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Complementation of the Mpg1 mutant phenotype in *Magnaporthe grisea* reveals functional relationships between fungal hydrophobins

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The functional relationship between fungal hydrophobins was studied by complementation analysis of an *mpg1*$^-$ gene disruption mutant in *Magnaporthe grisea*. *MPG1* encodes a hydrophobin required for full pathogenicity of the fungus, efficient elaboration of its infection structures and conidial rodlet protein production. Seven heterologous hydrophobin genes were selected which play distinct roles in conidiogenesis, fruit body development, aerial hyphae formation and infection structure elaboration in diverse fungal species. Each hydrophobin was introduced into an *mpg1*$^-$ mutant by transformation. Only one hydrophobin gene, *SC1* from *Schizophyllum commune*, was able partially to complement *mpg1*$^-$ mutant phenotypes when regulated by its own promoter. In contrast, six of the transformants expressing hydrophobin genes controlled by the *MPG1* promoter (*SC1* and *SC4* from *S.commune*, *rodA* and *dewA* from *Aspergillus nidulans*, *EAS* from *Neurospora crassa* and *ssgA* from *Metarhizium anisopliae*) could partially complement each of the diverse functions of *MPG1*. Complementation was always associated with partial restoration of a rodlet protein layer, characteristic of the particular hydrophobin being expressed, and with hydrophobin surface assembly during infection structure formation. This provides the first genetic evidence that diverse hydrophobin-encoding genes encode functionally related proteins and suggests that, although very diverse in amino acid sequence, the hydrophobins constitute a closely related group of morphogenetic proteins.

Keywords: appressorium development/conidiation/fungal pathogenicity/hydrophobin/rodlet layer

Introduction

Fungal hydrophobins are small secreted, hydrophobic proteins which are fundamental to the developmental biology of fungi. More than 20 hydrophobin-encoding genes have now been recognized and they may prove to be ubiquitous in filamentous fungi (Wessels, 1997). In most cases, hydrophobin genes have been identified as mRNAs abundantly transcribed during particular developmental processes such as sporulation, fruit body formation or fungal infection of plants and animals. Genetic studies have now implicated hydrophobins in all of these morphogenetic processes.

Hydrophobin genes have been mutated using targeted gene disruption and cause significant effects on development. In *Aspergillus nidulans* and *Neurospora crassa*, for example, null alleles at the *rodA* and *EAS* genes respectively cause spores to lose their surface hydrophobicity and clump together (Stringer *et al.*, 1991; Bell-Pederson *et al.*, 1992; Lauter *et al.*, 1992). This 'easily wettable' phenotype is due to the absence of a hydrophobin-encoded protein layer on the surface of conidia (Templeton *et al.*, 1995). In the mushroom *Schizophyllum commune*, four hydrophobin genes have been described—*SC1*, *SC3*, *SC4* and *SC6* (Wessels *et al.*, 1991; Wessels, 1997)—and are differentially expressed during development of fruit bodies. SC3, a hydrophobin which is produced during hyphal growth, is required for production of upwardly projecting aerial hyphae as *sc3*$^-$ gene disruption mutants are unable to form hydrophobic aerial structures (van Wetter *et al.*, 1996).

SC3 was the first hydrophobin to be purified, and this showed that hydrophobins can respond to interfaces between water and air, or between water and solid surfaces (Wösten *et al.*, 1993, 1994b, 1995). Exposure to such an interface caused spontaneous aggregation of SC3 monomers resulting in an amphipathic protein polymer. The hydrophobic side of aggregated SC3 hydrophobin possessed a rodlet architecture identical to that found on aerial hyphae of *S.commune* (Wösten *et al.*, 1993), suggesting that SC3 is secreted from fungal cells and forms the hydrophobic coating of hyphae after interfacial self-assembly (Wösten *et al.*, 1994a). Significantly, SC3 self-assembly also occurred in response to hydrophobic surfaces, and *sc3*$^-$ mutants were deficient in their attachment to hydrophobic substrates (Wösten *et al.*, 1994b, 1995).

In addition to development, hydrophobins play roles in fungal pathogenicity. Targeted gene replacement of *MPG1* in the rice blast fungus *Magnaporthe grisea* produced mutants with reduced pathogenicity (Talbot *et al*., 1993). This was correlated with a reduced ability to form infection structures called appressoria. *Magnaporthe grisea* appressoria are dome-shaped cells produced from the tips of fungal hyphae in response to the hydrophobic rice leaf surface (Hamer *et al.*, 1988; Talbot, 1995). Their role is to generate turgor and mechanically breach the rice cuticle (Howard and Valent, 1996; de Jong *et al*., 1997). Purification of MPG1 indicated that hydrophobin self-assembly occurs on the rice leaf surface and the resulting amphipathic hydrophobin layer then acts as a conformational cue for appressorium development (Talbot *et al.*, 1996). However, *MPG1* also encodes a spore wall rodlet protein (like *EAS* and *rodA*) and is required for efficient production of conidia. In *M.grisea*, therefore, it appears that a

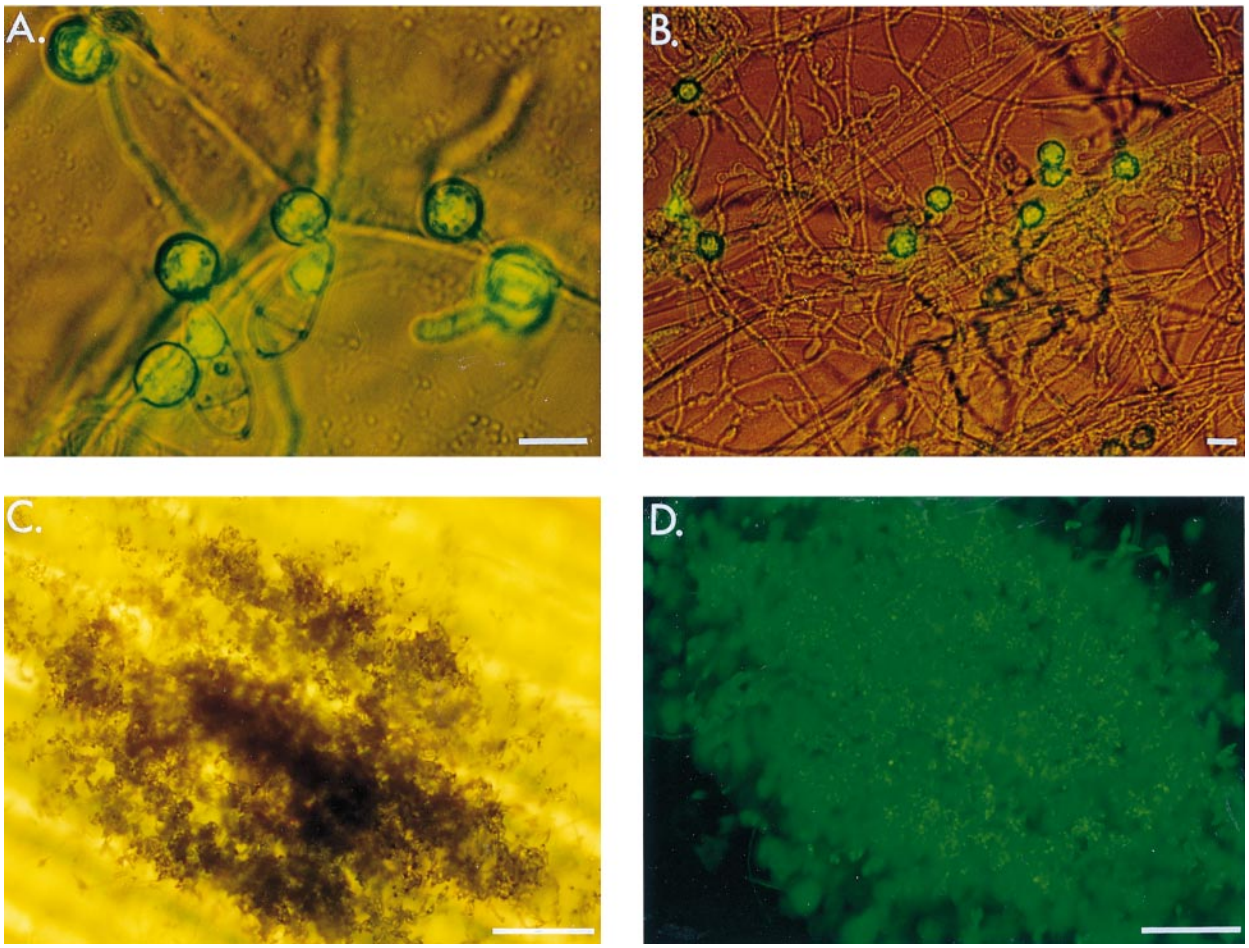

Fig. 1. Spatial regulation of *MPG1* expression during plant infection by *M.grisea*. Micrographs on the left are bright field images viewed with Hoffman modulation optics (Nikon). GFP fluorescence is shown on the right. Conidia of an *MPG1(p)::sGFP::Hph* transformant were incubated on sterile onion epidermis, and allowed to form appressoria and penetrate the epidermis. (**A**) Appressoria and penetration pegs after 24 h. (**B**) Infection hyphae after 48 h. Bar = 10 μm. (**C** and **D**) Aerially borne conidia from an *MPG1(p)::sGFP::Hph* transformant emerging from a rice blast disease lesion, 96 h after plant inoculation. Bar = 120 μm

hydrophobin functions during both conidiogenesis and appressorium development.

The diversity of hydrophobins described so far suggests that these morphogenetic proteins serve a large number of specific functions in filamentous fungi (Talbot, 1997; Wessels, 1997). Hydrophobins are indeed very diverse proteins, as the 20 hydrophobins described to date share only 4.3% amino acid identity (Wessels, 1997). They do, however, share a common hydropathy profile, and all hydrophobins have eight cysteine residues characteristically spaced in their amino acid sequences.

Two possibilities exist regarding the functional relatedness of hydrophobins. The different functions of hydrophobins may, for example, simply reflect the particular developmental stage at which they are produced. In this case, any hydrophobin gene would be able to complement the mutant phenotype of another, as long as it is expressed at the appropriate developmental stage. Conversely, it may be that the considerable divergence in hydrophobin sequence reflects real functional diversity. If this is the case, then complementation of hydrophobin mutant phenotypes would perhaps be restricted to only hydrophobins fulfilling the same function in different fungi. We decided to address these questions by complementation of the *mpg1*$^-$ mutant phenotypes in the rice blast fungus *M.grisea*.

In this report, we describe the introduction of seven hydrophobin-encoding genes into *M.grisea mpg1*$^-$ mutants. We show that six of the seven hydrophobins we tested could substitute, at least partially, for *MPG1* during fungal pathogenicity when expressed as *MPG1* promoter fusions. Complementation of *MPG1* mutant phenotypes consistently was associated with formation of a rodlet protein layer, indicating that hydrophobin self-assembly is fundamental to the function of *MPG1*. This provides the first evidence that hydrophobins are functionally related and suggests that although very diverse in amino acid sequence, they form a closely related group of morphogenetic proteins.

Results

In order to test functional relationships between the fungal hydrophobins, we selected diverse heterologous hydrophobin-encoding genes and introduced these individually into an *M.grisea mpg1*$^-$ null mutant. Complementation experiments were designed such that each hydrophobin-encoding gene was expressed in *M.grisea*,

Table I. Fungal hydrophobins used in this study

Name	Taxonomic class	Organism	Mutant phenotype determined	Biological function	Reference
MPG1	Ascomycetes	*Magnaporthe grisea*	+	Conidial spore wall protein. Involved in conidium and appressorium formation. Required for full pathogenicity	Talbot *et al*. (1993, 1996)
SC1[a]	Basidiomycetes	*Schizophyllum commune*	–	Unknown. Expressed in dikaryotic fruiting body	Schuren and Wessels (1990); Wessels *et al*. (1991)
SC3[a]	Basidiomycetes	*Schizophyllum commune*	+	Involved in aerial hyphae formation and able to attach to hydrophobic surfaces	Wösten *et al*. (1994); Van Wetter *et al*. (1996)
SC4[a]	Basidiomycetes	*Schizophyllum commune*	–	Lines gas channels in dikaryotic fruiting body	Schuren and Wessels (1990); Wessels *et al*. (1991); Wessels (1997)
ssgA[b]	Ascomycetes	*Metarhizum anisopliae*	–	Unknown. Expressed during appressorium development	St. Leger *et al*. (1992)
rodA[c]	Ascomycetes	*Aspergillus nidulans*	+	Conidial spore wall protein	Stringer *et al*. (1991)
dewA[c]	Ascomycetes	*Aspergillus nidulans*	+	Conidial spore wall protein	Stringer and Timberlake (1995)
EAS[d]	Ascomycetes	*Neurospora crassa*	+	Conidial spore wall protein	Bell-Pederson *et al*. (1992); Lauter *et al*. (1992)

Hydrophobin-encoding genes were obtained from: [a]Dr Frank Schuren (University of Groningen); [b]Dr Ray St. Leger (Cornell University); [c]Dr W.E.Timberlake (University of Georgia); [d]Drs Deborah Bell-Pederson and Jay Dunlap (Dartmouth Medical School).
All the hydrophobins belonged to class I.

either under the control of its own endogenous promoter or as a gene fusion to the *MPG1* promoter. In this way, the relative contribution of the regulated expression of *MPG1*, or the characteristics of its hydrophobin gene product to biological function, could be assessed.

Identification of a functional MPG1 promoter

A functional *MPG1* promoter initially was isolated for expression of alternative hydrophobin genes and to establish the precise pattern of expression of *MPG1* in relation to its known functions (Talbot *et al*., 1993, 1996). A 1.28 kb promoter fragment from the 5′ end of the *MPG1* gene was isolated and fused to a synthetic allele of the *GFP* reporter gene, encoding green fluorescent protein (Prasher *et al.*, 1992; Stearns, 1995; Chiu *et al*., 1996), providing a non-invasive reporter of *MPG1* expression. The *sGFP* gene was subcloned in-frame with the translation initiation codon at the 3′ end of the *MPG1* promoter and introduced into an *M.grisea* transformation vector conferring hygromycin B resistance. In a control experiment, *sGFP* was fused to the constitutively expressed *A.nidulans gpd* promoter and transformed into a wild-type *M.grisea* strain, Guy-11 (see Materials and methods). Transformants were selected and analysed by Southern blot to ensure that they contained single insertions. A total of eight *M.grisea* transformants containing single copy integrations of the plasmids were selected for further analysis, four containing the *MPG1(p)::sGFP::Hph* construct, and four the *gpd(p)::sGFP::hph* construct.

Conidia from *MPG1(p)::sGFP::Hph* transformants were incubated on rice leaves, or sterilized onion epidermis, for 24 h and infection structures were allowed to form. *GFP* expression could be observed clearly in appressoria of all *MPG1(p)::sGFP::Hph* transformants, but fluorescence did not migrate into infection pegs or hyphae which entered the plant epidermis (Figure 1A and B). This strongly indicates that *MPG1* expression is restricted to early infection-related development, prior to plant penetration. Rice leaf infections were incubated for a further 72 h in conditions of high humidity, allowing disease lesions to form and produce aerially borne conidia (Figure 1C). Conidia from *MPG1(p)::sGFP::Hph* showed extensive GFP fluorescence, indicating that high level *MPG1* expression also occurs during conidiogenesis (Figure 1D). Very little variation was observed between the four *MPG1(p)::sGFP::Hph* transformants examined. In control experiments, *gpd(p)::sGFP::Hph* showed GFP fluorescence at all stages of development (not shown).

Expression of diverse hydrophobin-encoding genes in an mpg1::Hph null mutant of M.grisea

Having isolated a functional *MPG1* promoter, seven alternative hydrophobin-encoding genes were selected for complementation tests. These were *SC1*, *SC3* and *SC4* from *S.commune*, the *rodA* and *dewA* genes from *A.nidulans*, *EAS* from *N.crassa* and the *ssgA* from *Metarhizium anisopliae*. Heterologous hydrophobin genes were selected to encompass the diversity of functions so far attributed to hydrophobins: SC1 and SC4 represent dikaryon-specific fruit body hydrophobins; rodA, dewA and EAS are all spore wall rodlet proteins; and ssgA has been implicated in appressorium development by the insect pathogen *M.anisopliae*, suggesting a function similar to MPG1. The origins and functions of all hydrophobins used in this study are given in Table I.

Genomic clones of *SC1*, *SC3*, *SC4*, *rodA*, *dewA* and *EAS* were selected containing all necessary components for expression in the corresponding native fungus (see Table II). Each genomic fragment was subcloned to an *M.grisea* transformation vector containing a selective marker conferring resistance to either bleomycin or bialophos (Table II). The resulting plasmids were then transformed into the *mpg1::Hph* gene replacement mutant 53-R-39 (Talbot *et al.*, 1996). Transformants were analysed by Southern blot to identify transformants carrying single-copy integrations of each hydrophobin gene (data not shown), and two recombinant transformants expressing each heterologous hydrophobin were selected for phenotypic analysis (Table II).

At the same time, genomic clones of *SC1*, *SC3*, *SC4*, *rodA*, *dewA* and *EAS*, and a cDNA clone of *ssgA* were selected for construction of translational fusions with the

Table II. Hydrophobin expression vectors generated in this study and corresponding *M.grisea* transformants

Hydrophobin gene	Promoter	Selectable marker	Recombinant construct	Recombinant strains	Genotype
SC1	SC1	bleomycin[a]	pNJT7	MJK7.8 MJK7.16	*mpg1::Hph;SC1(p)::SC1::ble*
SC3	SC3	bleomycin	pS330	SSC3.1 SSC3.3	*mpg1::Hph;SC3(p)::SC3 ::ble*
SC4	SC4	bleomycin	pMJK58	SSC4.5 SSC4.8	*mpg1::Hph;SC4(p)::SC4 ::ble*
rodA	rodA	bialophos[b]	pMJK20	ARODA.24 ARODA.29	*mpg1::Hph;rodA(p)::rodA::bar*
dewA	dewA	bleomycin	pNJT26	ADEWA. ADEWA.	*mpg1::Hph;dewA(p)::dewA::ble*
EAS	EAS	bleomycin	pTON5	NEAS. 5 NEAS.25	*mpg1::Hph;EAS(p)::EAS::ble*
SC1	MPG1[c]	bialophos	pNJT7	MSC1.2 MSC1.7	*mpg1::Hph;MPG1(p)::SC1::bar*
SC3	MPG1[c]	bialophos	pMJK85	MSC3.10 MSC3.13	*mpg1::Hph;MPG1(p)::SC3::bar*
SC4	MPG1[c]	bialophos	pMJK79	MSC4.3 MSC4.10	*mpg1::Hph;MPG1(p)::SC4::bar*
ssgA	MPG1[c]	bialophos	pMJK64	MSSGA.5 MSSGA.14	*mpg1::Hph;MPG1(p)::ssgA::bar*
rodA	MPG1[c]	bialophos	pMJK10	MRODA.4 MRODA.5	*mpg1::Hph;MPG1(p)::rodA::bar*
dewA	MPG1[c]	bialophos	pMJK146	MDEWA. 12 MDEWA.15	*mpg1::Hph;MPG1(p)::dewA::bar*
EAS	MPG1[c]	bialophos	pMJK55	MEAS.9 MEAS.13	*mpg1::Hph;MPG1(p)::EAS::bar*
MPG1	MPG1	bleomycin	pMJK26	MJK.13[d] MJK.16	*mpg1:Hph; MPG1(p)::MPG1::ble*

[a]Bleomycin resistance gene, *ble*, from pAN8-1 (Punt *et al.*, 1987).
[b]Bialophos resistance gene, *bar*, from pCB1265 (Sweigard *et al.*, 1996).
[c]All expressed as translational fusions under the control of a 1.2 kb *Pst*I–*Nco*I *MPG1* promoter fragment from pNJT190 (see Materials and methods).
[d]Transformant described previously by Talbot *et al.* (1996).

MPG1 promoter fragment. Each hydrophobin gene was subcloned in-frame to the *MPG1* promoter fragment (see Materials and methods) and introduced into the *M.grisea* transformation vector pCB1265 conferring bialophos resistance (Sweigard *et al.*, 1997). Bialophos-resistant transformants were selected and Southern blotted to identify those with single-copy integrations of each *MPG1* promoter gene fusion (not shown). Two transformants carrying single-copy integrations for each of the seven *MPG1* promoter–hydrophobin fusions were then selected for phenotypic analysis. Details of the 26 recombinant strains generated in this study are given in Table II.

RNA gel blot analysis was carried out to ensure *MPG1*-like patterns of expression in each gene fusion transformant. Starvation stress is known to induce *MPG1* expression (Talbot *et al*., 1993; Beckerman and Ebbole, 1996: Lau and Hamer, 1996) and, therefore, transformants were exposed to starvation stress by transfer of fungal mycelium to medium lacking either a nitrogen or carbon source for 12 h of growth. At this time, RNA was extracted, fractionated by gel electrophoresis, blotted and probed with the corresponding hydrophobin gene for each transformant. An example, showing expression of the 846 bp *rodA* transcript of transformant ARODA.24 during nitrogen and carbon source starvation, is shown in Figure 2. All transformants containing *MPG1* promoter fusions were tested in this way with identical results.

Expression of heterologous hydrophobin genes regulated by their own promoters was not detected by RNA gel blot analysis (using total RNA) in transformants either during infection-related development or during starvation stress (data not shown).

Complementation of the conidiation-deficient phenotype of an mpg1::Hph deletion mutant by heterologous hydrophobin-encoding genes

Conidiation is severely reduced in *mpg1::Hph* deletion mutants, and conidiating cultures have an 'easily wettable' phenotype (Talbot *et al.*, 1993). The easily wettable phenotype is due to loss of the *MPG1*-encoded rodlet protein layer found on the surface of *M.grisea* conidia (Talbot *et al.*, 1996). This also reduces the efficiency of conidiogenesis, allowing only a single conidium to differentiate from each conidiophore, in contrast to conidiophores of isogenic wild-type strains of *M.grisea* which produce 4–5 conidia in a sympodial array (Ou, 1985; Talbot *et al*., 1996). Deletion of *MPG1* also causes a reduction in pathogenicity, resulting in ~80% reduction in the usual number of disease lesions on infected rice seedlings (Talbot *et al.*, 1993, 1996). This is associated with reduction in the ability of *mpg1::Hph* mutants to elaborate appressoria (Talbot *et al.*, 1993, 1996). Recombinant transformants expressing each hydrophobin gene were therefore examined to determine their ability to substitute for *MPG1* in each of its attributed functions. As a control in these experiments, two transformants were also analysed routinely where *MPG1* had been reintroduced into an *mpg1*$^-$ mutant (Table II). We previously

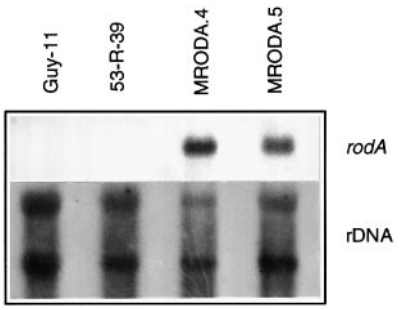

Fig. 2. *MPG1* promoter-regulated expression of *A.nidulans rodA* in an *M.grisea mpg1*$^-$ mutant. Total RNA was extracted from cultures of *M.grisea* which had been subjected to nitrogen starvation stress for 18 h and was then fractionated by gel electrophoresis, blotted and probed with a 4.3 kb *Xho*I–*Pst*I fragment of the *rodA* genomic clone, pMS10 (Stringer *et al*., 1991). A loading control hybridization was carried out with an *M.grisea* rDNA probe, pMG1. Lanes contain total RNA from wild-type strain Guy-11; *mpg1::Hph* mutant, 53-R-39; *MPG1(p)::rodA:: bar* transformant, MRODA.4; *MPG1(p)::rodA:: bar* transformant MRODA.5.

reported full complementation of all *mpg1*$^-$ mutant phenotypes in these transformants (Talbot *et al.*, 1996), which are phenotypically indistinguishable.

Conidiogenesis was assessed by taking plate cultures of *M.grisea* grown for a uniform time, flooding them with water and gently removing conidia with a glass rod. When transformants expressing hydrophobins under control of their own promoters were analysed, the only transformants to show partial complementation of conidial numbers were those expressing the *S.commune* hydrophobin, *SC1* (Figure 3A). Two transformants, MJK7.8 and MJK7.16 [*SC1(p)::SC1::ble*], produced mean conidial numbers of 9.5×10^5/ml compared with the wild-type strain Guy-11, which produced a mean of 8×10^6 conidia/ml, and the isogenic *mpg1::Hph* mutant 53-R-39 which produced a mean of 3×10^5 conidia/ml (Figure 3A). Both of the *SC1(p)::SC1::ble* transformants were also complemented for the easily wettable phenotype, being able to support a droplet of water on the culture surface (Figure 3C).

Transformants containing *MPG1* promoter gene fusions produced considerably greater numbers of conidia compared with the isogenic *mpg1*$^-$ mutant 53-R-39 (Figure 3B). The highest conidial numbers were found in recombinants expressing *MPG1(p)::EAS*, *MPG1(p)::dewA* and *MPG1(p)::SC1* gene fusions. Conidial numbers in these transformants were $>3\times10^6$/ml and therefore not significantly different from wild-type when compared by the Student's *t*-test ($P>0.05$). In contrast, *MPG1(p)::SC3::bar* transformants produced conidial numbers which were significantly different from wild-type ($t=35.1$; $P<0.001$; df = 5), but not significantly different from the isogenic *mpg1*$^-$ mutant ($t=0.4$; $P>0.05$; df = 5). Consistent with this, *MPG1(p)::SC3::bar* transformants were easily wettable while the remaining transformants were identical to wild-type *M.grisea* with respect to surface hydrophobicity (Figure 3C).

Introduction of heterologous hydrophobin-encoding genes restores pathogenicity of an mpg1::Hph deletion mutant

The ability of transformants to cause rice blast disease was determined by infecting rice seedlings of a susceptible rice cultivar CO-39. Conidial suspensions were prepared and adjusted to 1×10^4 conidia/ml. Fourteen-day-old rice seedlings were then sprayed with the suspension and the disease allowed to progress for 96 h. At this time, rice blast infections with the wild-type *M.grisea* strain Guy-11 produced large numbers of oval-shaped lesions on rice leaves. In *mpg1*$^-$ mutants, the frequency of lesions was between 10 and 20% of the number generated by an isogenic wild-type strain of *M.grisea* (Talbot *et al.*, 1993, 1996). To assess pathogenicity of transformants expressing alternative hydrophobins, the lesion number was recorded on 5 cm leaf sections as shown in Figure 4A and B. Pathogenicity was partially restored for *SC1(p)::SC1::ble* transformants, but none of the other transformants carrying hydrophobin genes expressed under endogenous promoters showed significant increases in lesion number compared with the *mpg1::Hph* mutant ($P>0.1$; df = 40) (Figure 4A and C). In contrast, six of the transformants expressing hydrophobin genes controlled by the *MPG1* promoter showed partial restoration of pathogenicity. The highest number of disease lesions was produced by *MPG1(p)::EAS* and *MPG1(p)::SC1* transformants which were not significantly different from lesion numbers generated by Guy-11 (Figure 4B and D). *MPG1(p)::SC3* transformants were not restored for pathogenicity and showed similar lesion numbers to the *mpg1::Hph* null mutant (Figure 4B). We conclude that *MPG1* promoter-driven expression of *dewA*, *rodA*, *SC1*, *SC4*, *EAS* and *ssgA* can restore the activity of *MPG1* in pathogenicity of *M.grisea*.

Restoration of appressorium development by expression of heterologous hydrophobins in an mpg1::Hph mutant

Reduced pathogenicity of *mpg1*$^-$ mutants is associated with a decrease in the ability of the fungus to elaborate appressoria (Talbot *et al.*, 1993). Transformants were therefore tested for their ability to develop appressoria on hydrophobic surfaces. Consistent with restoration of other Mpg1 mutant phenotypes, *SC1(p)::SC1::ble* transformants showed partial restoration of this ability (Figure 5A). None of the remaining transformants, however, produced significantly greater numbers of appressoria compared with the isogenic *mpg1::Hph* mutant. Transformants expressing hydrophobin genes under control of the *MPG1* promoter showed restoration of appressorium formation on hydrophobic surfaces, with the exception of *MPG1(p)::SC3* transformants. These produced numbers of appressoria which were not significantly different from the *mpg1*$^-$ mutant, 53-R-39 ($t=2.96$; $P>0.04$; df = 5). Appressoria were produced in the greatest numbers by *MPG1(p)::EAS*, *MPG1(p)::SC1* and *MPG1(p)::dewA* transformants (Figure 5B). Appressorium production was therefore restored to near wild-type levels by expression of *EAS*, *SC1* and *dewA* under control of the *MPG1* promoter, and partially restored by expression of *SC4*, *ssgA* and *rodA*.

A control experiment was carried out to determine whether the variation in appressorium formation was associated with introduction of the heterologous hydrophobins. Because transformation in *M.grisea* is integrative, it has the potential to cause mutations and, therefore, a control experiment was necessary to test whether appressorium deficiency was due simply to the insertion of plasmid DNA. Appressorium development in *M.grisea* can be

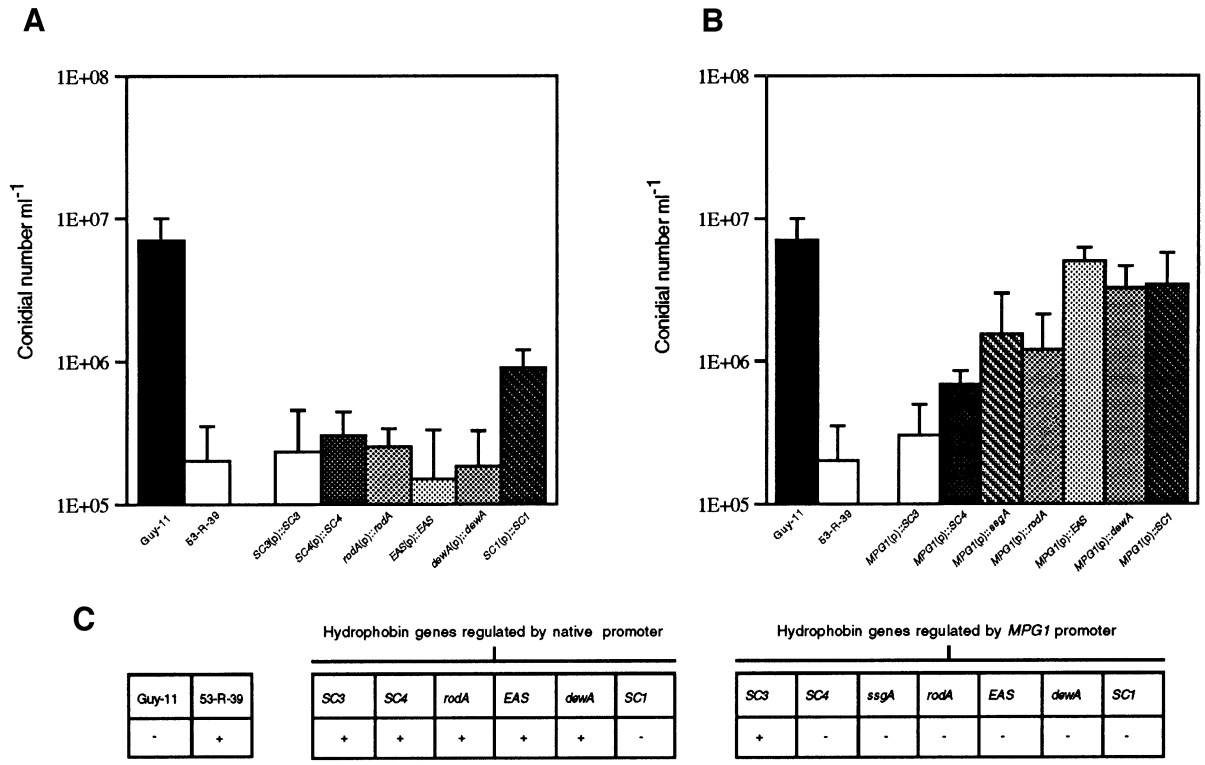

Fig. 3. Bar graph of numbers of conidia produced from cultures of an *mpg1*⁻ mutant 53-R-39 transformed with heterologous hydrophobin-encoding genes. Conidia were collected by flooding plate cultures and gently scraping aerial mycelium with a glass rod. Suspensions were filtered through cheesecloth to remove mycelial debris and counted. (**A**) Conidial numbers from *mpg1*⁻ transformants expressing hydrophobins regulated by their native promoters. (**B**) Conidial numbers from *mpg1*⁻ transformants expressing hydrophobins regulated by MPG1(p). Error bars indicate standard error of the mean ($n = 5$). (**C**) Wettability of transformant cultures assessed by their ability to support a 10 ml drop of water on the surface of conidiating cultures. (+ indicates wild-type, – indicates an easily wettable water-soaked phenotype).

induced by exogenous application of cAMP (Lee and Dean, 1993) and, previously, we and others showed that *MPG1* acts upstream of the cAMP signalling pathway as appressorium deficiency in *mpg1*⁻ mutants can be restored by exogenous cAMP (Beckerman and Ebbole, 1996; Talbot *et al*., 1996). A 10 mM aliquot of cAMP was therefore added to germinating conidia of all hydrophobin transformants. Under these conditions, all transformants generated in this study were able to form appressoria (data not shown).

Complementation of mpg1⁻ by heterologous hydrophobins is correlated with production of a rodlet protein

Rodlet proteins are well known ultrastructural characteristics of aerial structures of fungi and have been shown to be the products of hydrophobins by purification and peptide sequencing (Wösten *et al.*, 1993; Templeton *et al.*, 1995), immunolocalization (Wösten *et al.*, 1994a) or by their absence in hydrophobin null mutants (Stringer *et al.*, 1991; Bell-Pederson *et al.*, 1992; Talbot *et al.*, 1996). *MPG1* encodes a rodlet protein composed of 5–7 nm interwoven rodlets which coats the conidial cell wall (Talbot *et al.*, 1996), as shown in Figure 6. This rodlet layer is completely absent from *mpg1::Hph* mutants (Figure 6D) and distinct from the *rodA*-encoded rodlets found on *A.nidulans* conidia or *EAS*-encoded rodlets of *N.crassa* (Figure 6A and B). In both cases, the size of rodlets is greater than those of *M.grisea* and the arrangement is characteristic of each species.

When heterologous hydrophobins were expressed in *M.grisea* under control of their own promoters, a rodlet layer was only observed on conidia of *SC1(p)::SC1::ble* transformants (Figure 6E). In the remaining 10 transformants examined, the surfaces of conidia were indistinguishable from those of 53-R-39 (Figure 6D).

Expression of hydrophobin genes under control of the *MPG1* promoter led to partial restoration of a conidial rodlet layer for six of the seven hydrophobins tested (Figure 6G–L). In general, the conidial rodlet layers of transformants were indistinct compared with those of wild-type *M.grisea*, but it was possible to observe considerable variation in the size and arrangement of rodlets (Table III). Rodlets encoded by *dewA*, *SC1*, *SC4* and *ssgA* have not been described previously, and so characteristics of the layers observed on *M.grisea* conidia cannot be compared directly with rodlet layers in the host fungus. *EAS*-encoded rodlets and *rodA*-encoded rodlets, however, did appear to adopt broadly similar conformations in *M.grisea* transformants when compared with *N.crassa* and *A.nidulans*, respectively, although the rodlet layers were less distinct. We conclude that heterologous expression of hydrophobins in an *M.grisea mpg1*⁻ mutant can result in partial restoration of a rodlet layer.

Complemented mpg1::Hph mutants exhibit hydrophobin-mediated self-assembly during appressorium formation

The *MPG1* hydrophobin previously has been proposed to act as a developmental sensor of hydrophobic surfaces

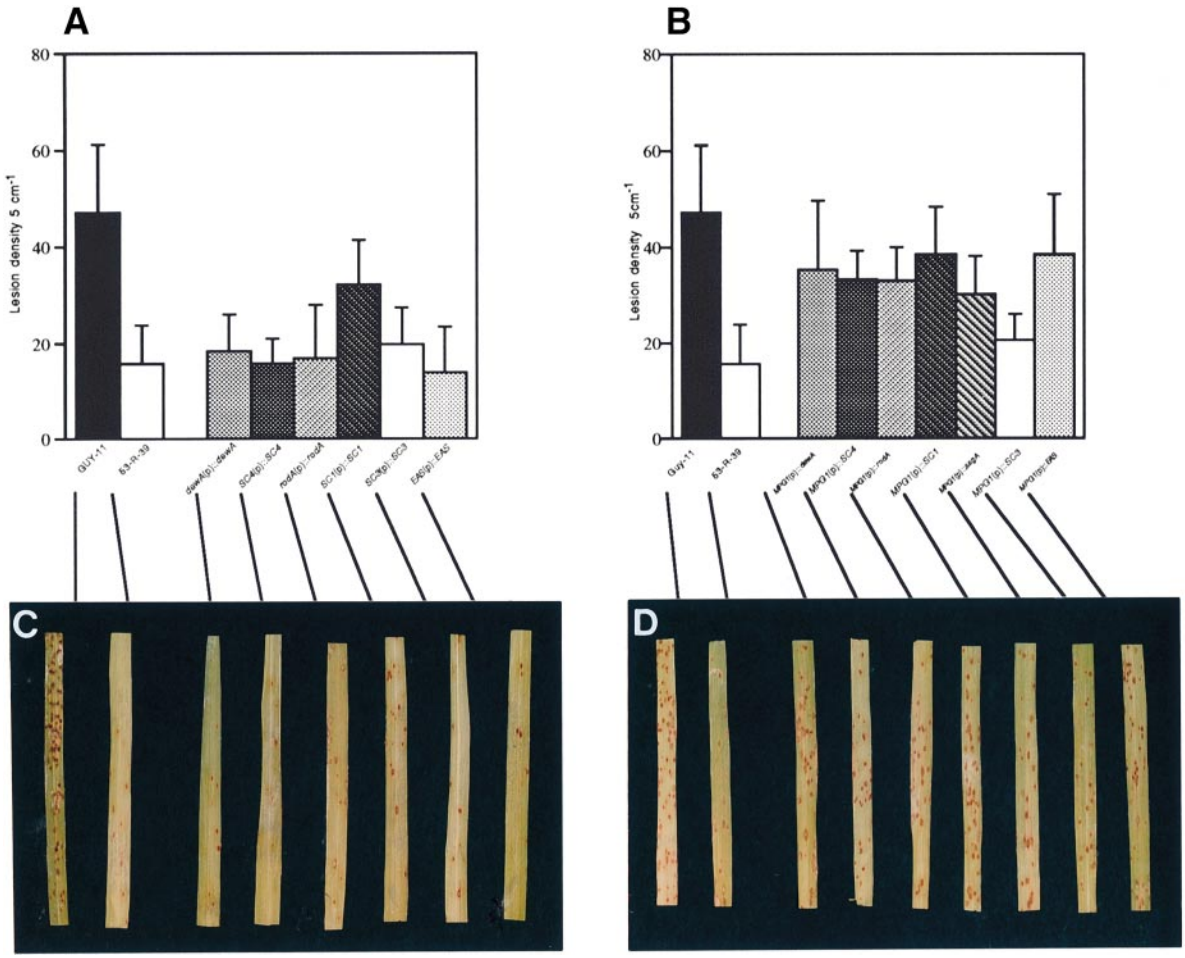

Fig. 4. Pathogenicity of *mpg1*⁻ mutant 53-R-39 transformed with heterologous hydrophobin-encoding genes. Fourteen-day-old rice seedlings of susceptible cultivar CO-39 were inoculated with conidial suspensions of *M.grisea* and allowed to develop rice blast symptoms for 4 days. (**A**) Bar graph of mean rice blast lesion density per 5 cm leaf tip from *mpg1*⁻ transformants expressing hydrophobins regulated by their native promoters. (**B**) Bar graph of mean rice blast lesion density per 5 cm leaf tip from *mpg1*⁻ transformants expressing hydrophobins regulated by *MPG1(p).* Error bar indicates standard error of the mean ($n = 40$). (**C** and **D**) Photographs of leaves from plants inoculated with each transformant indicated.

(Beckerman and Ebbole, 1996; Talbot *et al.*, 1996). Evidence for this role was based on assays of appressoria and germ tube adhesion to Teflon coverslips following washes in 60% ethanol and boiling in 2% SDS (Talbot *et al.*, 1996). Because assembled hydrophobins are insoluble in ethanol and hot SDS, while most proteins and carbohydrates are not, these washes remove fungal structures unless attached to the surface in a hydrophobin-dependent manner. In such an assay, wild-type *M.grisea* appressoria and germ tubes remain attached to hydrophobic surface while those of *mpg1*⁻ mutants do not (Talbot *et al.*, 1996), consistent with MPG1-mediated attachment.

In this study, hydrophobin adhesion assays were carried out for each hydrophobin transformant to determine whether remediation of appressorium deficiency and pathogenicity was due to surface self-assembly of the heterologous hydrophobin. Germ tubes and appressoria of complemented *MPG1(p)::EAS*, *MPG1(p)::dewA* and *MPG1(p)::SC1* transformants showed wild-type levels of attachment to the hydrophobic surface (Figure 7). However, germ tubes and appressoria of the non-complemented *MPG1(p)::SC3* were unable to attach strongly to the surface after treatment with ethanol and hot SDS. Taken together, these observations confirm that efficient formation of appressoria in *M.grisea* requires surface self-assembly of *MPG1*, and show that this function may be partially restored by correct expression of heterologous hydrophobins such as *EAS*, *dewA* and *SC1*.

Discussion

In this study, we have demonstrated that hydrophobins constitute a functional group of proteins and that diverse hydrophobins can partially complement *mpg1*⁻ mutant phenotypes if expressed at the appropriate developmental stage.

Our aim in these experiments was to establish whether regulated expression of a hydrophobin gene, or the particular characteristics of its encoded hydrophobin, were of greatest significance in determining its biological function. As only one hydrophobin gene, *SC1* from the basidiomycete *S.commune*, was able to complement *mpg1*⁻ when expressed under the control of its endogenous promoter, it would appear that regulated expression plays a role in determining hydrophobin function. This is consistent with the lack of detectable levels of mRNA expression in these transformants. The significance of gene regulation in determining hydrophobin function could be tested directly

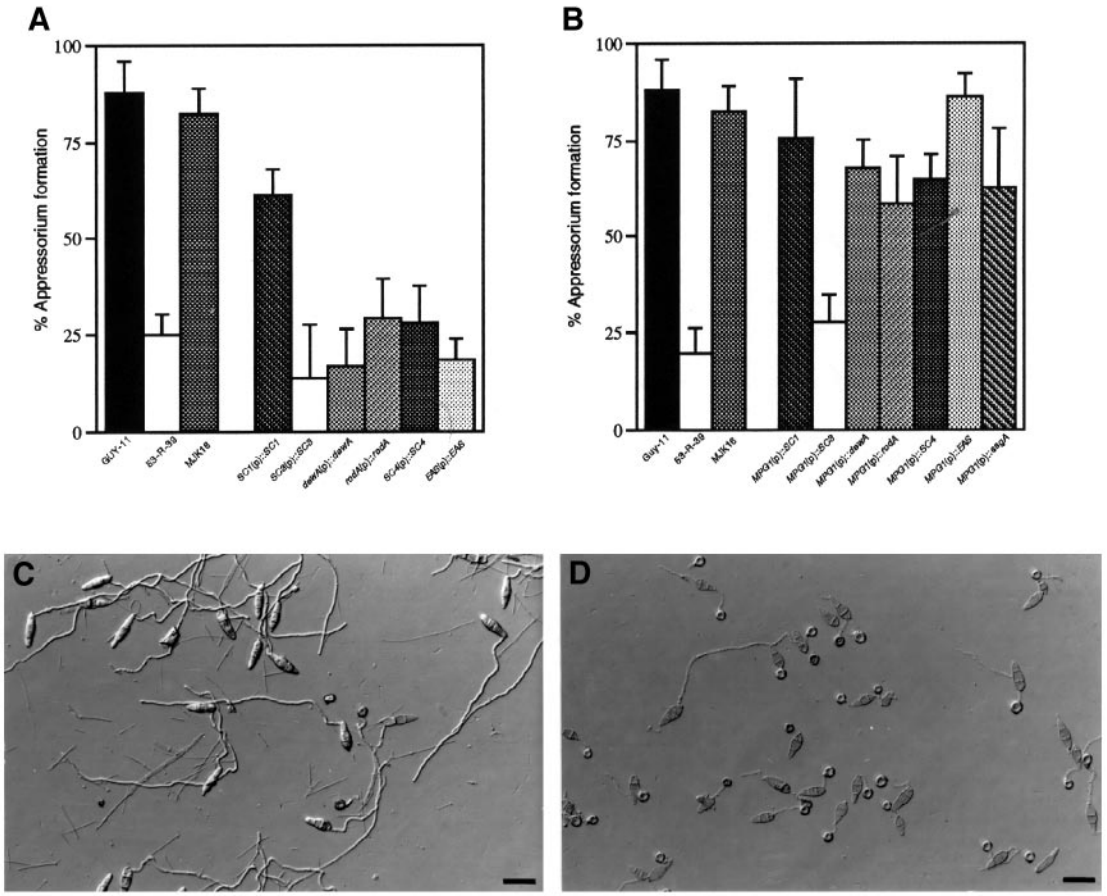

Fig. 5. Infection-related development in *mpg1*⁻ mutant 53-R-39 transformed with heterologous hydrophobin-encoding genes. Appressoria were allowed to form on hydrophobic Teflon membranes (DuPont) for 24 h. (**A**) Bar graph of mean appressorium formation by *mpg1*⁻ transformants expressing hydrophobins regulated by their native promoters. (**B**) Bar graph of mean appressorium formation by *mpg1*⁻ transformants expressing hydrophobins regulated by *MPG1(p).* MJK16 is an *mpg1*⁻ mutant complemented with MPG1 (Talbot *et al.*, 1996). The error bar indicates standard error of the mean ($n = 5$). (**C**) Appressorium development by *MPG1(p)::SC3* transformant, MSC3.10. (**D**) Appressorium development by *MPG1(p)::SC1* transformant, MSC1.2. Bar = 40 μm.

by expressing hydrophobin genes, including *MPG1*, under a constitutive *M.grisea* promoter. In this way, the effects of high level hydrophobin expression, in the absence of developmental changes in the fungus, could be investigated.

The failure of *EAS*, *dewA*, *rodA* and *ssgA* to complement *mpg1*⁻, when controlled by their native promoters, was somewhat surprising given that ascomycete promoters work well among related species (for a review, see Fincham, 1989) and that these hydrophobins are normally expressed during conidiation (Stringer *et al.*, 1991; Bell-Pederson *et al.*, 1992; Lauter *et al.*, 1992; Stringer and Timberlake, 1995) or appressorium development (St. Leger *et al.*, 1992).

The general lack of complementation by natively regulated hydrophobin genes highlighted the importance of specific regulation for the function of *MPG1* and the necessity for *MPG1* promoter fusions in any effective comparison of heterologous hydrophobins.

Functional relatedness of the fungal hydrophobins

Complementation analysis of *mpg1*⁻ with *MPG1* promoter-regulated hydrophobin genes suggests that hydrophobins are a closely related group of proteins in spite of their very considerable amino acid divergence. This indicates that conservation of eight cysteine residues and a similar distribution of hydrophobic and hydrophilic amino acids is sufficient for hydrophobin function, irrespective of low amino acid identity. Complementation tests did, however, define two functional groups of hydrophobins. Hydrophobin genes normally expressed in fruit bodies, conidia or infection structures, such as *rodA*, *dewA*, *EAS*, *ssgA*, *SC1* and *SC4* (Wessels, 1997), were all partially able to complement *mpg1*⁻ mutant phenotypes, whereas *SC3*, which is expressed in fungal mycelium and required for formation of aerial hyphae (van Wetter *et al.*, 1996) and surface attachment (Wösten *et al.*, 1994b), was unable to complement.

We cannot, at this stage, preclude that lack of complementation of *mpg1*⁻ by *SC3* was due to poor levels of secretion of the protein, perhaps due to the SC3 signal peptide being poorly recognized in *M.grisea*. Given the efficient expression and secretion of all other hydrophobins in *M.grisea* transformants, however, this seem unlikely. Interestingly, comparison of putative amino acid sequences for the seven hydrophobin genes does not reveal the putative functional relationship suggested by complementation tests. *SC3*, for example, is most homologous to the other *S.commune* hydrophobins *SC4* and *SC1* (Wessels, 1997), while *MPG1* shows greatest amino acid identity

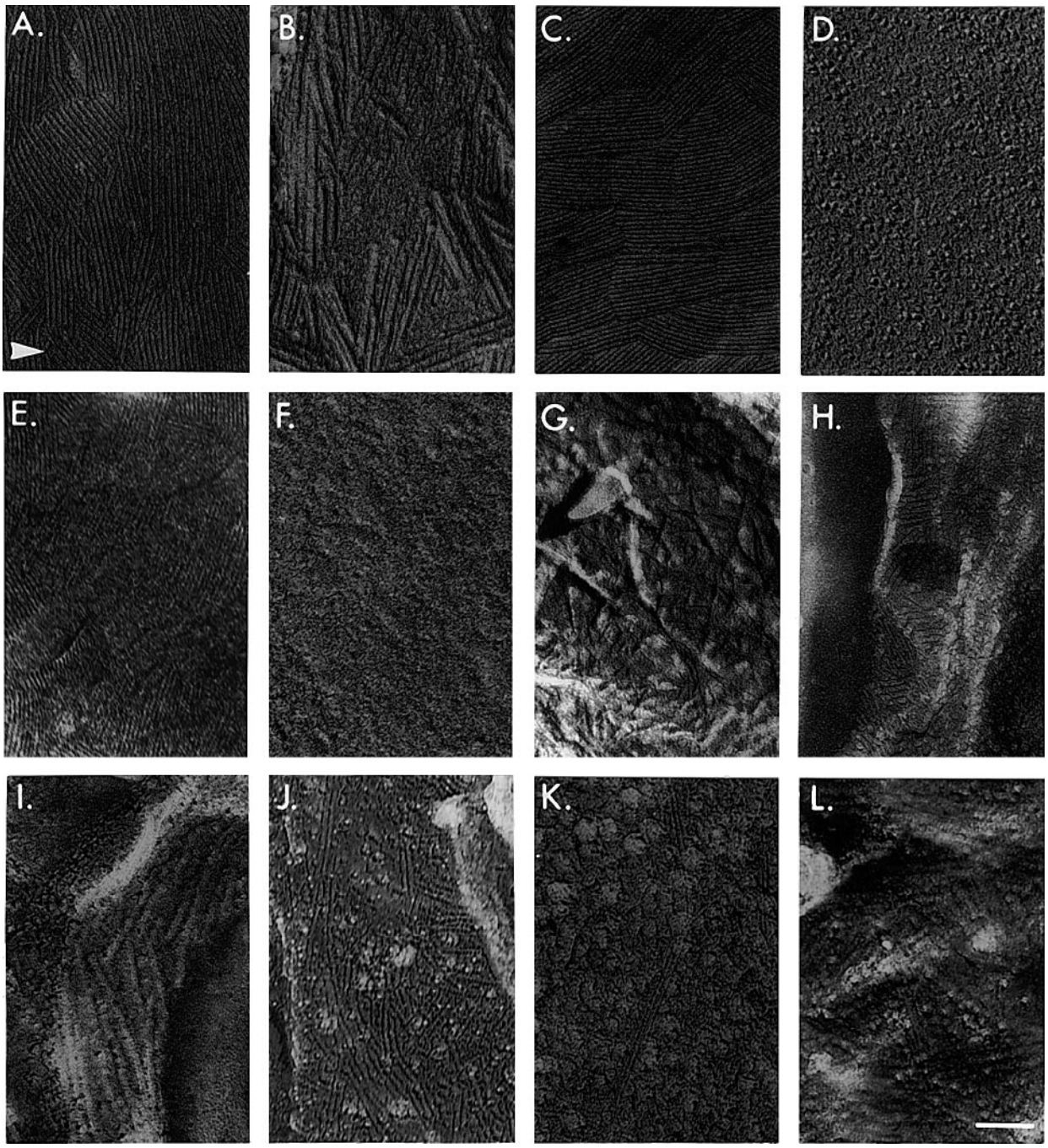

Fig. 6. Transmission electron microscopy of freeze-fractured conidium surfaces from the following strains: (**A**) *A.nidulans* RMSO11; (**B**) *N.crassa*; (**C**) *M.grisea* wild-type strain Guy-11; (**D**) *M.grisea mpg1*$^{-}$ mutant, 53-R-39; (**E**) *M.grisea SC1(p)::SC1::ble* transformant MJK7.8; (**F**) *MPG1(p)::SC3::bar* transformant MSC3.13; (**G**) *MPG1(p)::SC4::bar* transformant MSC4.3; (**H**) *MPG1(p)::SC1::bar* transformant MSC1.2; (**I**) *MPG1(p)::dewA::bar* transformant MDEWA.12; (**J**) *MPG1(p)::rodA::bar* transformant MRODA.4; (**K**) *MPG1(p)::ssgA::bar* transformant MSSGA.14; (**L**) *MPG1(p)::EAS::bar* transformant MEAS.9. The arrow in (A) indicates the direction of shadowing for all images shown. Bar = 100 nm.

with *ssgA* (Talbot *et al*., 1993). It may be worth speculating, therefore, that the function of a hydrophobin is determined mainly by its ability to link with the underlying fungal cell wall following self-assembly, perhaps mediated by carbohydrate interactions. This is consistent with lack of rodlet formation in *MPG1(p)::SC3* transformants. SC3 is known to be glycosylated (de Vocht *et al*., 1998), and other hydrophobins have sequence motifs consistent with N- and O-linked glycosylation (Stringer *et al*., 1991; Bell-Pederson *et al*., 1992). Correct glycosylation may, therefore, be essential for linkage to fungal cell walls and it may be that lack of complementation by *SC3* is due to incorrect glycosylation of the hydrophobin in *M.grisea*. This could also account for rodlet protein layers being only partially restored in heterologous hydrophobin transformants. A comparison of the glycosylation of purified hydrophobins is therefore likely to prove informative in comparison with the functional complementation data presented here.

Recently, two further hydrophobins expressed during

Table III. Characteristics of rodlet protein layers on *M.grisea* conidia in heterologous hydrophobin transformants

Transformant genotype	Heterologous hydrophobin expressed	Rodlet diameter[a]	Rodlet organization[b]	Surface coverage of rodlet layer[c]
mpg1::Hph	None	–	–	–
mpg1::Hph;SC1(p)::SC1::ble	SC1	5–7 nm	short, interwoven	25–50%
mpg1::Hph;SC3(p)::SC3 ::ble	SC3	–	–	–
mpg1::Hph;SC4(p)::SC4 ::ble	SC4	–	–	–
mpg1::Hph;rodA(p)::rodA::bar	rodA	–	–	–
mpg1::Hph;dewA(p)::dewA::ble	dewA	–	–	–
mpg1::Hph;EAS(p)::EAS::ble	EAS	–	–	–
mpg1::Hph;MPG1(p)::SC1::bar	SC1	5–7 nm	short, interwoven	25–50%
mpg1::Hph;MPG1(p)::SC3::bar	SC3	–	–[d]	–
mpg1::Hph;MPG1(p)::SC4::bar	SC4	10–2 nm	indistinct, short, interwoven	<10%
mpg1::Hph;MPG1(p)::ssgA::bar	ssgA	5–7 nm	indistinct, long	10–25%
mpg1::Hph;MPG1(p)::rodA::bar	rodA	10–12 nm	interwoven	10–25%
mpg1::Hph;MPG1(p)::dewA::bar	dewA	12–15 nm	indistinct, long	10–25%
mpg1::Hph;MPG1(p)::EAS::bar	EAS	12–15 nm	indistinct, long	25–50%
mpg1:Hph;MPG1(p)::MPG1::ble	MPG1	5–7 nm	short, interwoven	100%

[a]The rodlet diameter was determined from electron micrographs of conidial surface replicas of two transformants. In all cases, the diameter was estimated based on the distance across the rodlet from shadow to shadow.
[b]Rodlet organization is described in terms of the length and degree of interweaving of rodlets in electron micrographs of conidial surface replicas of two transformants.
[c]The surface coverage was estimated based on the frequency of observation of rodlets in the plane of freeze-fracture. At least two independent repetitions of the experiment were carried out for each of the 28 recombinants examined.
[d]Conidial surfaces of *mpg1::Hph;MPG1(p)::SC3* transformants were distinct from surfaces of *mpg1*$^-$ conidia.

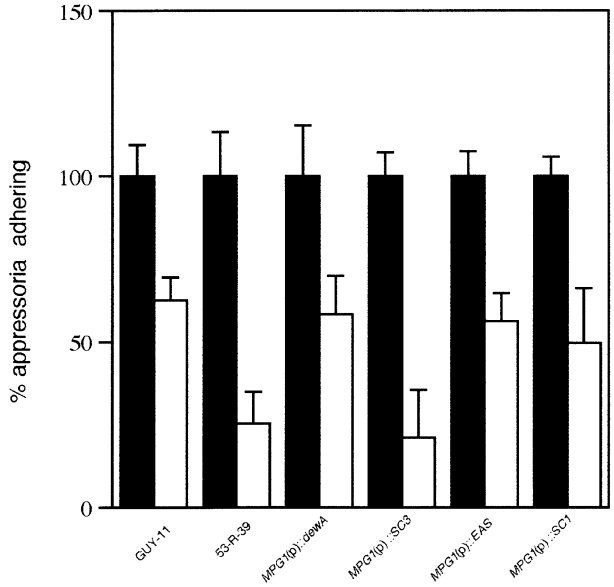

Fig. 7. Bar graph showing attachment of *M.grisea* recombinant strains transformed with hydrophobin-encoding genes to a hydrophobic surface during infection-related development. Conidia were germinated on hydrophobic PTFE-Teflon and appressoria allowed to form for 24 h. Teflon membranes were then washed in 60% ethanol and boiled in 2% SDS for 20 min (Talbot *et al.*, 1996). The percentage of appressoria remaining attached to the hydrophobic surface was determined. The error bar indicates standard error of the mean (n = 5).

mycelial growth of fungi have been described; the HCf-1 hydrophobin from *Cladosporium fulvum* (Spanu, 1997) and the CO-1 hydrophobin from *Coprinus cinereus* (Asgeìrsdottìr *et al.*, 1997). If SC3 does form a separate functional group of hyphal hydrophobins as suggested by this study, then these hydrophobins should also fail to complement *mpg1* and form a functional group with SC3. Experiments to test this idea currently are underway.

Evolution of fungal hydrophobins

The data presented show that very different heterologous hydrophobins can complement for a hydrophobin functioning in fungal pathogenesis. This suggests that hydrophobins have been co-opted into serving diverse developmental functions in fungi by evolution of specific developmental expression patterns. In the case of *MPG1*, for example, a single hydrophobin mediates efficient production of conidia, their surface hydrophobicity and the formation of infection structures, based largely on its regulated expression. The considerable divergence in hydrophobin sequence may, therefore, simply reflect a relative lack of selective pressure on hydrophobins as long as they are able to carry out interfacial self-assembly (Wösten *et al*., 1993) and linkage to the underlying fungal cell wall. This serves not only to emphasize developmental regulation of hydrophobin expression in conditioning function, but also the likely widespread conservation of the interfacial self-assembly process in the action of fungal hydrophobins.

Materials and methods

Fungal isolates

Strains of *M.grisea* used in this study are stored in the laboratory of N.J.Talbot. The fungus was grown routinely on complete medium (Talbot *et al.*, 1993) using standard procedures (Crawford *et al.*, 1986). Long-term storage of *M.grisea* was carried out by growing the fungus through sterile filter paper discs, desiccating these for 48 h and storing them at –20°C. The fertile rice pathogenic strain Guy-11 (Notteghem and Silue, 1992) was used as the wild-type strain in this study. The *mpg1*$^-$ mutant progeny 53-R-39 was selected from a cross between an original *mpg1::Hph* transformant, TM400-2, and a strain of opposite mating type strain, TH3 (Talbot *et al.*, 1993, 1996).

Construction of MPG1(p)::sGFP gene fusion

A 1.28 kb *Pst*I–*Nco*I restriction fragment from the 5′ end of *MPG1* was isolated from pNT800, a genomic subclone of *MPG1* previously shown to complement an *mpg1*$^-$ mutant (Talbot *et al.*, 1996). The *Nco*I site was introduced at the translation initiation codon by PCR using the

primer 5′-ACCTAATTCACCATGGTCTCCCTC-3′ and vector-specific T7 primer (Stratagene). The fragment was digested with *Pst*I and *Nco*I and cloned into pBS SK+ (Stratagene) to generate pNT190. The *MPG1* promoter fragment was sequenced by the dideoxy chain termination method using Sequenase (Amersham) and compared with the full DNA sequence of pNT800 to ensure fidelity with the genomic sequence.

The *sGFP* allele (Chiu *et al*., 1996) was obtained as a gene fusion under control of the *A.nidulans gpd* promoter and carrying the *A.nidulans trpC* terminator (Punt *et al*., 1987), kindly supplied by Dr Marc-Henri Lebrun (Université de Paris-Sud, France). A 3.75 kb *Eco*RI–*Hin*dIII fragment containing the *gpd(p)::sGFP* gene fusion was subcloned into pCB1004, an *M.grisea* transformation vector conferring hygromycin B resistance (Sweigard *et al*., 1997), to create pMJK-80. This construct subsequently was used in control transformation experiments to provide constitutive sGFP expression.

The *MPG1(p)::sGFP* expression vector, pMJK96, was generated by excision of *gpd(p)* from pMJK-80 as a 2.5 kb *Bst*XI–*Nco*I fragment and replacement with *MPG1(p)*, subcloned as a 1.3 kb *Bst*XI–*Nco*I fragment from pNT190.

Construction of hydrophobin expression vectors

Transformation vectors containing hydrophobin genes under control of their own promoter were constructed by subcloning the corresponding genomic clones into transformation vector pAN8.1 which confers bleomycin resistance (Punt *et al*, 1987), or into the vector pCB1265 which confers bialophos resistance (Sweigard *et al*., 1997).

Expression vectors containing hydrophobin genes as translational fusions to the *MPG1* promoter were generated by amplification of each hydrophobin gene coding sequence from genomic clones. Restriction sites were introduced during PCR amplification. Introduction at the start codon of an *Nco*I site (or sites compatible with *Nco*I) allowed each gene to be subcloned in-frame to the *MPG1* promoter at their translation initiation codon. At the 3′ end of each hydrophobin gene, a restriction site was introduced which was present in the transformation vector, but not internal to the *MPG1* promoter or the hydrophobin gene. In all cases, an *Apa*I (5′-GGGCCC-3′) site was thus generated. PCR amplification from plasmid templates was performed in a Perkin Elmer Gene Amp PCR 2400 with 25 cycles of amplification using the proofreading enzyme *Pfu* polymerase (Stratagene). This ensured fidelity of the amplified sequence. Each cycle consisted of 30 s denaturation at 94°C, 30 s annealing at 50°C and 1 min extension at 72°C. In all cases, a 3 min denaturation step at 94°C preceded the first amplification cycle and a 15 min extension at 72°C was performed after completion.

Oligonucleotides were designed according to the published sequence of each hydrophobin. For *rodA* (Stringer *et al.*, 1991) amplification, the primers used were RodA-5, 5′-CCACCTCTTCATCATGAAGTTCT-CCAT-3′, and RodA-3, 5′-CTTGGGCCCGAAGTAAAAGATAATAA-ACA-3′, to introduce sites for *Bsp*HI and *Apa*I, respectively (underlined). For amplification of *SC1* (Schuren and Wessels, 1990), primers used were SC1-5, 5′-AGCAACAACCATGGCCTTCTCGCTCGCC-3′, and SC1-3, 5′-CTTGGGCCCGTGCGTGTATACCGACCTT-3′, introducing sites for *Nco*I and *Apa*I, respectively. For *SC4* (Schuren and Wessels. 1990) amplification, primers used were SC4-5, 5′-CTTCATGAGATTCT-CGCTCGCTCTGCTTGCT-3′, and SC4-3, 5′-CTTGGGCCCGTCG-ACCACGTGCGCGCG-3′, to introduce sites for *Bsp*HI and *Apa*I. For amplification of *ssgA* (St. Leger *et al.*, 1992), primers used were SSGA-5, 5′-TCCTTCAACATGTTCAAGGCTCTCATCG-3′, and SSGA-3, 5′-CTTGGGCCCAAGAGACAGAGTAAATTTGT-3′, to introduce sites for *Afl*III and *Apa*I, respectively. For *EAS* (Bell-Pederson *et al*., 1992; Lauter *et al*., 1992) amplification, primers used were EAS-5, 5′-CCCAACCGCCATCATGAAGTTCACCAGC-3′, and EAS-3, 5′-CTT-GGGCCCAGGGTTAAGAGAGTGTATTA-3′, to introduce sites for *Bsp*HI and *Apa*I, respectively. For amplification of *dewA* (Stringer and Timberlake, 1995), primers used were dewA-5, 5′-AACTCAT-CAACATGTCCCGCTTCATCGTC-3′, and dewA-3, 5′-TGTGGGCC-CGAACAACACCAATTATTATT-3′, introducing sites for *Afl*III and *Apa*I, respectively.

In all cases, amplification products were excised and digested with appropriate restriction enzymes and subcloned into pNT190 to generate an *MPG1* promoter translational fusion at the *Nco*I site. Each gene fusion was then subcloned to pCB1265 (Sweigard *et al*., 1997) for *M.grisea* transformantion.

The subcloning of *SC3* (Wösten *et al*., 1994) into pCB1265 was carried out by ligation of a 874 bp *Nco*I restriction fragment containing the *SC3* open reading frame (Schuren and Wessels, 1990) directly into pNT190. Recombinant plasmids were restriction mapped to ensure the fragment was in the correct orientation. The *MPG1(p)::SC3* gene fusion was then excised as a 2.15 kb *Pst*I–*Apa*I fragment and subcloned into pCB1265. For details of all recombinant DNAs used in this study, see Table II.

Fungal transformations

Protoplast preparation and transformations were performed as described previously (Talbot *et al.*, 1993). Constructs were transformed into the Mpg1 mutant 53-R-39 and transformants selected for bleomycin resistance at 35 μg/ml bleomycin in the presence of 100 μg/ml caffeine, or for bialophos resistance at 35 μg/ml. Putative transformants were confirmed by DNA gel blot hybridization, and those carrying single plasmid integrations were selected.

Rice infections

Fourteen-day-old rice seedlings were infected with suspensions of *M.grisea* conidia prepared in 0.1% gelatin at a concentration of 1×10^4 conidia/ml. Two-week-old seedlings of the susceptible rice cultivar CO-39 were sprayed using an artists' airbrush (Badger Co., Franklin Park, IL). Plants were incubated in plastic bags for 18 h to maintain high humidity and then transferred to controlled environment chambers at 24°C, 84% relative humidity, with 900 $\mu E/m^2$ tungsten illumination and a 14 h day length. Plants were incubated for 96–120 h for full disease symptoms to become apparent. The first disease symptoms were observed 96 h after seedling inoculation. Lesion densities were scored routinely from 40 randomly chosen 5 cm leaf tips, and means and standard deviations determined. Results from pathogenicity assays were compared using the Student's *t*-test and two non-parametric tests; the Mann–Whitney two sample test and the Kruskal–Wallis test (Sokal and Rohlf, 1981).

Assays for infection-related morphogenesis

Appressorial development by *M.grisea* was observed on Teflon membranes (DuPont) as described by Hamer *et al*. (1988). A 200 μl drop of a conidial suspension at a concentration of 1×10^5/ml was placed on the surface of a Teflon coverslip and left in a humid environment at 24°C. The frequency of appressorium formation was determined by counting the number of appressoria that had developed from 300 conidia after 14 h (Talbot *et al.*, 1993). Remediation experiments with cAMP were carried out by incubation of conidia in 10 mM cAMP (Sigma) solutions as described by Lee and Dean (1993). Results from appressorium assays were compared using the Student's *t*-test.

Nucleic acid isolations and analysis

Genomic DNA was extracted from fungal mycelium using a CTAB (hexadecyltrimethylammonium bromide) procedure described by Talbot *et al*. (1993). Gel electrophoresis, restriction enzyme digestion and DNA gel blot hybridizations were all carried out using standard procedures (Sambrook *et al.*, 1989). DNA hybridization probes were labelled by 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). RNA gel electrophoresis and RNA gel hybridization were carried out using standard procedures (Sambrook *et al*., 1989).

Electron microscopy

The surface of *M.grisea* conidia were viewed as replicas made after freeze etching. For freeze etching, conidia were fixed in 3% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.0) and washed three times in buffer in a modification of the procedure of Stringer *et al.* (1991). Samples were then cryoprotected by sequential infiltration with 10% (for 1 h) and 20% glycerol (overnight). Conidia were then frozen in Freon 22 (ICI, Runcorn, UK) and nitrogen slush. Freeze-fracturing was carried out in a Baltzer's BA 301 (Baltzer Pfeiffer GmbH, Leichenstein) and conidia were then 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 electron microscope (Jeol, Tokyo, Japan).

Fungal attachment assays

Experiments to investigate hydrophobin-mediated attachment to hydrophobic surfaces were carried out by sequential treatment of germinated conidia–germ tube–appressoria combinations prepared on Teflon membranes (DuPont). Samples were treated with 60% ethanol for 20 min at room temperature with gentle shaking and then boiling in 2% SDS for

20 min in a water bath (Talbot *et al*., 1996). Teflon membranes were rinsed gently in sterile distilled water and examined by microscopy. Several independent experiments were carried out to determine the effect of each treatment singly. The percentage of adhering conidia–germ tube–appressoria combinations from each strain was then recorded.

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References

Asgeirsdottir,S.A., Halsall,J.R. and Casselton,L.A. (1997) Expression of two closely linked hydrophobin genes of *Coprinus cinereus* is monokaryon-specific and down-regulated by the *oid-1* mutation. *Fungal Genet. Biol*., **22**, 54–63.

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

Bell-Pederson,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.

Chiu,W., Niwa,Y., Zeng,W., 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 heterokaryotic and vegetative diploid phases of *Magnaporthe grisea. Genetics*, **114**, 1111–1129.

de Jong,J.C., McCormack,B.J., Smirnoff,N. and Talbot,N.J. (1997) Glycerol generates turgor in rice blast. *Nature*, **389**, 244–245.

de Vocht,M.L. *et al*. (1998) Structural characterization of the hydrophobin SC3, as a monomer and after self-assembly at hydrophobic/hydrophilic interfaces. *Biophys. J.*, **74**, 2059–2068.

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

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**, 288–290.

Howard,R.J. and Valent,B. (1996) Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annu. Rev. Microbiol.*, **50**, 491–512.

Lau,G. and Hamer,J.E. (1996) Regulatory genes controlling *MPG1* expression and pathogenicity in the rice blast fungus *Magnaporthe grisea*. *The Plant Cell*, **8**, 771–781.

Lauter,F.R., Russo,V.E.A. and Yanofsky,C. (1992) Developmental and light regulation of *Eas*, the structural gene for the rodlet protein of *Neurospora crassa*. *Genes Dev.*, **6**, 2373–2381.

Lee,Y.H. and Dean,R.A. (1993) cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *The Plant Cell*, **5**, 693–700.

Notteghem,J.L. and Silue,D. (1992) Distribution of the mating type alleles in *Magnaporthe grisea* populations pathogenic on rice. *Phytopathology*, **82**, 421–424.

Ou,S.H. (1985) *Rice Diseases*. Commonwealth Mycological Institute, C.A.B., Kew, Surrey, UK, pp. 109–201.

Prasher,D.C., Eckenrode,V.K., Ward,W.W., Prendergast,F.G. and Cormier,M.J. (1992) Primary structure of the *Aequoria victoria* green-fluorescent protein. *Gene*, **111**, 229–233.

Punt,P.J., Oliver,R.P., Dingemanse,M.A., Powels,P.H. and van den Hondel,C.A.M.J.J. (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene*, **56**, 117–124.

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

Schuren,F.H.J. and Wessels,J.G.H. (1990) Two genes specifically expressed in fruiting dikaryons of *Schizophyllum commune*: homologies with a gene not regulated by mating type genes. *Gene*, **90**, 199–205.

Sokal,R.R. and Rohlf,F.J. (1981) *Biometry*. W.H.Freeman and Co., San Francisco, CA, USA.

Spanu,P. (1997) HCF-1, a hydrophobin from the tomato pathogen *Cladosporium fulvum. Gene*, **193**, 89–96.

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.

Stearns,T. (1995) Green fluorescent protein—the green revolution. *Curr. Biol*., **5**, 262–264.

Stringer,M.A. and Timberlake,W.E. (1995) *dewA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. *Mol. Microbiol.*, **16**, 33–44.

Stringer,M.A., Dean,R.A., Sewell,T.C. and Timberlake,W.E. (1991) Rodletless, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Genes Dev.*, **5**, 1161–1171.

Sweigard,J.A., Chumley,F.G., Carroll,A., Farrall,L and Valent,B. (1997) A series of vectors for fungal transformation. *Fungal Genet. Newslett.*, **44**, 52–53.

Talbot,N.J. (1995) Having a blast: exploring the pathogenicity of *Magnaporthe grisea*. *Trends Microbiol*., **3**, 9–16.

Talbot,N.J. (1997) Fungal biology: growing into the air. *Curr. Biol.*, **7**, R78–R82.

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*. *The Plant Cell*, **5**, 1575–1590.

Talbot,N.J., Kershaw,M.J., Wakley,G.E., de Vries,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 by *Magnaporthe grisea. The Plant Cell*, **8**, 985–999.

Templeton,M.D., Rikkerink,E.H.A. and Beever,R.E. (1994) Small, cysteine-rich proteins and recognition in fungal–plant interactions. *Mol. Plant–Microbe Interact.*, **7**, 320–325.

Templeton,M.D., Greenwood,D.R. and Beever,R.E. (1995) Solubilization of *Neurospora crassa* rodlet proteins and identification of the predominant protein as the proteolytically processed *eas* (*ccg-2*) gene product. *Exp. Mycol*., **19**, 166–169.

Timberlake,W.E. (1980) Developmental gene regulation in *Aspergillus nidulans. Dev. Biol*., **78**, 497–510.

Van Wetter,M.-A., Schuren,F.H.J., Schuurs,T.A. and Wessels,J.G.H. (1996) Targeted mutation of the *SC3* hydrophobin gene of *Schizophyllum commune* affects formation of aerial hyphae. *FEMS Microbiol. Lett.*, **140**, 265–269.

Wessels,J.G.H. (1997) Hydrophobins: proteins that change the nature of the fungal surface. *Adv. Microbial Physiol.*, **38**, 1–45.

Wessels,J.G.H., de Vries,O.M.H., Ásgeirsdóttir,S.A. and Schuren,F.H.J. (1991) Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in *Schizophyllum. The Plant Cell*, **3**, 793–799.

Wösten,H.A.B., de Vries,O.M.H. and Wessels,J.G.H. (1993) Interfacial self-assembly of a fungal hydrophobin into a hydrophobic rodlet layer. *The Plant Cell*, **5**, 1567–1574.

Wösten,H.A.B., Ásgeirsdóttir,S.A., Krook,J.H., Drenth,J.H.H. and Wessels,J.G.H. (1994a) The fungal hydrophobin SC3p self-assembles at the surface of aerial hyphae as a protein membrane constituting the hydrophobic rodlet layer. *Eur. J. Cell Biol.*, **63**, 122–129.

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

Wösten,H.A.B., Ruardy,T.G., Van der Mei,H.C., Busscher,H.J. and Wessels,J.G.H. (1995) Interfacial self-assembly of a *Schizophyllum commune* hydrophobin into an insoluble amphipathic membrane depends on surface hydrophobicity. *Colloids and Surfaces B*, **5**, 189–195.

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