

Four conserved intramolecular disulphide linkages are required for secretion and cell wall localization of a hydrophobin during fungal morphogenesis

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Summary

Hydrophobins are morphogenetic proteins produced by fungi during assembly of aerial hyphae, sporulation, mushroom development and pathogenesis. Eight cysteine residues are present in hydrophobins and form intramolecular disulphide bonds. Here, we show that expressing eight cysteine–alanine substitution alleles of the *MPG1* hydrophobin gene from *Magnaporthe grisea* causes severe defects in development of aerial hyphae and spores. Immunolocalization revealed that Mpg1 hydrophobin variants, lacking intact disulphide bonds, retain the capacity to self-assemble, but are not secreted to the cell surface. This provides the first genetic evidence that disulphide bridges in a hydrophobin are dispensable for aggregation, but essential for secretion.

Introduction

Filamentous fungi occupy an enormous range of aquatic and terrestrial habitats and also cause important diseases of animals and plants. The versatility of fungi results from their ability to respond rapidly to environmental change and to their unique mode of nutrition, in which complex polymers are broken down outside fungal cells and the resulting simple sugars and amino acids transported into the advancing mycelium. One of the key characteristics of filamentous fungi – which allows them to move easily from solid to semi-solid substrates and to grow into the air – is their production and secretion of unusual surface-active proteins, known as hydrophobins (Wösten, 2001). These proteins are involved in many developmental processes carried out by fungi, including formation of aerial hyphae, spores and fruiting bodies

(Wessels, 1996; Kershaw and Talbot, 1998; Talbot, 1999; Wösten, 2001). Fungal hydrophobins are small, cysteine-rich proteins, which are secreted by fungi as monomers and undergo a process called interfacial self-assembly, whenever they encounter an interface between air and water, or between water and a hydrophobic surface (Wösten *et al.*, 1994; 1999). The resulting aggregated hydrophobins form an amphiphilic monolayer, which allows fungi to reduce surface tension and grow into the air, or to increase the wettability of hydrophobic surfaces, facilitating efficient surface attachment. Recently, the first crystal structure of a fungal hydrophobin, the HFBII class II hydrophobin from *Trichoderma reesei*, was reported (Hakanpää *et al.*, 2004) and revealed that hydrophobins contain a novel protein fold, consisting of one α -helix and four anti-parallel sheets (forming a β -barrel) built around four intramolecular disulphide bridges. The four disulphide bridges of HFBII are arranged symmetrically in the same plane, with two of them located entirely within the β -barrel and the other two found outside. Hydrophobins therefore adopt a very stable and compact structure. An interesting feature revealed by the crystal structure of HFBII is a hydrophobic surface patch formed by the exposure of hydrophobic residues present in two β -hairpins. This hydrophobic patch occupies 400 Å² (12%) of the total 3200 Å² surface area of the hydrophobin and is likely to be responsible for conferring the characteristic amphipathic properties that permit hydrophobin aggregation (Hakanpää *et al.*, 2004). Self-assembly of hydrophobins results in visible aggregations of protein rodlets on fungal structures (Wösten, 2001) and the unstructured β -sheet structure of assembled fungal hydrophobins is reminiscent of amyloid-like structures generated during Alzheimer's and prion diseases (Butko *et al.*, 2001; Mackay *et al.*, 2001).

In this study we have investigated the significance of intramolecular disulphide bridges of a fungal hydrophobin that is utilized by a pathogenic fungus during establishment of plant disease (Talbot *et al.*, 1993; 1996). The rice blast fungus *Magnaporthe grisea* expresses a hydrophobin, Mpg1, during formation of specialized infection structures, called appressoria, which are used to infect rice leaves (Talbot *et al.*, 1996). The *MPG1*-encoded hydrophobin is an important virulence determinant for *M.*

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grisea and is the most highly expressed fungal gene during plant infection (Matsumura *et al.*, 2003). We report here the generation of a series of *MPG1* alleles in which each of the eight cysteine-encoding codons has been substituted with an alanine codon to prevent disulphide bridge formation. These novel *MPG1* alleles have been expressed in a $\Delta mpg1$ mutant of the rice blast fungus. Analysis of the resulting transformants showed that hydrophobin self-assembly is still possible in the absence of the correct disulphide linkages, but hydrophobin secretion is severely compromised. These results have important consequences for our understanding of the role of hydrophobins in fungal morphogenesis.

Results

The eight conserved cysteine residues and associated intramolecular disulphide linkages in hydrophobins are shown schematically in Fig. 1A and B. The intramolecular disulphide linkages are based on the crystal structure of a class II hydrophobin (Hakanpää *et al.*, 2004). In order to determine the role of intramolecular disulphide linkages

in the Mpg1 hydrophobin, site-directed mutagenesis was used to substitute the eight cysteine codons, one-by-one, with alanine codons (Fig. 1C). The eight distinct *MPG1* cysteine–alanine (*MPG1^{C→A}*) substitution alleles were then introduced into a $\Delta mpg1$ mutant strain of *M. grisea*, 53-R-39 (Talbot *et al.*, 1996) and two independent transformants, carrying a single copy of each *MPG1^{C(p)A}* allele, selected for further study (Fig. 1C). To confirm expression of each *MPG1^{C→A}* substitution allele, RNA gel blot analysis was carried out, as shown in Fig. 1D. Expression of each of the novel *MPG1* alleles was equivalent when the fungus was exposed to nitrogen starvation, which is known to induce *MPG1* expression (Soanes *et al.*, 2002), indicating stable expression of the transgenes in the $\Delta mpg1$ null mutant.

Expression of MPG1 cysteine–alanine substitution alleles causes defects in aerial hypha development in M. grisea

Mpg1 hydrophobin mutants show several morphogenetic phenotypes, including poor levels of sporulation, reduced infection structure formation and attenuated virulence

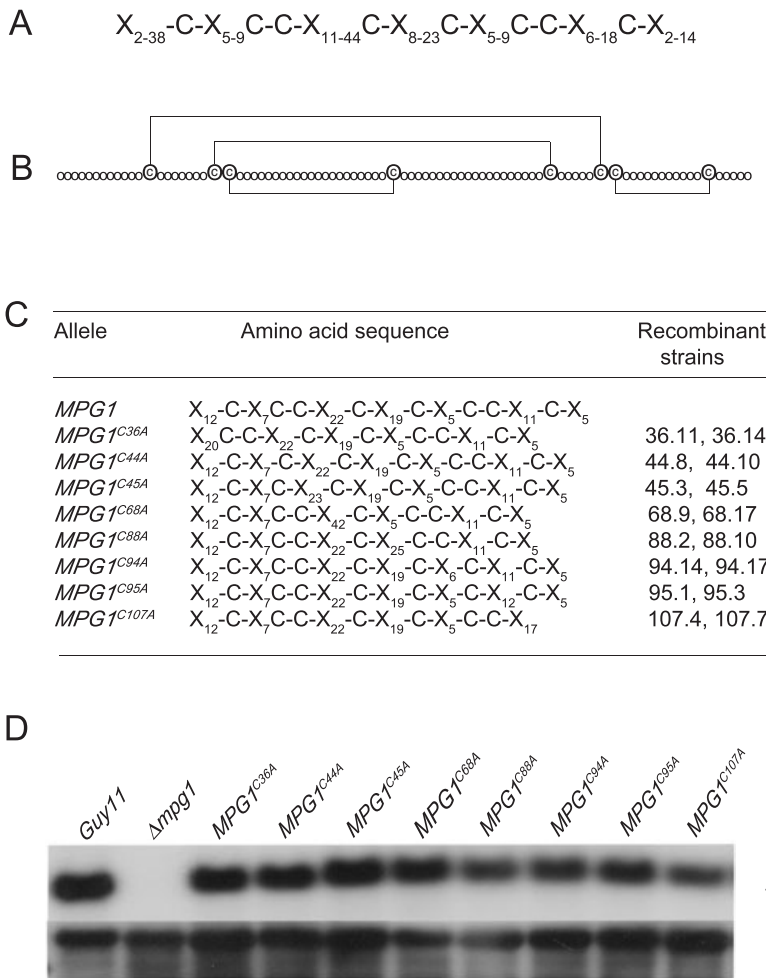


Fig. 1. A. Fungal hydrophobins share common spacing of eight cysteine residues within their amino acid sequence, which form four intramolecular disulphide linkages. B. Positions of the four intramolecular disulphide linkages of a fungal hydrophobin, based on structural analysis of the class II hydrophobin HFBII (Hakanpää *et al.*, 2004). C. Generation of eight distinct cysteine–alanine substitution alleles of the *MPG1* hydrophobin gene. Each allele was introduced into the $\Delta mpg1$ mutant 53-R-39, of *M. grisea* and two single-copy transformants selected. D. RNA gel blot showing expression of each *MPG1^{C→A}* allele in transformants of 53-R-39. *M. grisea* strains were subjected to nitrogen starvation for 48 h, RNA gel blots prepared and probed with a 3.75 kb *Xba*I–*Hind*III fragment of *MPG1*. Hybridization to the 18S rRNA gene from *M. grisea* is shown as a loading control.

(Talbot *et al.*, 1993; 1996). Each of the $\Delta mpg1$ mutant phenotypes can be complemented by reintroduction of a wild-type *MPG1* allele (Talbot *et al.*, 1996). To investigate whether *Mpg1* mutant phenotypes could be complemented by expression of *MPG1*^{C→A} substitution alleles, transformants were grown on solid agar medium and allowed to sporulate (Fig. 2A–F). The wild-type *M. grisea* strain Guy11 typically produced 1×10^7 conidia ml⁻¹ from a plate culture (Fig. 2I), and its three-celled pyriform conidia were produced on short-aerial hyphae in a sympodial array (Fig. 2A and D). In contrast, the $\Delta mpg1$ mutant 53-R-39 showed a reduction in aerial hyphae formation and typically produced 5×10^5 conidia ml⁻¹ (Talbot *et al.*, 1996; Kershaw *et al.*, 1998). Conidia of the $\Delta mpg1$ mutant 53-R-39 were also sympodially arrayed, but with normally only two or three conidia formed on each conidiophore (Fig. 2B and E). Strikingly, all of the $\Delta mpg1$ transformants expressing a *MPG1*^{C→A} substitution allele showed severe reduction in conidiation, with fewer than a thousand conidia typically produced in plate cultures (Fig. 2I). Where conidia were formed, they were only present as a single conidium per conidiophore (Fig. 2C). In addition, aerial mycelium was extremely sparsely produced, and aerial hyphae often collapsed and were misshapen (Fig. 2F). Cryoelectron microscopy further demonstrated that aerial hyphae of transformants expressing any of eight *MPG1*^{C→A} alleles were distorted and shrunken (Fig. 2H) when compared with those of a wild-type *M. grisea* strain (Fig. 2G). We conclude that expression of *MPG1*^{C→A} substitution alleles in a $\Delta mpg1$ mutant has developmental consequences that are more severe than complete absence of the hydrophobin.

The ability of each of the $\Delta mpg1$ transformants expressing *MPG1*^{C→A} substitution alleles to cause rice blast disease was assayed by infecting 14-day-old seedlings of the susceptible rice cultivar CO-39 with uniform suspensions of conidia. Because conidiation was so poor in *MPG1*^{C→A} transformants, this necessitated propagation of very large numbers of plate cultures (≈ 80 per strain) in order to generate sufficient conidia for rice infections. Rice leaves showing blast disease symptoms were removed and the density of disease lesions determined in 5 cm leaf tips (Fig. 2J and K). The wild-type strain Guy11 produced 60 ± 15.19 lesions per 5 cm, while the $\Delta mpg1$ mutant 53-R-39 showed 24 ± 10.67 lesions ($P < 0.01$). Lesion densities produced by transformants expressing *MPG1*^{C→A} substitution alleles, ranged from 19.1 ± 8.8 for seedlings inoculated with transformants expressing *MPG1*^{C45A}, to 28.4 ± 9.05 for leaves infected with transformants expressing *MPG1*^{C44A}. In all cases, the lesion numbers produced were not significantly different from infections with the $\Delta mpg1$ mutant 53-R-39 (Fig. 2J and K). Expression of *MPG1*^{C→A} alleles therefore does not restore full virulence to a $\Delta mpg1$ mutant.

Mpg1^{C→A} variants retain the capacity to form an aggregated rodlet layer

Hydrophobins aggregate into amphipathic monolayers on the surfaces of aerial fungal structures, such as spores and fruit bodies (Stringer *et al.*, 1991; Bell-Pederson *et al.*, 1992; Lauter *et al.*, 1992). The hydrophobic surface of aggregated hydrophobin layers can be visualized by transmission electron microscopy of fungal cell surfaces as interwoven rodlets, which have characteristic shapes and sizes depending on the particular hydrophobin (Kershaw *et al.*, 1998). Aggregated *Mpg1* hydrophobin can be visualized as a sheet of 5 nm diameter rodlets on the surface of conidia (Fig. 3A). This layer is completely absent in the $\Delta mpg1$ mutant 53-R-39 (Fig. 3B). When conidial surface replicas from $\Delta mpg1$ transformants expressing the eight *MPG1*^{C→A} substitution alleles were examined, fragments of the hydrophobic rodlet layer were observed in approximately 5% of the conidial surface replicas generated ($n = 60$) (Fig. 3C). The fact that rodlets could be observed – although very rarely – on the conidial surface of the *MPG1*^{C→A} transformants indicates that *Mpg1* hydrophobin self-assembly does not require the presence of intramolecular disulphide linkages. The scarcity with which the rodlet layer was observed, however, suggested to us that the hydrophobin was not being localized to the cell surface correctly and we decided to examine this possibility in more detail.

Intramolecular disulphide linkages are required for efficient secretion of the *Mpg1* hydrophobin

To investigate secretion of the *Mpg1* hydrophobin, a V5 epitope tag sequence was inserted at the C-terminus of the protein-coding sequence of a wild-type *MPG1* allele and the *MPG1*^{C45A} substitution allele. These alleles were transformed and expressed in the $\Delta mpg1$ mutant 53-R-39. Expression of the *MPG1*^{V5} allele led to full complementation of all $\Delta mpg1$ phenotypes (data not shown), indicating that the V5 epitope tag had no effect on the biological function of the hydrophobin. Immunofluorescence microscopy revealed localization of *Mpg1*^{V5} on the surface of conidia and around the appressorium (Fig. 4A). In contrast, transformants expressing the *MPG1*^{C45A-V5} alleles showed the same phenotypes as the *MPG1*^{C→A} transformants described above, and no cell surface fluorescence was observed during conidial germination and appressorium formation (data not shown). Detailed analysis of the distribution of the epitope-tagged hydrophobin was carried out by immunogold electron microscopy (Fig. 4B–F). Conidia and appressoria were prepared from each transformant, embedded in resin, and ultrathin sections produced. Sections from conidia expressing the *Mpg1*^{V5} hydrophobin showed accumulation of gold parti-

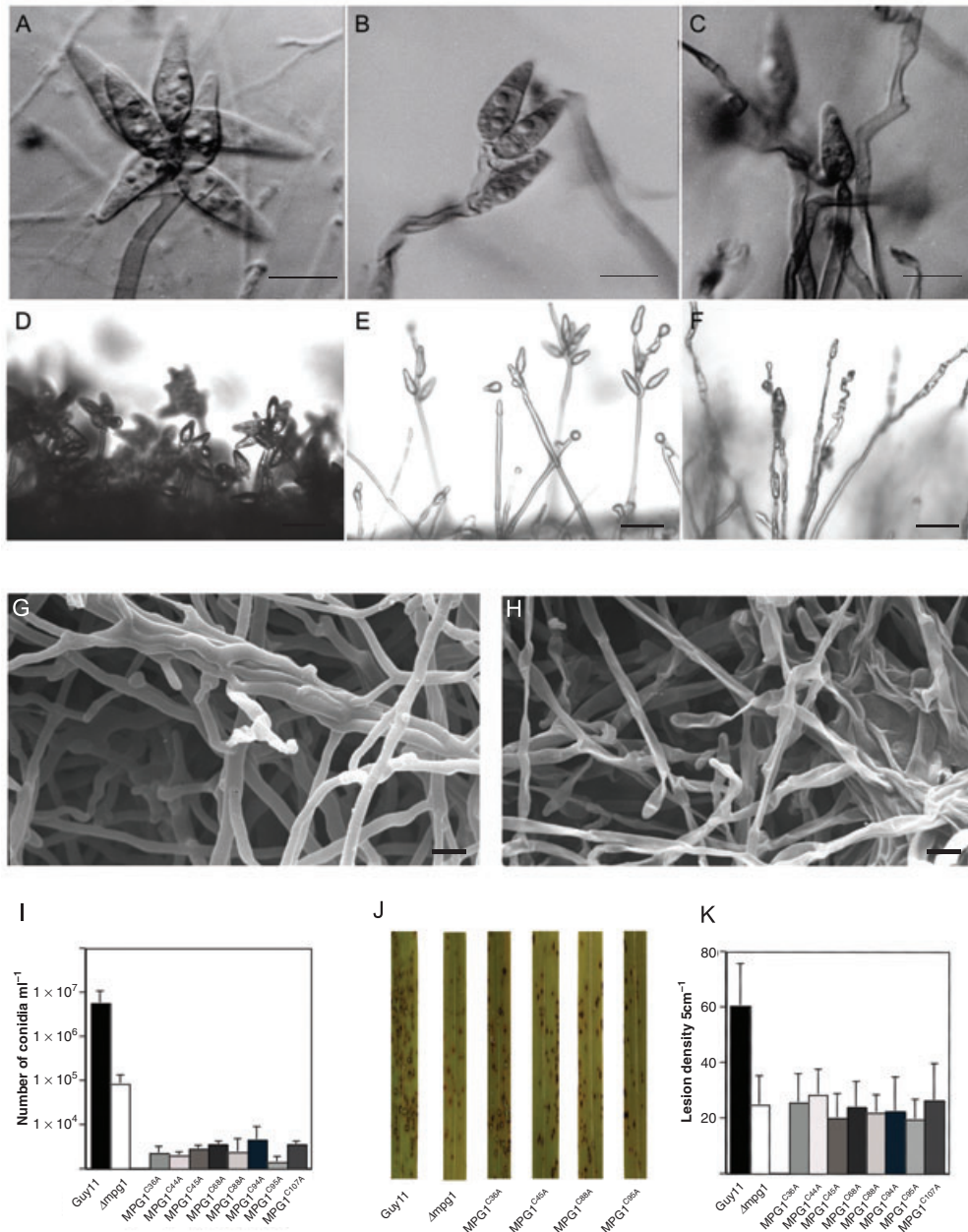


Fig. 2. The effect of expression of $MPG1^{C \rightarrow A}$ alleles in a $\Delta mpq1$ mutant of *M. grisea* on aerial hyphae development and sporulation. A–C. (A) Sympodial array of conidia produced by *M. grisea* wild-type Guy11, (B) Conidiophore and conidia of $\Delta mpq1$ mutant 53-R-39, (C) Conidiophores and aerial hyphae of transformant 45.3 of $\Delta mpq1$ mutant expressing $MPG1^{C45A}$. Bar = 10 μm . D–F. (D) Aerial conidiophores of Guy11, (E) Conidiophores of $\Delta mpq1$ mutant 53-R-39, (F) Conidiophores of $MPG1^{C45A}$ transformant 45.3. Bar = 30 μm . G and H. (G) Low-temperature scanning electron micrographs of aerial hyphae of Guy11 and (H) $MPG1^{C45A}$ transformant 45.3. Bar = 10 μm . I. Bar chart of numbers of conidia produced from cultures of *M. grisea* in Guy11, 53-R-39 and 53-R-39 transformants expressing each $MPG1^{C \rightarrow A}$ substitution allele. Value shown is mean of three replications of the experiment. Error bar is standard deviation. J. Rice seedlings of cultivar CO-39 were inoculated with conidial suspensions of *M. grisea* and allowed to develop rice blast symptoms for 4 days. Representative leaves from infected seedlings are shown. K. Bar graph of the mean disease lesion density per 5 cm leaf tip, from $\Delta mpq1$ transformants expressing $MPG1^{C \rightarrow A}$ substitution alleles. Error bar is standard deviation ($n = 40$).

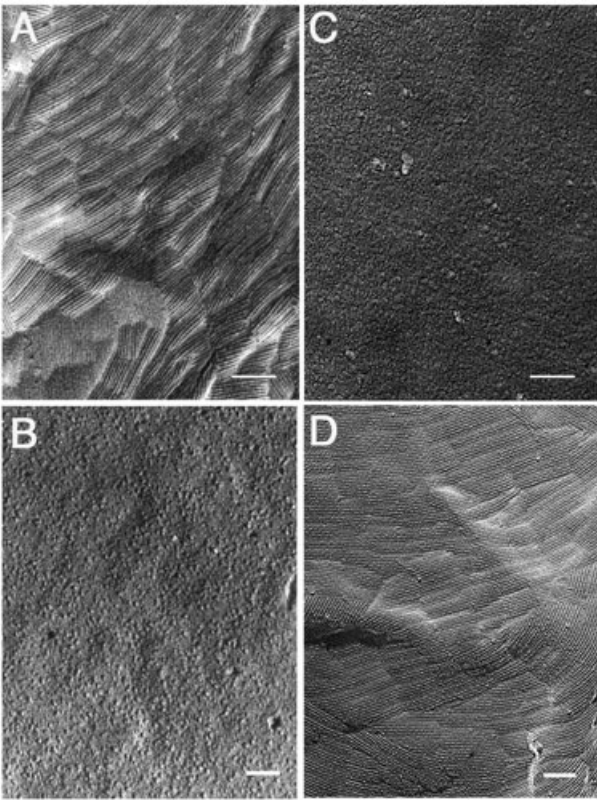


Fig. 3. Transmission electron microscopy of freeze-fractured conidium surfaces from (A) Guy11, showing rodlet protein layer, (B) $\Delta mpg1$ strain 53-R-39, which lacks Mpg1 rodlets, (C) $MPG1^{C45A}$ transformant 45.3. Most conidial samples observed (47/60) lacked rodlets (D) $MPG1^{C45A}$ transformant 45.3, showing rodlets, which were rarely observed in conidial replicas (3/60). Bar for all panels = 100 nm.

cles predominantly to the cell wall (Fig. 4C and D). Sections of conidia from transformants expressing the $Mpg1^{C45A-V5}$ hydrophobin showed gold particles throughout the cell interior, and only rarely in the cell wall (Fig. 4E and F). Quantitative analysis of the distribution of gold particles in replicate sections of conidia indicated that the $Mpg1^{V5}$ hydrophobin was more efficiently secreted to the cell wall than the $Mpg1^{C45A-V5}$ hydrophobin (Fig. 4B).

One possible reason for the lack of cell surface localization of the $Mpg1^{C45A-V5}$ hydrophobin could be a general defect in protein secretion, perhaps due to accumulation of the misfolded hydrophobin. To investigate this idea, plugs of mycelium were recovered from hyphal cultures of transformants expressing either the $MPG1^{V5}$ or $MPG1^{C45A-V5}$ alleles (Fig. 5A), and used to prepare colony immunoblots of growing hyphae (Fig. 5B and C). The anti-V5 antibody could readily detect secretion of the $Mpg1^{V5}$ hydrophobin from aerial hyphae, but not the $Mpg1^{C45A-V5}$ hydrophobin (Fig. 5B, panel 1). In contrast, secretion of a constitutively expressed extracellular enzyme, glucoamylase, was detected in immunoblots of all transformants (Fig. 5B, panel 2), indicating that general secretion was not affected

by expression of $Mpg1^{C45A-V5}$. This was consistent with measurements of the amount of total protein recovered from culture filtrates of each transformant, which were not significantly different from that of the wild-type strain Guy11 (data not shown).

To quantify secretion of the $Mpg1^{V5}$ hydrophobin, an enzyme-linked immunosorbent assay (ELISA) was performed. Conidia of the epitope-tagged strains, together with a Guy11 control strain of *M. grisea*, were germinated in the wells of a microtitre plate and appressoria allowed to form. ELISA was carried out at four time points during conidial germination and appressorium development. The $Mpg1^{V5}$ hydrophobin was detected on the outside of fungal structures throughout conidial germination and appressorium development, peaking in abundance 6 h after germination when appressoria were starting to form (Fig. 5D). In contrast, in $Mpg1^{C45A-V5}$ transformants the protein was not present at a high level on external fungal structures (Fig. 5D). When considered together with immunolocalization studies, we conclude that absence of intramolecular disulphide linkages in the Mpg1 hydrophobin impairs its secretion and localization to the cell surface.

Discussion

Fungal hydrophobins are strongly amphiphilic biomolecules, which undergo self-assembly and aggregation when exposed to boundaries between air and liquids, or liquids and solids (Wösten, 2001). Expression of eight distinct cysteine–alanine substitution alleles of $MPG1$ in a $\Delta mpg1$ mutant allowed two important conclusions to be made regarding the role of disulphide bonds in hydrophobins. First, we found that secretion of the Mpg1 hydrophobin was severely impeded in the absence of all disulphide linkages. This was demonstrated by the rarity with which aggregated hydrophobin rodlets were observed on conidia of *M. grisea* transformants expressing any of the $MPG1^{C\rightarrow A}$ alleles, and by immunolocalization studies which showed the $Mpg1^{C45A-V5}$ hydrophobin predominantly located inside fungal cells. The very low concentration of $Mpg1^{C\rightarrow A}$ hydrophobin at the cell surface also explains why the virulence phenotype of a $\Delta mpg1$ mutant could not be complemented. The role of Mpg1 in appressorium formation is a consequence of secretion of the hydrophobin during infection-related development on the plant surface, where it increases wettability of the hydrophobic leaf surface, which aids attachment and promotes subsequent development of appressoria. The relatively inefficient production of appressoria is consistent with the reduction in disease lesion numbers observed in $\Delta mpg1$ mutants (Talbot *et al.*, 1996; Soanes *et al.*, 2002).

The second important conclusion from this study results from the observation that, in a small number of preparations, a conidial rodlet layer could still be seen in $\Delta mpg1$

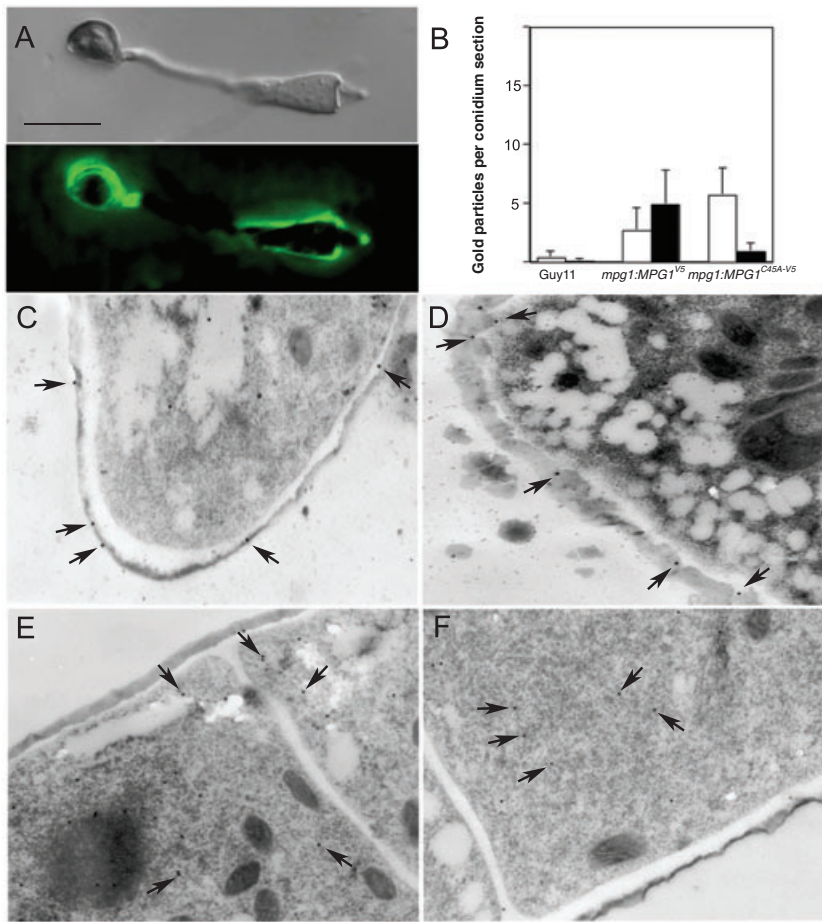


Fig. 4. Immunolocalization of epitope-tagged Mpg1 hydrophobin.

A. Bright-field micrograph and immunofluorescence of $\Delta mpg1$ transformant, MV5.4, expressing Mpg1^{V5} hydrophobin. Epifluorescence image is in the lower panel. Bar = 10 μ m.

B. Bar chart showing the cellular location of gold particles in immunogold electron microscopy of conidial sections of Guy11 (negative control), Mpg1^{V5} transformant MV5.4, and Mpg1^{C45A-V5} transformant 45V5.3. White bars indicate the mean number of gold particles per section observed inside cells. Black bars indicate the mean number of gold particles per section, found within the cell wall. $n = 50$ sections from three independent experiments.

C and D. Immunogold electron microscopy of transverse section of a conidium of transformant, MV5.4, showing localization of Mpg1^{V5} to cell wall. E and F. Transverse sections of conidium of transformant, 45V5.3, showing predominantly intracellular localization of Mpg1^{C45A-V5}. Bar for C–E = 200 nm.

transformants expressing $MPG1^{C \rightarrow A}$ alleles. This provides evidence that self-assembly of Mpg1 can still take place even without all of the correct disulphide linkages. Our results are consistent with *in vitro* studies using the purified SC3 hydrophobin from *Schizophyllum commune* (De Vocht *et al.*, 2000) in which disulphides were reduced with dithiothreitol, and free thiols blocked with iodoacetamide. The SC3 hydrophobin unfolded after this treatment, but after drying down the protein, or exposing it to a hydrophobic surface, the hydrophobin was still able to aggregate (De Vocht *et al.*, 2000). Our results are consistent with this biochemical study and show that *in vivo* self-assembly of hydrophobins does not require every disulphide bond to be intact. When considered with the recently reported hydrophobin structure, our results indicate that disruption of any one of the disulphide bonds (either within, or outside the proposed β -barrel hydrophobin structure) is insufficient to prevent surface exposure of the hydrophobic patch of amino acid residues, proposed to facilitate interfacial self-assembly (Hakanpää *et al.*, 2004), because surface rodlets were observed in rare instances in all $MPG1^{C \rightarrow A}$ transformants. It will be important to carry out systematic mutagenesis studies to

alter hydrophobicity of the group of residues which form the hydrophobic patch, to determine whether this surface feature is required for the surfactant activity of hydrophobins. The compact globular β -barrel structure of hydrophobin monomers – a consequence of the formation of the disulphide linkages – also appears to be fundamental to the correct localization of monomers to the cell surface. The results presented here support the hypothesis presented by De Vocht *et al.* (2000) in which hydrophobin disulphide linkages were proposed to prevent self-assembly within the cell, before secretion.

A striking consequence of expressing $MPG1^{C \rightarrow A}$ alleles in *M. grisea* was the severe effect on aerial growth and sporulation. Expression of the aberrant hydrophobins had a much greater impact on development, for instance, than complete absence of the hydrophobin in a $\Delta mpg1$ mutant. As suggested previously, one role of hydrophobin disulphide bonds may be to prevent premature self-assembly of the proteins before encountering an interface (De Vocht *et al.*, 2000; Wösten, 2001), and it is possible that misfolded Mpg1^{C→A} hydrophobins undergo aggregation inside the cell before secretion. Currently, we cannot preclude the possibility that Mpg1^{C→A} variant proteins are

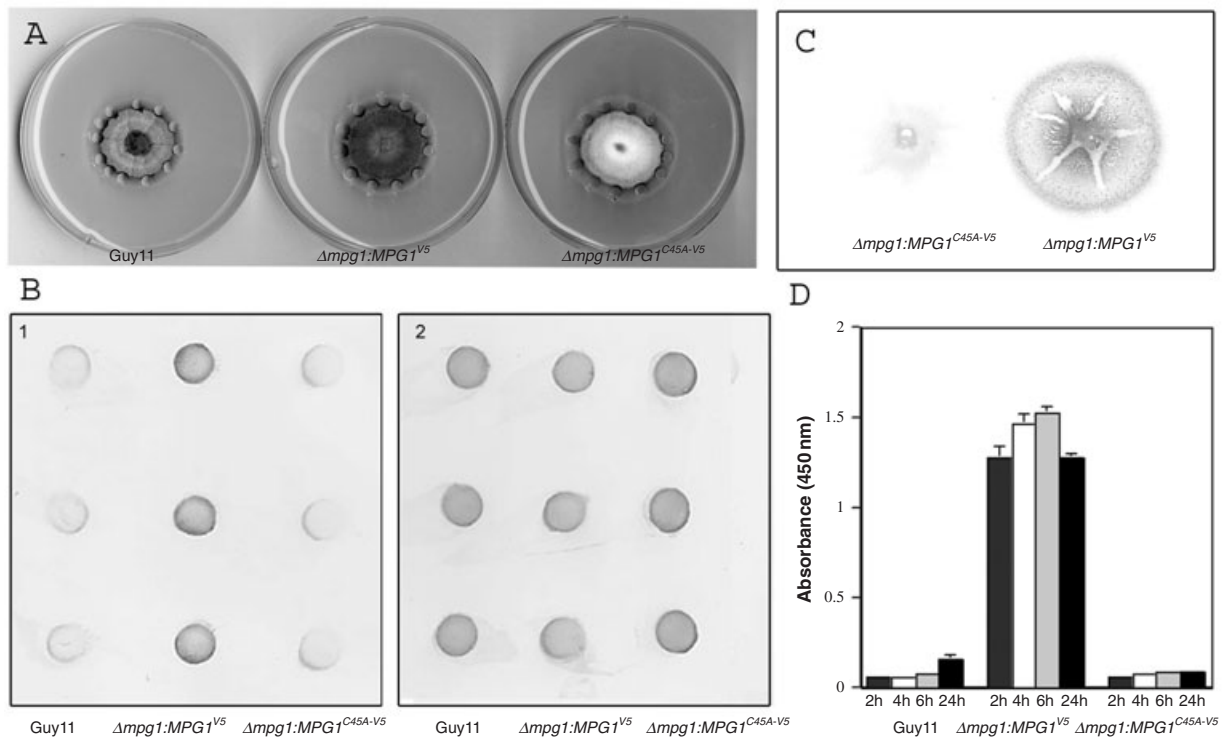


Fig. 5. Colony immunoblots to study secretion of epitope-tagged Mpg1 hydrophobin.

A. Plate cultures of *M. grisea* Guy11, $\Delta mpg1$ transformant, MV5.4, expressing Mpg1^{V5} hydrophobin, and transformant, 45V5.3, expressing Mpg1^{C45A-V5}. Hyphal plugs were taken from the actively growing margins of 5-day-old mycelium.

B. Colony immunoblots were prepared by placing hyphal plugs on PVDF membranes for 8 h (see *Experimental procedures*). Panel 1 shows immunoblot treated with anti-V5 antibody. Panel 2 shows immunoblot treated with anti-glucoamylase antibody. In both panels three replications of the experiment are shown.

C. Colony blot of whole 5-day-old *M. grisea* mycelial colony of transformants MV5.4 and 45V5.3 treated with anti-V5 antibody.

D. Enzyme-linked immunosorbent assay (ELISA) of Mpg1^{V5} secretion during spore germination and appressorium development. Conidia of Guy11, and transformants expressing Mpg1^{V5} or Mpg1^{C45A-V5} epitope-tagged hydrophobin were germinated in microtitre plates and allowed to form appressoria for 24 h. ELISA was carried out using anti-V5 antibody. Error bar represents standard error of the mean ($n = 4$).

turned over more quickly and do not accumulate to the same concentration as the wild-type MPG1 hydrophobin. However, the immunolocalization data presented in Figs 4 and 5 indicate that accumulation of these aberrant hydrophobins does occur within fungal cells, arguing against an effect on translational efficiency or protein stability. Such an accumulation of aggregated, amyloid-like, hydrophobin deposits within a cell would, very likely, be harmful to hyphal growth and conidial development. Alternatively, there may be wider consequences of hydrophobin misfolding. Protein folding in eukaryotes involves oxidation of a flavin adenine dinucleotide (FAD)-dependent enzyme, Ero1, by molecular oxygen and its action as an oxidant of protein disulphide isomerase, which then directly oxidizes disulphide bonds in folding proteins within the endoplasmic reticulum (Tu and Weissman, 2004). Because Mpg1 is an abundantly secreted protein during conidiogenesis, its misfolding may potentially disrupt this process, leading to oxidative stress in the cell. The presence of an unpaired cysteine residue in each misfolded hydrophobin could also cause non-native disulphide bond formation with

other proteins, including perhaps other hydrophobins. The *M. grisea* genome sequence indicates the presence of six genes that potentially encode hydrophobins in the fungus (<http://www.broad.mit.edu/annotation/fungi/magnaporthe/>). Interestingly, when one of the *MPG1*^{C→A} alleles was expressed in a wild-type strain of *M. grisea*, conidiation was also reduced (data not shown) indicating that the mutation may have a dominant negative effect, even in the presence of native Mpg1 hydrophobin.

In summary, disulphide linkage within fungal hydrophobins fulfil a structural role essential for efficient secretion from fungal hyphae. The self-aggregation process, however, can occur *in vivo* even in the absence of all intramolecular disulphide bonds.

Experimental procedures

Growth and maintenance of M. grisea

Fungal isolates used in the study are stored at the University of Exeter. *M. grisea* strains used were a rice pathogen, Guy11 (Leung *et al.*, 1988), and the $\Delta mpg1$ mutant 53-R-39

(Talbot *et al.*, 1996). Growth and storage of the fungus were carried out as reported previously (Talbot *et al.*, 1993).

Construction of vectors for site-directed mutagenesis

Mutations at each cysteine-encoding codon (TGC) of *MPG1* were created by a two-step mutagenic polymerase chain reaction (PCR) technique (Sarkar and Sommer, 1990), leading to substitution by an alanine-encoding codon (GCC). The PCR was performed in a Perkin Elmer Gene Amp 9600 thermal cycler using *Pfu* polymerase (Stratagene) to ensure fidelity of the amplified sequence. Oligonucleotides were designed based on the published sequence for *MPG1* (Talbot *et al.*, 1993). Primers used were C36A, 5'-GCTCAGCAGAAGGCCGGTGCCGAGAAGGTC-3'; C44A, 5'-AAGGTCGTCTCTGCCTGCAACAGCAA GGAG-3'; C45A, 5'-GTCGTCTCTTGCGCCAACAGCAAG GAGCTT-3'; C68A, 5'-CTCAGCGGCGAGGCCAAGAACATC CCGATC-3'; C88A, 5'-ATCAACAACCTCGCCTCGGACACC GTTTCGTGC-3'; C94A, 5'-GACACCGTTTCGGCCTGCTC CGGCGAGCAG-3'; C95A, 5'-ACCGTTTCGTGCGCCTC CGGCGAGCAGATT-3', and C107A, 5'-GTCAACATCCAG GCCACTCCTATCCTG-3'. Mutations were confirmed by DNA sequence analysis. Each *MPG1* cysteine-alanine substitution allele was excised as a 3.75 kb *HindIII*-*XbaI* fragment, containing a functional *MPG1* promoter sequence, protein-coding region and terminator (Soanes *et al.*, 2002), and subcloned into pCB1532 which carries a sulphonylurea resistance gene for selection of *M. grisea* transformants (Sweigard *et al.*, 1997). Transformation of *M. grisea* was carried out as described previously (Talbot *et al.*, 1993) and transformants were selected using 50 µl ml⁻¹ sulphonylurea (Greyhound Chromatography, Birkenhead, UK). Putative transformants were confirmed by DNA gel blot hybridization and only those carrying single integrations of the plasmid were selected. Genomic DNA and total RNA were extracted from fungal mycelium, as described previously (Talbot *et al.*, 1996). Gel electrophoresis, restriction enzyme digestion, DNA gel blot hybridizations, gel electrophoresis and RNA gel blot hybridizations were performed using standard procedures (Sambrook *et al.*, 1989).

Rice infections and microscopy

Fourteen-day-old rice seedlings of the susceptible, dwarf indica cultivar, CO-39 were infected with suspensions of *M. grisea* conidia prepared in 0.2% gelatin, at a concentration of 1 × 10⁴ conidia ml⁻¹, using an artist's airbrush. Plants were incubated in plastic bags for 24 h to maintain high humidity and then transferred to controlled environment chamber at 24°C, 90% relative humidity for 96 h for full disease symptoms to develop. Disease symptoms were scored from 40 randomly chosen 5 cm leaf tips. Light microscopy was carried out using a Zeiss Askioskop. Low temperature scanning electron microscopy was carried out as described by Balhadère and Talbot (2001). Conidial surface replicas were prepared by freeze fracture, freeze etch, in a Balzer 301 evaporator (Balzer Pfeiffer GmbH, Germany) and shadowed with carbon and platinum at 45°C (Kershaw *et al.*, 1998). A backing layer of pure carbon was added at 90°C and the replicas were

floated onto distilled water. Replicas were cleaned overnight in 50% chromic acid, washed in distilled water, before being picked up onto copper grids and viewed with a Jeol 100C electron microscope (Jeol, Tokyo, Japan).

Generation of V5 epitope-tagged Mpg1 hydrophobin

A V5 epitope tag-encoding sequence (Southern *et al.*, 1991) was inserted at the 3' end of the coding sequence of the *MPG1* wild-type allele and *MPG1*^{C45A} allele using inverse PCR. Primers used were MPG1v5rev, 5'-CGTGGAGTCCA GACCCAGCAGAGGATTAGGGATAGGCTTACCGCTCAGG ATAGGAGTGCCTGGATGTTGACCAGACCCTG-3', which incorporated the viral V5 epitope sequence (underlined, encoding GKIPNPLLGLDST), and Mpg1S, 5'-TAAATG GCTTAATGTCTTTACCGA-3'. Plasmids pNT800 (Talbot *et al.*, 1996) and pMJKC45A were used as templates. Incorporation of the V5 tag was verified by DNA sequencing. The V5 tag has previously been utilized to study the localization of fungal hydrophobins (Whiteford *et al.*, 2004).

Immunolocalization studies

For immunofluorescence microscopy, conidia were germinated for between 1 h and 24 h at 24°C in multitest 10-well glass slides (ICN). Material was air-dried, fixed (Thornton, 2001) and treated with anti-V5 antibody (Invitrogen), diluted 1:500, for 1 h, and subsequently incubated with FITC-labelled anti-mouse antibody (Sigma), diluted 1:40 in PBS, for 1 h before being observed using a Zeiss Axioskop microscope. For immunogold electron microscopy, conidia were harvested from 10-day-old plate cultures of *M. grisea* and either fixed directly, or allowed to form appressoria on the hydrophobic surface of Gelbond (FMC) for 24 h. Material was fixed and immunolabelled, as described previously (Thornton, 2001). Anti-V5 antibody was used at a 1:200 dilution, with a 20 nm gold conjugate goat anti-mouse antibody (British Biocell International) as the secondary reporter molecule. In control experiments, the primary antibody was omitted. Sections were post-stained with 2% (w/v) aqueous uranyl acetate (5–8 min) and lead citrate (10 min) before observation using a JEOL 100C transmission electron microscope (Tokyo, Japan). For colony blots, plugs of mycelium were taken from the leading edge of 5-day-old plate cultures of *M. grisea* and placed on the surface of PVDF blotting membrane (Bio-Rad). In a separate experiment, whole colonies were overlaid with PVDF membrane. After 8 h incubation at 24°C in a moist environment, membranes were removed and processed according to Thornton (2001). For detection of V5 antigen, membranes were treated with anti-V5 antibody diluted 1:2500, followed by goat anti-mouse IgG alkaline phosphatase conjugate (Sigma) at 1:1000. For detection of glucoamylase, membranes were treated with anti-glucoamylase antibody (Acris GmbH, Hiddenhausen, Germany) diluted 1:2000, followed by rabbit anti-goat IgG alkaline phosphatase conjugate at 1:5000. For ELISA, conidial suspensions were allowed to germinate in the wells of microtitre plates (Thermo Labsystems) in the presence of hexadecanediol, an inducer of appressorium formation, at 50 ng ml⁻¹. The assay was carried out with anti-V5 antibody

(1:500) followed by a 1:1000 dilution of goat anti-mouse polyvalent peroxidase conjugate (Sigma).

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