast extract individually by addition to minimal medium did not repress *MPG1* (data not shown).

Because *MPG1* is required for efficient appressorium formation on rice tissue or Teflon film (Talbot et al. 1993), we investigated the effect of nutrient sources. Conidia germinated in 2% yeast extract, but failed to form appressoria (Table 1). Nutrient sources that failed to repress *MPG1* expression did not inhibit formation of appressoria (Table 1; Fig. 2). We were unable to identify any specific component(s) of yeast extract responsible for repression of *MPG1* or inhibition of appressorium formation. Addition of yeast extract to germinating conidia of the *MPG1* deletion mutants on Teflon or the hydrophobic side of GelBond film also resulted in a reduction of appressorium formation relative to a water control (data not shown).

It was previously reported that addition of cAMP to conidia germinated on noninductive hydrophilic surfaces induced appressorium formation (Lee and Dean 1993). We found that cAMP is able to reverse the inhibition of appressorium formation caused by 2% yeast extract (Table 1; Fig. 2). cAMP also induced efficient appressorium formation in the *MPG1* deletion mutants TM400-2 and TM400-5 inoculated on Teflon film (not shown).

Time course of appressorium formation.

We exploited the fact that yeast extract inhibits appressorium formation and that cAMP induces appressorium formation to examine whether *M. grisea* becomes committed to appressorium development at an early time during spore germination on a hydrophobic surface. Conidia were germinated in water and at subsequent times the water was replaced with yeast extract to inhibit appressorium formation. We found that addition of yeast extract at any time prior to the initiation of appressorial morphogenesis (6 to 8 h) blocked later development (Table 2).

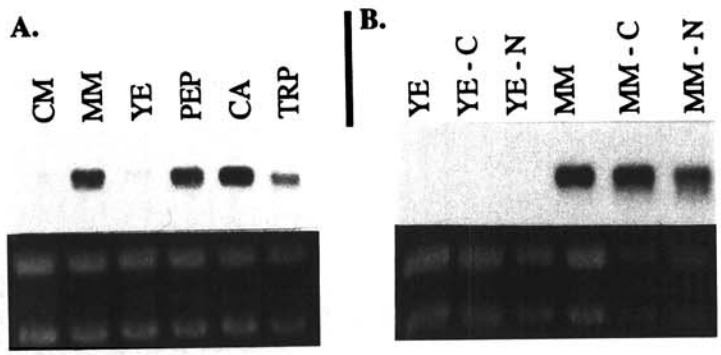

Fig. 1. Analysis of *MPG1* expression in response to nutrients. Top panel: Northern blot probed with *MPG1* clone. Bottom panel: Ethidium bromide stained gel showing amount of ribosomal RNA present in each lane. **A,** Repression of *MPG1* by yeast extract. Washed mycelium was added to the following: Complete medium (CM), Minimal medium (MM), MM + 2% yeast extract (YE), MM + 2% Peptone (PEP), M + 2% Casamino Acids (CA), MM + 2% Tryptone (TRP). **B,** Effect of carbon and nitrogen. Washed mycelium was added to minimal medium + 2% YE, MM + 2% YE without exogenous glucose (YE-C), MM + 2% YE without inorganic nitrogen (YE-N), MM, MM without glucose (MM-C), MM without inorganic nitrogen (MM-N). Cultures were incubated for 24 h at 25°C with shaking

In a similar time course experiment, conidia were initially grown in the presence of yeast extract to repress development. The yeast extract was replaced with water or with yeast extract containing 10 mM cAMP to determine whether development could be induced after an initial period of inhibition. In both cases, media replacement up to 4 h resulted in high levels of appressorium formation demonstrating that at this time germlings were still competent to initiate development. A decrease in appressorium formation was observed after 6 h when the yeast extract was replaced by water. Yeast extract amended with cAMP was capable of full restoration of appressorium formation at the 6-h time point (Table 2). At 8 h, both treatments were relatively ineffective in inducing development and this trend continued to the 12-h time point, at which virtually no appressorium formation was observed (Table 2).

Effect of substrate on appressorium formation in wild type and *MPG1* mutants.

There is disagreement in the literature concerning the substrate characteristics that are most important for development. Several reports suggest that appressorium formation is induced on hydrophobic, but not hydrophilic surfaces (Howard et al. 1991; Lee and Dean 1993; Lee and Dean 1994), while others have suggested that surface hardness is more important than hydrophobicity (Xiao et al. 1994b), or that there is variability between strains but that no clear relationship exists between hydrophobicity and appressorium formation (Howard 1994; Jelitto et al. 1994). Although all of the surface characteristics critical for inducing development are not known, there is a clear response of the fungus to specific surfaces, i.e., conidia from 10-day-old cultures of strain Guy11 form appressoria on Teflon and not on the hydrophilic side of Gelbond film (Table 3). We found that appressoria of Guy11 did not form on glass coverslips manufactured by Corning Co., but appressoria did form efficiently on glass coverslips obtained from Fisher Co., even after cleaning with 10 N sodium hydroxide (Table 3). However, treatment of the Corning Co. coverslips with 10% chromic acid or 50% hydrofluoric acid allowed appressoria formation similar to that observed on Fisher Co. coverslips (Table 3). The acid treatments did not substantially alter the hydrophobicity of the glass surfaces.

Approximately 5% of *MPG1* mutant germlings produced appressoria on Teflon or Parafilm. Surprisingly, approximately 60 to 70% of conidia formed appressoria on the hydrophobic side of GelBond film and on acid-treated glass coverslips (Table 3). However, this was lower than the 90% appressorium formation found with Guy11 on these substrates (Table 3). Teflon was reproducibly more hydrophobic than GelBond film in our assays (hydrophobicity index of 92 and 87, respectively). Fisher Co. and Corning Co. glass coverslips treated with chromic acid were similar in hydrophobicity and somewhat more hydrophobic than hydrofluoric acid treated glass (Table 3). However, there was no significant difference in the efficiency of appressorium formation on these acid treated glass surfaces.

Table 1. Effect of different media on appressorium formation

Compound tested	% Appressoria formation
Water	96 ± 3[a]
2% Yeast Extract	3 ± 2
2% Casamino Acids	97 ± 3
2% Peptone	97 ± 4
2% Tryptone	96 ± 2
2% Yeast Extract + 10mM cAMP	84 ± 12

[a] A suspension of 10^4 conidia in 10 µl was inoculated onto Teflon and percentage of appressorium formation was quantitated after 16 to 20 h.

Mpg1p acts in *trans*.

Hydrophobins in other fungi are secreted proteins and *MPG1* encodes a polypeptide containing a typical secretion signal peptide. We therefore expect that Mpg1p is secreted into the medium and is equally available for interaction with any germ tube present. An experiment was performed to test for complementation of appressorium formation of the *mpg1* strains by coinoculation with Guy11. Mixtures of the germ-

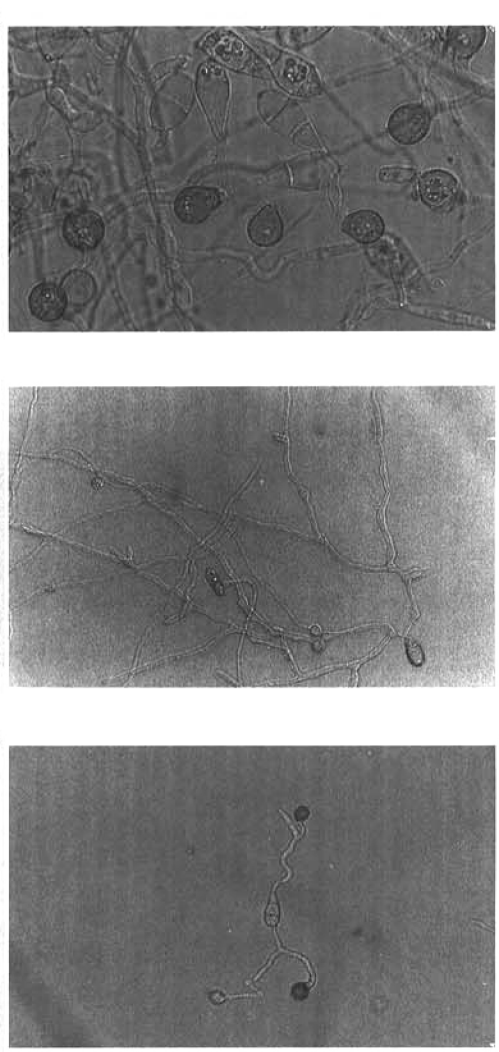

Fig. 2. Effect of yeast extract and cAMP on appressorium formation. **A,** Conidia of wild type were germinated on the hydrophobic side of GelBond film in the presence of sterile water, 400× magnification. Conidia are tear-drop shaped, appressoria are darker and spherical; **B,** 2% yeast extract, 100×. Conidia germinate and grow as vegetative hyphae; **C,** 2% yeast extract with 10 mM cAMP, 100×. Formation of appressoria from multiple germ tubes of a single conidium is common. Suspensions of conidia were incubated for 16 h as described in Materials and Methods.

lings in the ratios of 3:1, 1:1, and 1:3 would be expected to result in roughly 75, 50, and 25% appressorium formation in the absence of interaction based on the control levels of 95% for Guy11 and an average of 6% for the deletion mutants (Fig. 3). Mixing conidia in a 3:1 ratio (wild-type/deletion mutants) resulted in approximately 95% appressorium formation. A 1:1 mixture of wild-type/deletion mutant conidia yielded greater than 80% appressorium formation versus an expected 50% if no extracellular complementation occurred. Analysis of variance indicated a significant ($P = 0.0001$, Fisher's LSD) deviation for both the 3:1 and 1:1 ratios from the expected level if there was no interaction between wild-type and the *mpg1* mutants. (SAS) (Statistical Analysis System 1987). The 1:3 mixture of Guy11:mutant conidia exhibited a high degree of variability in formation of appressoria.

DISCUSSION

Nutrient source and control of *MPG1* expression and appressorial development.

During pathogenesis, *MPG1* mRNA is detected early during infection of rice leaves at a time corresponding to appressorium formation. During colonization of rice leaf tissue *MPG1* expression is not detected in abundance until late in infection (Talbot et al. 1993). This late expression of *MPG1* may be due to nutrient limitation at the time of lesion delimitation, to initiation of conidiophore development, or in response to accumulation of specific plant-derived compounds. Nutrient limitation has been correlated with the formation of appressoria in the insect pathogen *Metarhizium anisopliae* (St. Leger et al. 1992). A fungal hydrophobin, *ssgA*, was obtained from a cDNA library representing a nutrient-deprived culture of the *M. anisopliae*, and expression of *ssgA* was found to be regulated by nutrients (St. Leger et al. 1992). In our growth experiments we found that *MPG1* expression is repressed in the presence of yeast extract and is not repressed by other complex nutrients, such as tryptone and peptone. However, we did not analyze the effect of light, temperature, or other environmental signals that may influence expression (Jelitto et al. 1994). If the apparent repression of *MPG1* expression in planta is due to nutrient availability, then *MPG1* responds to nutrients in a complex fashion that may depend on a specific combination of nutrients rather than simple carbon or nitrogen catabolite repression. Tryptone medium can support luxurious growth of the mycelium but is unable to repress appressorium formation. Hence, availability of nutrients per se is not a signal for appressorium development. Another interpretation is that yeast extract contains compounds unique to fungi that signal repression of *MPG1* and block development.

We reasoned that yeast extract might repress *MPG1* expression in wild-type germlings and mimic the phenotype of the *mpg1* strains. We found that 2% yeast extract was sufficient to repress appressorium formation in vitro. Recently, yeast extract was shown to repress appressorium formation of *C. gloeosporioides* (Kolattukudy et al. 1995). Attempts to inhibit appressorium formation in *M. grisea* using simple nutrients, including 5% glucose (Lee and Dean 1993), have been unsuccessful. The active compound(s) in yeast extract (Difco) does not appear to be an amino acid, purine, pyrimidine, or other abundant component present in Casamino Acids, peptone, or tryptone. It will be of interest to identify the component(s) of yeast extract that inhibits appressorium formation and determine whether the compound(s) could be present in host tissue during colonization.

MPG1 protein is one component involved in initiation of development.

MPG1 deletion mutants are no longer able to efficiently recognize Teflon or rice leaves as inductive surfaces. However, this defect is not absolute since a low level (5 to 10%) of appressorium formation occurs on these surfaces in the *mpg1* strain. We found that yeast extract could further reduce appressorium formation in *mpg1* strains. Similarly, appressorium formation of both the *MPG1* deletion mutants and wild type is inhibited on the hydrophobic surface of Gelbond film by yeast extract indicating that yeast extract influences factors in addition to *MPG1* expression.

Our results support the findings of others (Howard 1994; Jelitto et al. 1994; Xiao et al. 1994) that there is no absolute correlation between surface hydrophobicity and efficacy in inducing appressorium formation. However, development on hydrophilic glass surfaces could be affected by the type of glass and glass treatments (i.e., coatings, age, washing, and heat) that can affect surface characteristics. The glass coverslips manufactured by Corning Co. were a poor substrate for induction of appressorium formation. However, acid washes revealed the glass to be as inductive as the glass obtained from Fisher Co. This observation may help to explain the disparate results previously reported with glass substrates (Jelitto et al. 1994; Lee and Dean 1993; Xiao et al. 1994b). Although these apparently hydrophilic surfaces can support development, measurement of hydrophobicity may not be accurate for surfaces that possess microscale variation. The presence of microscale hydrophobic patches would likely not be detected by standard methods of estimating hydrophobicity, such as the assay employed here (Lee and Dean 1993; Lee and Dean 1994) or measuring surface contact angle (Hazen 1990). Interestingly, the two substrates that did not support appressorium

Table 2. Effect of medium replacement during appressorium development on Teflon

Treatment[a]	Hours incubated prior to media replacement						
	0	2	4	6	8	10	12
Replace water with YE[b]	4 ± 2	11 ± 5	32 ± 16	39 ± 25	77 ± 13	78 ± 24	96 ± 3
Replace YE with water[c]	97 ± 2	94 ± 2	92 ± 4	51 ± 12	33 ± 24	16 ± 3	4 ± 2
Replace YE with YE + cAMP[d]	84 ± 2	78 ± 7	77 ± 4	83 ± 1	43 ± 15	15 ± 6	2 ± 1

[a] Appressorium formation of all samples was quantitated 12 to 16 h following the final time point.
[b] Water was replaced with 2% yeast extract.
[c] 2% yeast extract was replaced with water.
[d] 2% yeast extract was replaced with 2% yeast extract + cAMP.

formation displayed a high degree of variability in our hydrophobicity assay even though the average hydrophobicity was similar to the acid-treated glass surfaces (Table 3).

Mpg1p hydrophobin is responsible for determining the range of substrate hydrophobicity that induces development, with loss of the hydrophobin resulting in an inability to recognize the most hydrophobic of substrates (i.e., Teflon, Parafilm, and rice leaves). Surprisingly, both a relatively hydrophobic surface (GelBond-hydrophobic) and the hydrophilic glass surfaces, also supported development of appressoria of *MPG1* deletion mutants. This shows that sufficient interactions occur through other factors in the absence of *MPG1* protein to induce development on these surfaces.

MPG1 hydrophobin functions through an initially soluble form.

Examination of rodlet structures in electron micrographs indicates a definite orientation of assembled hydrophobin with the "rodlets" facing the hydrophobic side (substrate), and an amorphous surface facing the hydrophilic side (cell wall) (Wösten et al. 1994). Recent findings indicate that *MPG1* protein forms rodlets on the surface of *M. grisea* conidia and that *MPG1* mutants adhere slightly less tightly to Teflon than wild type (N. J. Talbot, M. J. Kershaw, G. F. Wakley, O. M. H. de Vries, J. G. H. Wessels, and J. E. Hamer, unpublished). During germ tube growth on an inductive surface, a cluster of cytoplasmic vesicles is observed near the cell apex, adjacent to the substrate (Howard 1994), rather than at the forefront of the hyphal tip as seen in vegetative hyphae. Conceivably, these substrate-oriented vesicles could contain Mpg1p, and if so, the interaction of hydrophobin with the cell wall and substrate could occur in *cis* by direct secretion of hydrophobin onto the substrate in contact with the cell wall. Alternatively, the interaction of Mpg1p with hyphae could occur in *trans* by assembly of hydrophobin from the surrounding medium at the substrate-hypha interface or by assembly of rodlets first at the water-substrate interface. We found that mixing wild-type and *mpg1* conidia resulted in rescue of appressorium formation of the mutant. The variation observed in the frequency of appressorium development in the 1:3 (wild-type:*MPG1* deletion mutant) mixture may reflect a limiting level of hydrophobin production. This suggests that Mpg1p acts in *trans*, and that hydrophobin may become limiting at approximately 25% of wild-type levels in the experimental protocol employed here. Because Mpg1p can apparently be titrated by *mpg1* mutant hyphae, we suggest that Mpg1p assembles directly at the hypha-substrate interface rather than coating the substrate surface for subsequent discovery by wandering hyphal tips.

Table 3. Percent appressorium formation from germlings of wildtype and MPG1 deletion mutants on different substrates

	% Appressorium formation		
Substrate	Wild type	*MPG* mutant	Hydrophobicity index
Teflon FEP	98 ± 2[a]	8.7 ± 1.7	92 ± 1
Parafilm	98 ± 2	2.8 ± 2.8	92 ± 1
GelBond-hydrophobic	93 ± 17	58 ± 16	87 ± 2
GelBond-hydrophilic	0	0	60 ± 15
Corning Glass	0	0	75 ± 10
Corning Chromic[b]	87 ± 6	67 ± 13	77 ± 3
Corning HF[c]	97 ± 2	75 ± 10	65 ± 2
Fisher Glass	92 ± 12	68 ± 28	69 ± 3
Fisher Chromic[b]	89 ± 6	75 ± 9	76 ± 2
Fisher HF[c]	90 ± 5	68 ± 9	67 ± 2

[a] Percent of germ tubes that gave rise to appressoria ± standard deviation.
[b] Treated for 24 h with 10% chromic acid.
[c] Treated for 5 min with 50% hydrofluoric acid.

A model for *MPG1* function.

Our data support the model that Mpg1p acts by assembling at hypha-substrate interfaces to promote surface recognition. If the properties of Mpg1p are similar to the better-characterized hydrophobin Sc3 (Wösten et al. 1994), it is possible that Mpg1p acts through enhancing adhesion. However, it is clear that other factors also contribute to adhesion. On some surfaces, such as Teflon, Parafilm, or rice leaves, Mpg1p is of critical importance to signal development. Our data do not directly prove that it is Mpg1p that acts in *trans* to rescue development. Conceivably Mpg1 could function in production of cAMP or some other factor that could act in *trans* to induce development. The fact that high levels of cAMP (10 mM) are needed to induce development in *M. grisea* makes this alternative unlikely. The biochemical properties of hydrophobins make them difficult to manipulate for direct addition of Mpg1p to *mpg1* cells. Further studies with purified Mpg1p and localization using Mpg1p-specific antibodies will be needed for proof of the role of Mpg1p in appressorium development.

Lee and Dean (1993) have proposed that surface recognition leads to a cAMP signaling pathway that causes development of appressoria. We found that cAMP or inducing conditions must be present approximately 8 h following germination or the ability to efficiently form appressoria is lost. It was found that appressorium formation was not substantially stimulated by a 2-h exposure to cAMP at 0 to 2 or

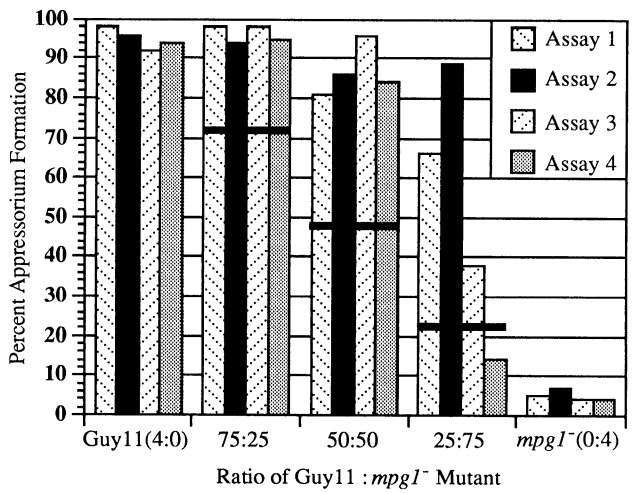

Fig. 3. Appressorium formation following co-inoculation of Guy11 and *MPG1* mutant spores. Conidia of Guy11 and *MPG1* mutant TM400-L2 (Assays 1 and 2) or TM400-L5 (Assays 3 and 4) were mixed in ratios of 3:1, 1:1, 1:3 (Guy11:mutant). Bars represent percent appressorium formation in each of the four assays. Appressorium formation of pure culture controls of Guy11 (4:0) and the *MPG1* mutants (0:4) for each assay are shown. The solid black lines across the bars indicate the level of appressorium formation expected based on the average amount of appressorium formation observed with Guy11 and the *MPG1* mutants and assuming no interaction between germlings.

2 to 4 h following spore inoculation (Lee and Dean 1993). However, appressorium formation was stimulated by pulses of cAMP during 4 to 6 and 6 to 8 h following spore inoculation. This timing of the response to cAMP is consistent with our finding of a window of developmental competence in which cells are able to respond to inducing environments or added cAMP.

Lee and Dean (1993) observed that cAMP could restore appressorium formation to conidia germinated on noninductive surfaces. Our finding that cAMP can bypass the inhibitory effect of both yeast extract and the absence of Mpg1p in the null mutant indicates that cAMP acts downstream of *MPG1* function and the other functions affected by yeast extract.

Lee and Dean (1993) also reported that hyphae of older mycelia could be induced to form appressoria by compressing mycelial balls between plastic coverslips or between noninductive glass coverslips in the presence of cAMP. How this treatment might restore developmental competence is unclear. Howard (1994) observed variation in appressorium formation in response to different surfaces using different strains. It is likely that both strain-specific differences and differences in apparently similar substrates could contribute to variation in the behavior of conidia germinated on some surfaces. We have been careful to use conidia of the same age and use consistent spore harvesting conditions in this study.

Jellito et al. (1994) found that the length of germ tubes with appressoria and the efficiency of appressorium formation were influenced by environmental factors including light. Interestingly, expression of the hydrophobin of *Neurospora crassa* is regulated in response to light and the circadian clock (Bell-Pedersen et al. 1992; Lauter et al. 1992). In preliminary experiments with *M. grisea* (DJE and JLB, unpublished), no light or clock regulation of *MPG1* was detected. However, the conditions employed for those assays were based on procedures developed for *N. crassa* and were not optimized for studies with *M. grisea*.

Control of both *MPG1* expression and appressorium development by nutrients and other factors may provide a mechanism to the fungus to forego a pathogenic lifestyle in favor of a saprophytic one in response to environmental conditions. The existence of a window of developmental competence suggests that under typical conditions, *M. grisea* attempts to identify a potential host plant. If a potential host is not identified within approximately 8 h, the fungus enters a saprophytic mode of growth.

These results support a model in which Mpg1p is involved in detecting surface characteristics and acts as a morphogenetic signal for induction of cellular differentiation on the host surface. Hydrophobins have been identified in phytopathogenic, entomopathogenic, zoopathogenic, and wood-decaying fungi (Wessels 1994). In addition to the several other roles of hydrophobins, these small proteins may serve generally as part of the surface recognition systems of fungi.

MATERIALS AND METHODS

Fungal strains and production of conidia.

M. grisea strain Guy 11, and *MPG1* deletion mutants TM400-L2 and TM400-L5 have been described (Talbot et al. 1993). Cultures were maintained on oatmeal agar (OMA) (50 g of oatmeal per liter) and grown at 24°C under constant fluorescent light to promote conidiation. Conidia were collected from 10- to 14-day old cultures by agitation in 1% Tween-80 (Sigma, St. Louis, MO) and then washed twice in sterile distilled water. Conidia were quantitated using a hemacytometer and suspended to a final concentration of 1×10^4 to 1×10^5 conidia ml^{-1}. *MPG1* deletion mutants were suspended at a final concentration of 1×10^4 conidia ml^{-1}.

Culture conditions.

A 1-cm^2 patch of *M. grisea* mycelium was excised from a plate culture and added to complete medium (CM) containing 1% glucose, 0.2% peptone, 0.1% Casamino Acids, 0.1% yeast extract, 0.1% (v/v) trace elements solution, 0.1% (v/v) vitamin supplement, 0.6% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4$, and 0.15% $KH2PO_4$, pH 6.5 (Crawford et al. 1986), and grown overnight at 200 rpm at 24°C with constant illumination. The culture was fragmented in a Waring blender and grown overnight as described above. This culture was transferred to a sterile 50-ml conical tube, centrifuged, and washed with water twice prior to inoculation into CM, minimal medium (MM) (CM lacking Casamino Acids, peptone, and yeast extract) or MM with 2% (w/v) of either: yeast extract, Casamino Acids, tryptone, or peptone (Difco, Detroit, MI) and incubated for 24 h as described above prior to harvesting for RNA extraction. To examine the effect of carbon and nitrogen limitation, the washed starter culture was added to MM with 2% yeast extract, MM with 2% yeast extract without glucose, MM with 2% yeast extract without inorganic nitrogen, MM, MM without glucose, and MM without inorganic nitrogen.

RNA extraction.

RNA was isolated from mycelia or conidia by disruption with 0.5-mm glass beads in a mini-beadbeater (Biospec Products, Bartlesville, OK) in the presence of phenol/choroform/lysis buffer in a ratio of 1:1:1.375, as described (Sachs and Yanofsky 1991), except 10 mM EDTA was added to the buffer. Twenty micrograms of RNA was separated by electrophoresis in a 1.2% formaldehyde-agarose gel and blotted onto Zeta probe nylon membrane (BioRad) according to manufacturer's instructions. Radiolabeled *MPG1* probes were prepared by random primed synthesis from a cDNA clone, pMC2 (Sambrook et al. 1989).

Appressorium formation assays.

Conidia were suspended in sterile water, modified Fries medium (LeBrun et al. 1990), complete medium, or water containing 2% of the following: yeast extract, Casamino Acids, tryptone, peptone, or yeast extract plus 10 mM cAMP (Sigma, St. Louis, MO). Ten 10-µl drops of spore suspension were placed on the the hydrophobic side of GelBond film (FMC, Rockland, ME) in a disposable petri dish containing a moistened 70 mm Whatman #1 filter. Petri dishes were wrapped with Parafilm and incubated at 24°C overnight. The percentage of germinating conidia induced to form appressoria was determined by direct microscopic examination of 100 conidia per 10-µl drop, with a minimum of three 10-µl drops counted in each experiment. Experiments were repeated a minimum of three times.

Hydrophobicity assay.

An estimation of substrate hydrophobicity was made using a modification of the assay described by Lee and Dean (Lee

and Dean 1993). Five 10-µl drops of water with 0.1% cotton blue were placed on the test substrate and allowed to dry. The area of the dried cotton blue was scanned, digitized (Nikon Coolscan Electronic Imaging, Nikon, Melville, NY), and quantitated using ImagePro Plus (Media Cybernetics, Silver Spring, MD). The hydrophobicity index was determined by subtracting the area of the drop (mm^2) from 100. The following substrates were tested: Teflon FEP (Dupont, Wilmington, DE), Parafilm, the hydrophobic and hydrophilic sides of GelBond Film, and glass coverslips from Corning (Corning, NY) and Fisher (Fisher Premium Gold, Pittsburgh, PA). Coverslips were cleaned by soaking 24 h in 10% chromic acid or etched for 5 min in 50% hydrofluoric acid. Hydrophobicity determinations were repeated three times. Appressorium formation on these substrates were tested as described above.

Time course of appressorium formation.

For each time point, conidia were suspended in sterile water or 2% yeast extract and five 10-µl drops of suspension were placed on the hydrophobic side of of GelBond film. Conidia were allowed to adhere and, at each time point, the liquid of each droplet was removed from the samples and immediately replaced with water, yeast extract, or yeast extract plus 10 mM cAMP. The frequency of appressorium formation was quantitated approximately 12 to 16 h after the final medium replacement (24 to 28 h after the conidia were initially deposited on the substrate). As controls for each treatment, conidia suspended in water or yeast extract were included for comparison. These experiments were perfomed a minimum of two times.

***MPG1* complementation assay.**

To determine if production of Mpg1p by wild-type germlings could rescue the null mutant, conidia of the two strains were mixed in water in known ratios. Conidia were harvested and quantitated as above. Conidia of Guy11 and TM400-L2 or TM400-L5 were mixed in ratios of 3:1, 1:1, 1:3, or pure culture controls. Ten-microliter drops of the suspensions were added to Teflon and incubated overnight. Appressoria formation was quantitated from a minimum of three groups of 100 germinated conidia per experiment. This experiment was repeated four times. Statistical analyses of the data were computed utilizing Statistical Analysis System (SAS) computer program (Statistical Analysis System 1987) to examine statistical significance using Fisher's LSD.

ACKNOWLEDGMENTS

We thank Karen Scholtoff, Herman Scholtoff, and Doug Cook for critical review of the manuscript. We thank Peter Christie, David Griffin, John Hamer, and Nick Talbot for insightful discussions and Robert Butchko for assistance with photography. This work was supported by the Texas Advanced Research Program and NIH Grant R29 GM47977.

LITERATURE CITED

Beever, R. E., and Dempsey, G. P. 1978. Function of rodlets on the surface of fungal spores. Nature 272:608-610.

Bell-Pedersen, D., Dunlap, J. C., and Loros, J. J. 1992. The *Neurospora* circadian clock-controlled gene, *ccg-2*, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer. Genes Dev. 6:2382-2394.

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.

Hamer, J. E., Howard, R. H., Chumley, F. G., and Valent, B. 1988. A mechanism for surface attachment in spores of a plant pathogenic fungus. Science 239:288-290.

Hazen, K. C. 1990. Cell surface hydrophobicity of medically important fungi, especially *Candida* species. Pages 249-295 in: Microbial Cell Surface Hydrophobicity. R. J. Doyle and M. Rosenberg, eds. American Society for Microbiology, Washington, D.C.

Hoch, H. C., Staples, R. C., Whitehead, B., Comeau, J., and Wolf, E. D. 1987. Signaling for growth orientation and cell differentiation by surface topography in *Uromyces*. Science 235:1659-1662.

Howard, R. J. 1994. Cell biology of pathogenesis. Pages 3-22 in: Rice Blast Disease. R. S. Zeigler, S. A. Leong, and P. S. Teng, eds. CAB International, Wallingford, UK.

Howard, R. J., Ferrari, M. A., Roach, D. H., and Money, D. H. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures. Proc. Natl. Acad. Sci. USA 88:11281-11284.

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.

Kolattukudy, P. E., Rogers, L. M., Li, D., Hwang, C.-S., and Flaishman, M. A. 1995. Surface signaling in pathogenesis. Proc. Natl. Acad. Sci. USA 92:4080-4087.

Lauter, F.-R., Russo, V. E. A., and Yanofsky, C. 1992. Developmental and light regulation of *eas*, the structural gene of the rodlet protein of *Neurospora*. Genes Dev. 6:2373-2381.

LeBrun, M. H., Dutfoy, F., Gaudemer, F., Kunesch, G., and Gaudemer, A. 1990. Detection and quantification of the fungal phytotoxin tenuazonic acid produced by *Pyricularia oryzae*. Phytochemistry 29:3777-3783.

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. 115:71-76.

Sachs, M. S., and Yanofsky, C. 1991. Developmental analysis of mRNA levels for genes involved in conidiation and amino acid biosynthesis in *Neurospora crassa*. Dev. Biol. 148:117-128.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

St. Leger, R. J., Staples, R. C., and Roberts, D. W. 1992. Cloning and regulatory analysis of starvation-stress gene, ssgA, encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae*. Gene 120:119-124.

Statistical Analysis System. 1987. SAS User's Guide: Statistics. 5th ed. SAS Institute, Cary, NC.

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 5:1575-90.

Wessels, J. G. H. 1994. Developmental regulation of fungal cell wall formation. Annu. Rev. Phytopathol. 32:413-437.

Wessels, J. G. H., de Vries, O. M. H., Asgeirdottir, S. A., and Shuren, F. H. J. 1991. Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in *Schizophyllum*. Plant Cell 3:793-799.

Wösten, H. A. B., Asgeirsdottir, S. A., Krook, J. H., Drenth, J. H. H., and Wessels, J. G. H. 1994. The fungal hydrophbin Sc3p self assembles at the surface of aerial hyphae as a protein membrane constituting the hydrophobin rodlet layer. Eur. J. Cell Biol. 63:122-129.

Wösten, H. A. B., Schuren, F. H. J., and Wessels, J. G. H. 1994. Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces. EMBO J. 13:5848-5854.

Wynn, W. K. 1976. Appressorium formation over stomates by the bean rust fungus: Response to a surface contact stimulus. Phytopathology 66:136-146.

Xiao, J., Ohshima, A., Kamakura, T., Ishiyama, T., and Yamaguchi, I. 1994a. Extracellular glycoprotein(s) associated with cellular differentiation in *Magnaporthe grisea*. Mol. Plant-Microbe Interact. 5:639-644.

Xiao, J. Z., Watanabe, T. Kamakura, T., Ohshima, A., and Yamaguchi, I. 1994b. Studies on cellular differentiation of *Magnaporthe grisea*. Physico-chemical aspects of substratum surfaces in relation to appressorium formation. Physiol. Mol. Plant Pathol. 44:227-236.