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# Surface Attachment and Pre-Penetration Stage Development by Plant Pathogenic Fungi

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■ **Abstract** Fungal pathogens cause many of the most serious crop diseases. One of the principal reasons for the success of this group is their ability to locate and perceive appropriate host surfaces and then to elaborate specialized infection structures. Here we review the processes implicated in surface attachment, germ tube elongation, and development of appressoria. The involvement of surface-acting proteins such as fungal hydrophobins and integrins in these processes is evaluated, along with a description of studies that have revealed the existence of conserved signaling pathways that regulate appressorium formation. Finally, we anticipate the prospect of genome-level analysis of fungal pathogens and the key research questions that will need to be addressed.

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## INTRODUCTION

Plant diseases caused by foliar fungal pathogens are initiated when spores attach to host surfaces and germinate. The resulting germ tubes attach tenaciously to their hosts, and in some species mechanisms have evolved to perceive leaf surface topology and thereby locate natural openings such as stomata (99, 100). A subset of fungal pathogens has also evolved the ability to elaborate specialized infection structures that enable direct penetration of the plant cuticle. These so-called appressoria are normally swollen dome-shaped cells that differentiate from the ends of fungal germ tubes (29). There has been an extensive survey of infection strategies by fungal pathogens, and the morphology of the fungal infection process is well-documented for many species (29, 99, 100). The underlying molecular basis of the mechanisms that mediate spore attachment, germination, and infection cell development, however, remains obscure. Recently, the use of molecular genetic and cell biological methods has revealed aspects of the physiology of the infection process and the identity of at least some of the significant genetic components. This review describes recent progress in understanding fungal development during the pre-penetration stage of plant disease. We concentrate on a relatively limited number of fungal species, where the most significant advances have been made, or where effective comparisons in infection strategies can be made. The review then examines the prospect for a more systematic and detailed analysis of plant infection processes using the new tools of functional genomics.

## SPORE ATTACHMENT MECHANISMS

Adhesion of fungal spores to the plant surface is the first step in committing a pathogen to the establishment of disease. Following dissemination from the primary host, a conidium enters a hostile environment and even after landing on a new host plant, factors such as rainfall, wind, and competition from other pathogens can result in displacement from the infection court (99, 100). Primarily, adhesion serves to anchor the propagule to the plant surface (51) but it may also be required for host recognition and subsequent fungal development.

Although adhesion to plant surfaces is common among fungal species, there is significant variation in the apparent composition of the adhesive materials between species and in the environmental cues that induce the development of spore adhesiveness (38). Fungal adhesives are typically water-insoluble glycoproteins (135, 172) and labeling with lectins has been used to predict both their composition and distribution (51, 66). Lipids and polysaccharides have also been detected in adhesive material and, in some fungi, can clearly be shown to contribute to adhesion (108, 109). The precise composition of fungal adhesives is therefore very heterogeneous, and there is no evidence for a common adhesive compound or mechanism of attachment in phytopathogenic species (29, 66).

The environment encountered by the spore when it lands on the plant surface is often pivotal in triggering its attachment and germination. In the case of the

rice blast fungus *Magnaporthe grisea*, moist air or dew is required for hydration and extrusion of a spore tip mucilage (STM), which serves to attach the conidium, by its apex, to the hydrophobic plant surface (51, 66). The adhesive contains $\alpha$-linked-mannosyl and glucosyl residues in addition to protein and lipid components and is immediately released upon hydration (51, 66). Adhesion in *M. grisea* is therefore a passive process involving the release of a preformed adhesive. Such a process may obviously be beneficial because it allows for rapid attachment to the host without the expenditure of metabolic energy. Also, apical attachment of the pyriform conidia produced by *M. grisea* allows them to resist the flow of water, and as water is essential for later stages of development in this fungus, this may be a useful adaptive mechanism to maintain water droplets around the attached conidium (51). Although similar mechanisms of spore attachment are likely to be widespread among pathogenic fungi (11, 72, 112), conidium attachment by the barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* and many other powdery mildews differs markedly from the situation in *M. grisea*, largely because free water is not absolutely required for pre-penetration phase development (16, 17, 29). Attachment and appressorium formation in *B. graminis* are accompanied by release of cutinase from the spore. This enzyme appears not only to cement the conidium to the leaf surface but also to make the surface of both the attachment zone and the conidium more hydrophilic, favoring further attachment and development of primary and appressorial germ tubes (82, 108, 109). For a number of fungi, a thigmotropic response is also essential before release of spore adhesives (129, 130; see below).

In contrast to the passive release of preformed adhesive from conidia of *M. grisea*, spore attachment in other species can involve energy-requiring metabolic processes. In the corn anthracnose pathogen *Colletotrichum graminicola*, for example, spores do not attain maximum adhesion to the surface until 30 min after landing. Inhibitors of glycoprotein processing and Golgi function (brefeldin A), or protein synthesis (cycloheximide) reduced conidial adhesion in *C. graminicola* by 30% and 50%, respectively, indicating that *de novo* glycoprotein synthesis is involved in the adhesion of ungerminated conidia in this fungus (101, 102, 135). Similarly, spores of the pea wilt fungus *Nectria haematococca* are nonadherent after hydration (71) and require respiration and protein synthesis before spores become adhesive.

The precise mechanism of spore attachment can involve a very specific interaction with a host plant surface, which can be achieved through lectins (64), ionic interactions (110), or hydrophobic contact with the plant cuticle. Many fungi, including *M. grisea*, *C. graminicola*, and the bean rust pathogen *Uromyces appendiculatus*, adhere tenaciously to inert hydrophobic substrata (29, 51, 66, 140), indicating that in these cases primary attachment is stimulated by physical rather than chemical signaling.

In addition to secreted specific adhesives, spores of many fungal species are surrounded by an extracellular matrix that can also play a role in attachment and preparation of the host surface for penetration (66). Cutinase and other esterases are present in the matrix that surrounds uredospores of the broad bean rust fungus

*Uromyces viciae-fabae* (31) and form an adhesion pad with the underlying plant cuticle. When these surface-localized enzymes were separated by non-denaturing gel electrophoresis and then added to autoclaved spores, which normally exhibit only low levels of adhesion to broad bean cuticle, they restored adhesion to normal levels (100). The presence of such enzymes in adhesive material also provides a means of ensuring that cuticle breakdown products are immediately available as nutrients for the most active part of the fungus, the advancing germ tube (161). Secretion of esterases could also act at this stage by causing alteration of the conformation of the plant cuticle, thereby producing an area with different adhesive properties. This was demonstrated when highly concentrated extracellular material released by conidia of *B. graminis* was applied to the surface of barley leaves, resulting in erosion of the plant surface (109).

Attachment of conidia to leaf surfaces is obviously affected by prevailing environmental conditions. Temperature changes, for example, can alter the adhesion properties of conidia (71, 126). Fluctuations in temperature obviously also influence respiration and metabolic rate, which could thus impair spore adhesion, pleiotropically. However, sodium azide, a mitochondrial respiration inhibitor, had no apparent effect on the adhesion of *C. graminicola* conidia (101). Conidial adhesion properties can also be adversely affected when conidia age. The ability of *C. graminicola* conidia to germinate gradually declined as cultures aged beyond 30–35 days (89), and the adhesive competence of ungerminated conidia also decreased over this time period (102).

Spore adhesion is clearly an essential prerequisite for infection by many pathogenic fungi (99, 100, 129). However, the adhesive compounds and the environmental cues that trigger their release are extremely diverse and have not been systematically compared. The level of transcriptional control required for release of adhesives is also highly variable, making adhesion either an apparently active or passive metabolic process, depending on species. Such dramatic global differences in the response of conidia to surface attachment and germination will undoubtedly provide fascinating comparisons when analyzed by genome-level approaches in the future. Spore adhesion influences subsequent developmental events by holding a spore in proximity to the host surface, and the act of attachment seems often to be required for transmission of morphogenetic signals for germ tube extension and infection structure development (29, 66). Further investigation of the protein components of adhesives and corresponding genes could thus provide insight into how surface sensing is mediated and how complex external signals are transmitted through the cell wall and acted upon by signal transduction pathways. Fundamental studies of fungal cell wall biochemistry have already revealed that many proteins located within the wall do not have an exclusively structural role (14, 56, 72, 78, 161, 162). Some secreted proteins, for example, play an important role in determining adhesive properties of fungi, whereas others, including most notably the hydrophobins, may be required for fungal growth through diverse environments and during developmental transitions. The roles of these proteins are discussed below in the context of host-surface perception, but their potential role in initial spore attachment should not be discounted.

## SPORE GERMINATION AND HOST SURFACE PERCEPTION

Following successful attachment of a fungal propagule to the host surface, a series of developmental events begins that determines whether successful infection will occur. The precise mechanism by which germination is triggered is not considered here in detail, but nutrient availability in necrotrophic pathogens and related saprotrophic species is clearly an important stimulus and, conversely, nutrient deprivation may be important in some biotrophic and hemibiotrophic species (29). Physical stimuli and hydration also play a role in germination, and in *M. grisea* hydration alone seems to be sufficient to induce spore germination. Contact stimulation is clearly an important signal in *B. graminis* f. sp. *hordei*, where elegant studies using silk threads from spiders' webs have shown that the point of contact of a conidium with a surface can dictate the orientation and emergence of the primary germ tube and subsequent appressorial germ tube (170). The conidial response to surface stimulation in *B. graminis* is also immediate and coincident with uptake by the spore of low-molecular-weight anionic material from the host surface (107). Conidia may also possess mechanisms to prevent germination until such stimulation, or when in proximity to other spores. For example, in *M. grisea* lipophilic self-inhibitors of germination are present in conidial washes and inhibit germination and appressorium development when high concentrations of conidia are placed on surfaces (57). Although the stimuli may be diverse, the process of germination in all fungal spores requires mobilization of storage reserves—lipids, polyols, and carbohydrates such as trehalose—accompanied by polarization and rapid membrane and cell wall biosynthesis during germ tube extension. The germ tube that emerges is a specialized structure and is distinct from vegetative fungal hyphae (66), growing sometimes for only a very short distance (for example, in *C. graminicola* and *B. graminis*) before differentiating into an appressorium (100). The germ tube is normally also the site and developmental stage in which perception of the host surface occurs (28, 130, 144, 161). If the appropriate environmental signals are not perceived correctly, the germ tube will remain undifferentiated and will eventually arrest growth upon nutrient depletion. If appropriate physical and chemical signals are detected by the germ tube, then a complex morphogenetic program is induced, resulting in appressorium formation. Germ tube extension and differentiation can occur in response to a number of signals including surface hardness, hydrophobicity, plant signals, and surface topography.

### Surface Hardness and Thigmotropism

Phytopathogenic fungi often respond to contact stimuli through a directional change, reorientation of the direction of germ tube growth, or by direct differentiation of appressoria (129–131). Wynn was among the first to demonstrate that the directional growth of rust germ tubes was due to physical features of the leaf surface (171). This was a significant development because, by using plastic replicas of the leaf surface, he confirmed that no chemical stimulus was involved in the surface sensing process of rust fungi. Germ tubes of a number of fungal species grow

across the leaf surface at right angles to the parallel arrangement of anticlinal plant cell walls, including the wheat stem rust fungi *Puccinia graminis* f. sp. *tritici* and *Uromyces appendiculatus* (2). This provides an increased chance of encountering a stoma, as these are arranged in longitudinal rows over the leaf surface. *U. appendiculatus* not only recognizes a precise series of ridges (between 0.5 and 30 $\mu$m in height) but a ridge of 0.5 $\mu$m, similar to those encountered at the edges of stomatal guard cells, resulted in maximum differentiation of appressoria on polystyrene replicas (148). The mean height of the guard cell lip was measured and found to be 0.487 $\mu$m (63), providing evidence that this is the inductive signal for cellular differentiation in *U. appendiculatus*. This process, thigmotropism, involves the sensing of surface topography and has been studied primarily in biotrophic fungi. Appressorium formation was further enhanced in *U. appendiculatus* by scratches on inert surfaces or by the hydrophobic characteristics of the contact surface (130, 148).

The region involved in perceiving the signal for appressorium formation has been identified in *U. appendiculatus* by using micropipettes on glass slides to perturb growth. The most receptive area is located within 10 $\mu$m of the germ tube apex and only the cell surface in contact with the substratum is responsive (20, 21). Patch clamp analysis has been used in *U. appendiculatus* to show the presence of a mechano-sensitive ion channel that can transport a number of cationic substrates including $Ca^{2+}$ ions (44, 182). Membrane stress during the encounter with a stomatal ridge may therefore cause a $Ca^{2+}$ ion flux, acting as a cue for appressorium formation (2, 161). Consistent with this idea, application of $Ca^{2+}$ ions to *U. appendiculatus* uredospore germ tubes induces appressorium formation (63). It has been postulated that a temporary depolymerization, or break, in the continuity of the cytoskeleton in the region of the germ tube overlying the signal is involved in appressorium differentiation in *U. appendiculatus* (62). Involvement of elements of the cytoskeleton in signal transduction is well known, particularly for integrin-mediated processes (45). In *U. appendiculatus*, appressorium differentiation is inhibited when microtubules, but not actin filaments, are depolymerized in the tip region of the germ tube (128). Within 4 min of signal perception in *U. appendiculatus*, the cytoskeleton and the vesicles in the apex of the hypha are reorganized along the cell wall (83, 84). Germling microtubules have been shown to be oriented parallel to the direction of scratches on an artificial substrate and the cell wall was found to be significantly thinner in the region of the cell in direct contact with the substrate (8, 63, 129). This suggests that transmembrane proteins might link extracellular sensing proteins with cytoplasmic proteins adjacent to the plasmalemma (8). The finding that protease-treated germlings of *U. appendiculatus* failed to recognise topographical signals and form appressoria is consistent with this hypothesis (38, 39).

Surface hardness and hydrophobicity are both important stimuli for appressorium development in *M. grisea* and *Colletotrichum* species (30, 51, 70, 88), and these infection cells form readily on hydrophobic plastic surfaces such as poly tetrafluoro-ethylene (Teflon). Surface hardness is probably the single most

important cue for appressorium formation in *M. grisea* (172) as other requirements can be bypassed (28, 46, 150), whereas in *C. gloeosporioides*, specific surface hardness-induced gene expression occurs prior to appressorium formation (80). In addition to surface signals, a number of pathogenic fungi clearly respond to plant signals that stimulate appressorium formation. An isolate of *C. gloeosporioides*, for example, which was pathogenic on avocado, formed appressoria in response to wax extracted from its host (115), while *M. grisea* has been shown to react to wax components, including most notably 1,16-hexadecanediol (46). The plant-ripening hormone ethylene has also been shown to induce appressorium development in *C. gloeosporioides*. Transgenic tomato plants unable to synthesize ethylene owing to antisense expression of 1-amino cyclopropane-1-carboxylic acid synthase did not stimulate appressorium development as efficiently as wild-type plants (40).

## Integrin-Mediated Interactions

Integrin and integrin-like proteins have been studied in most detail in mammalian cells (45). They have also been identified in plants, algae, and fungi either directly, by detection of the protein with anti-integrin antibodies on immunoblots or immunocytochemically, or indirectly, by inhibition of cell function or cell adhesion after masking the extracellular domains of the integrin receptor with Arg-Gly-Asp (RGD) containing peptides (42, 47, 65, 75, 118, 123, 132, 159). In mammalian cells, RGD-containing peptides are found in the extracellular matrix (ECM) and operate by binding to the extracellular RGD-receptive site of the membrane-spanning integrin (19, 117). Exogenous application of RGD peptides can block a variety of surface-mediated functions in many cell types, for example, adhesion, cell spreading, and differentiation into fibroblasts (12, 68, 69, 158). As adhesion, growth, and differentiation are important stages in the development of fungal germlings during the pre-penetration phase, it is plausible that a similar mechanism for sensing the environment may operate in fungi. Mammalian studies have shown that integrins are a large family of cell surface receptors composed of two subunits, $\alpha$ and $\beta$, and each $\alpha\beta$ combination has its own binding specificity and signaling properties. Most integrins recognize several ECM proteins (68), and it has been suggested that in some fungi, proteinaceous components of the ECM may be involved in signal recognition (39). Most integrins appear to be glycosylated (20, 21, 74) and can signal through the cell membrane in either direction, as extracellular binding activity of integrins is regulated from inside the cell, while binding of the ECM elicits signals that are transmitted into the cell (45).

Evidence to implicate integrins in appressorium development has come primarily from studies in *U. appendiculatus* (20, 21). Seven different RGD peptides were applied to uredospore germ tubes to see if they inhibited appressorium formation. Five of the peptides were inhibitory to infection cell formation, while the two peptides that had no effect were thought to have adopted inappropriate structural confirmations of the ligand (20, 21). Removal of the inhibitory peptides reversed the inhibition, consistent with an integrin interaction (21). Different

affinity constants between integrin and various RGD and non-RGD peptides have been reported in mammalian cells, depending on the presence of specific cations and the physiological state of the cell (68, 69). The presence of integrins in *M. grisea* has also been inferred, based on immunoblot analysis with polyclonal antisera to human vitronectrin and fibronectrin. This revealed vitronectrin-like (95-kDa) and fibronectrin-like (60-kDa) proteins in infection-stage preparations of *M. grisea*. Addition of the same antisera to conidial suspensions prevented both conidial attachment and appressorium formation but did not affect germination. The vitronectrin-like protein immunolocalized predominantly to the spore tip mucilage-containing conidial apex, consistent with a role in surface attachment (29, 30).

In mammalian cells a phenomenon known as integrin clustering has been identified (45). Because the cytoplasmic tails of integrins are generally short and always devoid of enzymatic features, they transduce signals by associating with adaptor proteins that connect the integrin to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors (45). Integrin signaling and assembly of the cytoskeleton are therefore intimately linked. As integrins bind to ECM they become clustered in the plane of the cell membrane and associate with a cytoskeletal and signaling complex that promotes the assembly of actin filaments. In this capacity, integrins also facilitate adhesion and cause reorganization of actin filaments into larger stress fibers enhancing further integrin clustering. As a result, ECM proteins, integrins and cytoskeletal proteins assemble into aggregates on each side of the membrane, known as focal adhesions or ECM contacts, and can be detected by immunofluorescence microscopy (13). In this manner, integrins serve as integrators of the ECM and cytoskeleton (45), the property for which they were named, making this form of signaling a likely mechanism to regulate the morphogenetic transitions associated with appressorium development and plant infection. Integrins were found closely associated with the growing tip of the oomycete *Saprolegnia ferax* in patches associated with the plasma membrane (74), similar to the focal adhesions described above.

In *U. appendiculatus*, when RGD-binding proteins were separated from microsomal fractions, electrophoresis revealed the presence of 12 proteins (20, 21), a number similar to those in elution profiles from human placenta (117). Using two different antibodies prepared to different antigens of $\beta$1-integrin, a common peptide of 95 kDa was revealed (21). This is smaller than the integrins described for mammalian cells (110–140 kDa) but integrins from various other sources have molecular masses reported to range from 72–240 kDa (58, 59).

The critical role proposed for integrins in *U. appendiculatus* is mediating the interaction of a wall-encased germ tube with plant surface topology. The cell wall in the thigmoresponsive region of the germling at the plant interface is very thin ($\leq$20 nm) (83). The approximate length of a $\beta$1-integrin molecule outside the plasma membrane is also approximately 20 nm (55). This length could thus conceivably span the width of the fungal cell wall and allow the ligand to extend partially into the cell wall (21). Hoch and colleagues have postulated a model for

integrin-mediated signaling in *Uromyces*, which may involve an integrin-clustering stage in the region of inductive topography. In this model, clustering of integrin would only occur when the cell is in a receptive state. In *Uromyces*, clustering could be induced by RGD-containing ligands in the ECM laid down by the germlings, as in mammalian cells, except that in *Uromyces* ECM can be seen accumulated with the recesses along the inductive ridges of the plant (21, 83, 84). In this way, integrins could assemble large signaling complexes, as in mammalian systems, and activate multiple signaling pathways, acting as a class of "master regulators" of cellular differentiation (45). Consistent with a role for integrin-based signaling in *U. appendiculatus*, a gene showing homology to fibronectin has been identified in the fungus (177).

## Fungal Hydrophobins

Filamentous fungi produce specialized proteins that are involved in the development of aerial structures and in the interactions of hyphae with hydrophobic surfaces. These proteins are known as fungal hydrophobins and have been implicated in several morphogenetic processes carried out by fungi, including spore formation, fruit body development, and appressorium formation. Hydrophobins have been classified into two distinct groups, Class I and II, based on their biochemical characteristics and the spacing of conserved cysteine residues in their amino acid sequences (37, 77, 141, 161, 162, 164). Class I hydrophobins have been studied most extensively in the basidiomycete fungus *Schizophyllum commune*, where a hydrophobin gene, *SC3*, is implicated in control of aerial attachment and hyphal/surface interactions, and three hydrophobin genes, *SC1*, *SC4*, and *SC6* are involved in fruit-body development (37, 161). Targeted deletion of *SC3* produced Δ*sc3* mutants that were deficient in their ability to produce aerial hyphae. This was particularly apparent when plate cultures were sealed to create a humid environment; under such conditions, Δ*sc3* mycelial cultures were entirely devoid of aerial growth (155). SC3 also plays an important role in attachment of *S. commune* to hydrophobic surfaces, and Δ*sc3* mutants are severely impaired in their ability to produce hyphae that are capable of attaching to hydrophobic surfaces (168). Moreover, the distribution of SC3 hydrophobin was shown by immunolocalization to be associated with the interface between the hyphae and the hydrophobic surface during hyphal attachment (168).

Hydrophobins carry out these biological functions because the proteins are able to undergo a process called interfacial self-assembly, as shown in Figure 1. Hydrophobins are small, secreted hydrophobic proteins with eight cysteine residues characteristically spaced throughout the amino acid sequence (77). Structural studies on the SC3 hydrophobin showed that the protein is secreted as a monomer with four intramolecular disulphide bridges (27, 163, 164). Monomers of SC3 spontaneously polymerize when exposed to an interface between water and air, or between water and a hydrophobic surface (165, 166). Self-assembled SC3 hydrophobin forms an amphipathic membrane-like structure

that is extremely stable and can only be disrupted by extraction with agents such as trifluoroacetic acid (26, 27). The amphipathic nature of SC3 results from an increase in $\beta$-sheet structure (164), and assembled SC3 hydrophobin is extremely hydrophobic on one side, producing contact angles with water of approximately 110° (165). This is similar in hydrophobicity to the nonstick surface poly tetrafluoro-ethylene (PTFE), or Teflon (DuPont). In contrast, the hydrophilic face of assembled SC3 aggregates produces contact angles with water of 36° (165), which is at least in part due to SC3 glycosylation and the exposure of mannose residues on the hydrophilic side of aggregated SC3. The role of SC3 in aerial growth of hyphae and in surface attachment processes may be due to the fact that the protein acts as a surfactant, inducing a large fall in surface tension of water during self-assembly. Addition of purified SC3 was found to lower the normal surface tension of water (72 mJ $m^{-2}$) to as low as 24 mJ $m^{-2}$. This feat means that SC3 is among the most powerful surface-active proteins known (154) and that fungal secretion of hydrophobins at high concentrations can lower surface tension dramatically, allowing hyphae to escape the liquid and grow into the air (141). However, continued secretion of SC3 is also important for sustained aerial growth (Figure 1). Adding SC3 to the growth medium of a Δ*sc3* mutant, for example, produces hydrophilic aerial hyphae that lack the hydrophobic coating produced by the wild type (169). This means that SC3 production by hyphae occurs throughout aerial growth and is consistent with gene expression studies, which showed that the *SC3* transcript was abundant during aerial morphogenesis (34, 124, 163). The surfactant activity of SC3 is among the highest reported (154). This is an unusual characteristic for a protein, because most natural surfactants are lipids or lipid-associated proteins. Structural studies have shown, however, that SC3 lacks a close association with lipid in either its monomeric or assembled form (169) but an associated polysaccharide, schizophyllan, may be involved in the ability of SC3 to undergo interfacial self-assembly (97).

Fungal hydrophobins appear to be ubiquitous among ascomycete and basidiomycete fungi, and their attributes suggest an involvement in pre-penetration phase development by pathogenic fungi. In two cases, so far, this has indeed been the case. The MPG1 class I hydrophobin was first identified as a differentially expressed gene during infection of rice by the blast fungus *M. grisea*. A targeted deletion produced mutants that were impaired in their ability to cause disease symptoms, and this was associated with a reduction in the frequency of appressorium development (143). *MPG1* was not found to be absolutely required for appressorium development, because some infection cells were still made by Δ*mpg1* mutants. Instead, Δ*mpg1* mutants appeared like a strain of *M. grisea* that had been incubated on a surface that does not efficiently induce appressorium formation. Thus, it appeared that the hydrophobin might be playing a role in surface perception, prior to appressorium formation. *MPG1* was found to encode a class I hydrophobin, which also forms the hydrophobic rodlet protein that coats conidiospores of *M. grisea* (144). Consistent with such a dual role, *MPG1* is highly expressed during both conidiogenesis and appressorium development, and Δ*mpg1*

mutants produce approximately tenfold less conidia than wild-type strains of *M. grisea. MPG1* thus is involved in two distinct pathways: conidium and appressorium morphogenesis (143, 144). Disruption of spore rodlet proteins in *A. nidulans* and *N. crassa* resulted in a reduction of surface hydrophobicity but not in conidial number (6, 86, 133, 134), demonstrating the diversity of function shown by class I hydrophobins (77). The *MPG1*-encoded hydrophobin appears to undergo self-assembly at hydrophobic surface interfaces (Figure 1). This hypothesis is based on experiments showing that an SDS-insoluble hydrophobin contributes to the attachment of appressoria to Teflon surfaces in wild-type strains of *M. grisea* but not in Δ*mpg1* mutants (144). The role of *MPG1* in appressorium development may therefore be due simply to secretion of the hydrophobin during germ tube formation, which would induce self-assembly directly at the hyphal/rice leaf surface interface. As a consequence of this process, MPG1 would form an amphipathic layer with the hydrophilic side facing the developing germ tube, thus increasing the relative wettability of the leaf surface. This is potentially important because most adhesives produced by *M. grisea* are hydrophilic molecules such as mucilages, as described previously (66, 172). The *MPG1* hydrophobin may therefore act as a primer for the action of such molecules, allowing strong attachment of the germ tube tip prior to appressorium differentiation (77, 144). This attachment process in turn appears to act as a developmental signal for cellular differentiation and is impaired in Δ*mpg1* mutants (143). Appressorium differentiation in *M. grisea* is known to be regulated, at least in part, by a cyclic AMP-dependent signaling pathway, and the fact that Δ*mpg1* mutants can be induced to undergo appressorium formation in the presence of exogenous cAMP is consistent with the action of MPG1 prior to cellular differentiation (37, 144). Interestingly, it appears that many class I hydrophobin genes are able to substitute for *MPG1* during appressorium formation and to form rodlet proteins on the surface of conidia. In a comprehensive set of cross-species complementation experiments, it was shown that *SC1*, *SC4*, *ssgA*, *EAS*, *dewA*, and *rodA* could all substitute for *MPG1*, at least in part, restoring the pathogenicity and ability to make appressoria to a Δ*mpg1* mutant when introduced under the control of the *MPG1* promoter (78). The important conclusion of this work is to suggest that the action of *MPG1* is probably fairly nonspecific and is likely to be a simple consequence of its ability to self-assemble at hydrophobic surface interfaces (78).

The second example of a hydrophobin involved in pre-penetration phase development is the class II hydrophobin cerato-ulmin (CU), which appears to be an attachment and parasitic fitness factor in *Ophiostoma ulmi*. CU was first identified approximately 25 years ago as a protein secreted by the Dutch elm disease fungi *O. ulmi* and *O. novo-ulmi* (137, 138). It was originally thought to possess characteristics of a wilt toxin, because injection or exposure of elm cuttings to purified CU resulted in wilting and other disease symptoms associated with inoculation of the pathogen into trees (137). The two *Ophiostoma* species also show different levels of CU production, with the more aggressive *O. novo-ulmi* producing more CU (9, 10, 137). *CU* gene expression and colony hydrophobicity was also found to be much higher in aggressive isolates (145). However, when Δ*cu* loss-of-function

mutants were generated, they showed no visible difference in virulence. CU does, however, appear to contribute to parasitic fitness by mediating attachment to the bark beetles that transmit the fungus to new hosts (146). It was demonstrated that Δ*cu* mutants showed reduced attachment frequencies to bark beetle cuticle, consistent with such a role. CU is produced in large amounts during spore development (137) and contributes to hydrophobicity, thus enhancing dispersal, consistent with the role of a number of hydrophobins in mediating adhesion (96, 127, 136, 168). Electron microscopy showed that CU in low quantities produced a diffuse web-like coating, and in higher concentrations produced a thicker, sheath-like membrane on cell surfaces (145). This layer may also act to protect spores from desiccation (145). All of these factors would help to protect the spore while it is being carried on its vector to a new host. Recent evidence has implicated CU as a host-range determinant for disease on Dutch elm, perhaps with a direct effect on pathogenesis and a somewhat CU-related protein, cerato-platanin from *Ceratocystis fimbriata* f. sp. *platani*, has been found to be phytotoxic (113), once more implicating small hydrophobic proteins as pathogenicity factors (147).

A class II hydrophobin, cryparin, has also been identified in the chestnut blight fungus *Cryphonectria parasitica*; it is found on the surface of aerial hyphae, in fruiting bodies, and as a free protein when the fungus is grown in liquid media (180). The cryparin-encoding *Crp* gene is expressed as the most abundant mRNA during the late-growth phase of *C. parasitica* (15). As protein excretion occurs through the cell wall, it is likely that cryparin binds to the cell wall as it is being secreted and that saturation of binding sites within the wall results in release of excess cryparin into the culture fluid. However, in a pulse-labeling study by McCabe & van Alfen (94), the bulk of labeled cryparin was first found to be free in the culture fluid and only later to be bound to the cell wall. Protein secretion in filamentous fungi normally occurs at the growing hyphal tip (161, 167) and excretion of Sc3, for example, occurs in this region (165). McCabe & van Alfen suggest that cryparin is secreted at the hyphal tip but does not initially bind to the cell wall during secretion because the cell wall targets for cryparin binding are only present in the mature hyphal wall away from the hyphal tip (94). Cryparin has lectin-like properties unlike the other class II hydrophobins; this may explain why cryparin binds to the cell walls of submerged hyphae under conditions lacking a hydrophobic-hydrophilic interface (94). Different binding specificities, if they exist, may explain why cryparin can be readily extracted from cell walls with 60% ethanol whereas Sc3 must be treated with trifluoroacetic acid. An unusual trimeric hydrophobin from the ergot fungus *Claviceps fusiformis* is related to cryparin but contains three hydrophobin units linked by glycine-asparagine tracts within a single polypeptide. The protein appears to be wall-localized, and its distribution is similar to that of cryparin (27).

Hydrophobins are involved in many different processes both within single fungal species and between different fungi (125). This diversity in function is possible in spite of the limited homology observed in hydrophobins identified so far and their ability to functionally substitute for one another (78, 156). The plasticity in

hydrophobin function may therefore result from the considerable variability in gene expression profiles seen in different hydrophobin genes (77, 161, 164). Hydrophobins are normally differentially expressed and appear to be under complex transcriptional regulation to allow expression at various times during the life cycle of a fungus. Thus, the *EAS* gene in *Neurospora crassa* is regulated by the circadian clock, blue-light, and by nutrient availability, whereas *MPG1* is regulated during conidiogenesis, appressorium formation, and during starvation stress (6, 86, 143). Hydrophobins may therefore also prove useful as morphogenetic target genes for identification of upstream signaling components that regulate pathogenicity (85).

# TRANSMISSION OF INDUCTIVE SIGNALS FOR APPRESSORIUM MORPHOGENESIS

The perception of inductive signals from plant surfaces by pathogenic fungi has received considerable attention mainly as a consequence of being able to clone and characterize genes encoding signaling components based on homology to genes from *Saccharomyces cerevisiae* and other model fungi (29, 30). The conservation of such signaling molecules among eukaryotes means that it has been relatively straightforward to test the involvement of a given signal transduction pathway in infection-related development by engineering a null mutant using targeted gene replacement. The results of these studies have been significant because within a short period of time, cyclic AMP and mitogen-activated protein kinase (MAPK)-mediated signaling pathways have been directly implicated in regulating infection-related development in diverse pathogenic fungi (29, 173).

## Signal Perception and Stimulation of Secondary Messengers

The first process in signal transduction is the perception of an extracellular signal and its transmission via the plasma membrane, resulting in accumulation of intracellular signaling molecules and induction of a phosphorylation cascade. A putative membrane-bound sensor or receptor has been identified in *M. grisea* that appears to respond to the presence of hydrophobic surfaces, transmitting a cue for appressorium formation. This receptor is present in the plasma membrane and encoded by the *PTH11* gene (28). *PTH11* was identified by characterizing a set of insertion mutants in *M. grisea*—the *pth11* mutant failed to make appressoria efficiently on hydrophobic surfaces. As the *pth11* mutant could be complemented by exogenous addition of cAMP, it appears to operate upstream of (or convergent with) a signaling pathway leading to cAMP accumulation (28). The most likely route for transmission of a PTH11-perceived surface signal is via a GTP-binding protein signaling mechanism. No direct evidence for such an interaction has yet been found, although *M. grisea* possesses at least three distinct G-protein $\alpha$-subunit-encoding genes of which one, *MAGB*, affects appressorium formation and pathogenicity. *MAGB* encodes an inhibitory group I (G$\alpha$i) protein (92), and it

is not clear how this interacts with other signaling cascades. The fact that Δ*magB* mutants can be complemented by exogenous cAMP, however, indicates that *MAGB* operates upstream of the generation of a cAMP signal for appressorium formation (29, 92). Since *MAGB* encodes an inhibitory type class III $\alpha$-subunit protein (7), it seems plausible that the reduction in virulence in a Δ*magB* loss-of-function mutant may be due to repression of adenylate cyclase activity, perhaps by the corresponding $\beta\gamma$ subunit. The importance of a cAMP signal for infection structure formation in *M. grisea* is evident from the fact that a Δ*mac1* adenylate cyclase mutation causes complete loss of appressorium formation (18). This mutation is, however, unstable in certain strain backgrounds, and a bypass suppressor mutation, *sum1-99*, in the regulatory subunit of protein kinase A was able to restore appressorium development (1). This is consistent with a role for cAMP and PKA signaling in appressorium formation, although the role of cAMP may also be central to the maturation and function of appressoria in *M. grisea*, as discussed below. G$\alpha$ subunit proteins have also been implicated in pathogenicity of the corn smut fungus *Ustilago maydis* (119) and the chestnut blight fungus *Cryphonectria parasitica* (43), among others (7).

Transmission of the cAMP signal proceeds via cAMP-dependent protein kinase A (PKA) activity and subsequent phosphorylation of target proteins. In *M. grisea* the CPKA gene, which encodes a catalytic subunit of protein kinase A (PKA-c), is required for pathogenicity of the fungus. Appressorium morphogenesis is delayed significantly and small nonfunctional appressoria are produced, which are unable to penetrate cuticle layers (103, 174). The production of appressoria by Δ*cpkA* mutants, in contrast to the situation in Δ*mac1* adenylate cyclase mutants, indicates that there may be further PKA-encoding genes that play a part in regulating the development of appressoria. The major PKA activity in developing germ tubes is, however, encoded by the *CPKA* gene (1) and it is clearly pivotal to the production of functional appressoria (52). This may be in large part due to its role in regulating mobilization of carbohydrate and lipid stores to the appressorium (151), as discussed below. The *M. grisea* Δ*cpkA* phenotype is very similar to the results of a targeted deletion of a PKA-c gene from *Colletotrichum trifolii* (178). The resulting PKA-c mutants were able to produce appressoria but these were unable to penetrate cuticles and cause disease (178). In the powdery mildew fungus *B. graminis*, cAMP signaling plays a role in initiation of appressorium development. Intracellular cAMP levels were shown to rise during conidial differentiation on barley leaf surfaces and appressorium germ tube emergence (49, 50). Levels of cAMP then fall as the germ tube extends, and at this point application of cAMP inhibits further development. This suggests that in *B. graminis* cAMP signaling is required during the initial differentiation process, leading to emergence of the specialized appressorium germ tube (after anchoring of the conidium and primary germ tube emergence). This is consistent with PKA activity, which has been measured in developing conidia and appressorium germ tubes (49), and the presence of a PKA-c encoding gene in *B. graminis* (49, 50), which is functionally related to *CPKA* from *M. grisea* (L. Bindslev, R.P. Oliver, M.J. Kershaw & N.J. Talbot, unpublished).

The second potential signal transduction pathway involved in infection-related morphogenesis is the phospholipase C pathway leading to generation of diacylglycerol and inisitol-1,4,5-triphosphate from phosphatidyl inositol-4-5-bisphosphate. This in turn would lead to generation of a $Ca^{2+}$ signal and stimulation of calcium-/calmodulin-dependent protein kinase signaling. Evidence has been presented to indicate that this pathway may be involved in infection-related development in *M. grisea* (93), where exogenous application of diacylglycerol stimulated appressorium formation on normally noninductive surfaces (150). In *C. trifolii* and *C. gloeosporioides*, calmodulin genes were highly expressed in response to contact of conidia with hard surfaces (80, 111).

## Mitogen-Activated Protein Kinase Signaling Pathways for Infection-Related Development

Mitogen-activated protein kinase (MAPKs) and their upstream regulatory kinases comprise a functional unit that transmits input signals from the cell periphery to the nucleus to elicit appropriate gene expression (48, 61). The role of MAP kinases in filamentous fungi has been reviewed recently (173), and here we focus only on their roles in the pre-penetration phase of development by pathogenic species. MAPKs are regulated by a MAPK kinase or MEK (for MAPK/ERK kinase), which in turn is activated by a third kinase termed MAPKKK or MEKK (for MEK kinase). The budding yeast *S. cerevisiae* has six identified MAPK pathways that regulate the transition to filamentous growth, the response to mating pheromones, cell integrity, hyperosmotic stress adaptation, and ascospore formation (48, 61). The MAPK module consisting of the three kinases is in some cases held together by a scaffold protein. In the pheromone response pathway this function is fulfilled by STE5, and in the hyperosmotic stress adaptation pathway (high osmolarity glycerol–HOG pathway), some evidence suggests that the MAPKK PBS2 acts as a scaffold (48).

In phytopathogenic fungi, the role of MAPK signaling in regulation of appressorium formation was first proposed following isolation of the *PMK1* MAP kinase gene from *M. grisea. PMK1* encodes a functional homolog of *FUS3*, a MAPK gene from budding yeast involved in the response to mating pheromone (176). A targeted deletion of *PMK1* generated mutants that were unable to make appressoria and were nonpathogenic. *PMK1* is specifically involved in the regulation of appressorium formation in response to a surface signal but is also necessary for invasive growth or viability in rice plants (176). Pmk1 mutants are unable to form appressoria, but conidial germ tubes do respond to treatment with exogenous cAMP undergoing pronounced hooking and swelling. Based on this observation, it has been proposed that *PMK1* operates downstream of the original cAMP signal for appressorium formation, although a direct interaction between components of these pathways has yet to be confirmed (29, 173). Other components of the PMK1 pathway have not been identified, but as *PMK1* complements *fus3* in the yeast pheromone response pathway, it is likely that they will be related to Ste7 and Ste11 kinases from yeast (48, 120).

The identification of *PMK1* in *M. grisea*, and its pronounced mutant phenotype, has stimulated parallel studies in other foliar pathogens. In *Colletotricum lagenarium*, the causal agent of anthracnose of cucumber, a *PMK1*-related MAPK gene *CMK1* (*Colletotricum* MAP Kinase 1) was identified following a PCR-based screen to identify putative MAPK homologs. It shares significant similarity (96% amino acid identity) with the *M. grisea* PMK1 MAPK (139). *CMK1* also shares 59% identity with *S. cerevisiae* Fus3 and Kss1 and contains all 11 conserved protein kinase domains (48, 54). To determine whether *CMK1* was functionally homologous to *PMK1*, a complementation experiment was carried out. *CMK1* was introduced into a *M. grisea* Δ*pmk1* strain and shown to restore appressorium formation (139). Disruption of *CMK1* in *C. lagenarium* was carried out, and the resulting mutants were significantly impaired in conidiation and attachment of conidia to glass surfaces. Δ*cmk1* strains were unable to cause disease lesions on cucumber leaves, and it was found that conidia from mutant strains do not germinate on host leaves. This suggests that loss of pathogenicity in the Δ*cmk1* strains is mainly due to deficiency in the pre-penetration process as both conidial germination and appressorium formation are affected (139). When manual inoculation was performed through wound sites, lesions still failed to develop so *CMK1* is required for invasive growth but dispensable for vegetative growth, which is consistent with the phenotype of a *M. grisea* Δ*pmk1* strain (176). A MAPKK-encoding gene from *C. gloeosporioides* was also found to be required for appressorium differentiation, consistent with a similar signaling mechanism being involved in both *Colletotrichum* species (79).

A *PMK1*-related MAPK gene has been isolated from the necrotrophic, gray mold fungus *Botrytis cinerea* (181). Named *BMP1*, the predicted gene product is 94% identical and 95% similar to that of *M. grisea PMK1*. To examine the function of *BMP1* in *B. cinerea*, Δ*bmp1* gene replacement mutants were isolated and showed both a reduced rate of vegetative growth and loss of pathogenicity following inoculation onto host plants. As only very localized necrosis was observed following direct injection of spores from a Δ*bmp1* gene replacement mutant, it is proposed that *BMP1* is also important for fungal growth after initial plant penetration by *B. cinerea* (181). Scanning electron microscopy (SEM) revealed that Δ*bmp1* conidia germinate but the germ tube grows into a thin undifferentiated mycelium on the plant surface without penetration. While *B. cinerea* does not produce well-defined appressoria like *M. grisea*, the germ tubes have to arrest tip growth and change their direction of growth to penetrate plant cells. Germ tube tips usually swell slightly and can form appressorium-like structures in *B. cinerea* (3, 35, 121). *BMP1* may play a role in expression and secretion of cell wall–degrading enzymes such as the endopolygalacturonase gene reported to be involved in pathogenesis (55). The KSS1 MAPK pathway, for example, regulates the expression of an endopolygalacturonase gene, *PGU1*, in yeast (95). *B. cinerea* and *M. grisea* clearly share related MAPK pathways for plant infection, even though they cause different diseases and have different infection mechanisms (176, 181).

A *PMK1*-related MAPK gene has recently been identified from *Cochliobolus heterostrophus*, the causal agent of southern corn leaf blight. Spores of *C. heterostrophus* adhere to leaves, germinate, and produce small appressoria, which are distinct from the large melanized appressoria produced by *M. grisea* and *Colletotrichum species*. These small appressoria are not essential for plant penetration, which can occur directly through the cuticle or through stomata. A MAPK homolog named *CHK1* was identified in *C. heterostrophus* and shares 90% identity to *PMK1* (90). Targeted disruption of *CHK1* was carried out, and Δ*chk1* mutants showed altered colony morphology with poorly developed aerial hyphae (90). They were also completely impaired in production of conidia. Hyphal tips of wild-type *C. heterostrophus* respond to the presence of a glass or plastic surface by swelling to form small appressoria. As the Δ*chk1* strains did not conidiate, mycelial fragments were inoculated onto glass slides in the presence of a rich nutrient medium. Under these conditions, appressoria did not form; instead the hyphae continued to grow, often forming loops and coils (90). When mycelial suspensions of Δ*chk1* mutants were inoculated onto corn plants, the mutant was much less virulent than an isogenic wild-type strain, although pathogenicity was not completely lost in any Δ*chk1* mutant tested. Mutation of *CHK1* causes pleiotropic phenotypes, which suggests the MAPK is involved in regulating several developmental pathways such as the formation of appressoria, conidia, and aerial hyphae. However, all of these morphological states are likely to require cytoskeletal reorganization and alterations in polarized tip growth, and this indicates that the primary function of *CHK1* may be in regulating such morphological transitions (90). The differences in phenotype between Δ*chk1* and Δ*pmk1* mutants in *C. heterostrophus* and *M. grisea*, respectively, emphasizes the different use of similar signaling components, even in two ascomycete foliar pathogens (90, 176). Interestingly, although *CHK1* is most similar to *PMK1*, it shows significant similarities in mutant phenotype to a second *M. grisea* MAP kinase encoded by the *MPS1* gene (175), discussed below. Thus, a single MAPK cascade in *C. heterostrophus* may be sufficient to regulate developmental processes controlled by two distinct pathways in *M. grisea*. Identification of the *MPS1* homolog in *C. heterostrophus*, if present, will be significant in determining the interplay between the two pathways and the degree of comparability between genetic regulatory mechanisms in both fungi.

Further *PMK1*-related MAPK genes have now been isolated from *Fusarium oxysporum* f. sp. *lycopersici*, *Pyrenophora teres*, *Gaeumannomyces graminis* (32, 36, 91, 122) and where tested by construction of targeted loss-of-function mutations, have effects on development of conidia, infection structures, and pathogenicity. (For summary see Table 1.)

The most detailed analysis of the interplay between the cAMP response pathway and MAP kinase signaling during pre-penetration phase development in a fungal phytopathogen has been carried out in the corn smut fungus *Ustilago maydis* and has been extensively reviewed recently (73, 81). In this basidiomycete fungus, sporidia of opposite mating types encounter one another on the surface of a corn leaf and mate to form an infectious, filamentous dikaryon. The initial

**TABLE 1** FUS3/KSS1-like MAP kinase genes identified in plant pathogenic fungi

| Gene | Source | Mutant phenotype | Reference |
|---|---|---|---|
| *PMK1* | *Magnaporthe grisea* | Does not elaborate appressoria. Nonpathogenic even after introduction through wounds or by injection into plant tissue. Required for penetration and invasive growth | 176 |
| *BMP1* | *Botrytis cinerea* | Nonpathogenic. Required for penetration and invasive growth | 181 |
| *GMP1* | *Gaeumannomyces graminis* | Not determined. Functional homolog of *PMK1* | 36 |
| *CMK1* | *Colletotrichum lagenarium* | Impaired in conidiogenesis, conidial attachment, and germination. Nonpathogenic, required for invasive growth | 139 |
| *CHK1* | *Cochliobolus heterostrophus* | Completely impaired in conidiogenesis. Required for full virulence | 90 |
| *FMK1* | *Fusarium oxysporum* f. sp. *lycopersici* | Required for pathogenicity. Required for either production of vascular wilt symptoms or fruit rot. Not impaired in conidiogenesis | 32 |
| *Kpp2/ubc3* | *Ustilago maydis* | Reduced in virulence. Reduced in filamentous growth in solopathogenic haploid strains. Deficient in tumor formation and tumor size | 98, 106 |
| *PTK1* | *Pyrenophora teres* | Deficient in conidiogenesis. Nonpathogenic. Required for appressorium formation and invasive growth | 122 |

cell fusion is triggered by pheromone signaling controlled by the *a* mating-type loci, whereas subsequent filamentous hypha formation and pathogenesis are controlled by the multiallelic *b* loci (73, 81). The cellular response to pheromone exposure is transmitted via a MAP kinase cascade involving the PMK1-related MAPK, Kpp2 (Ubc3). Loss-of-function Δ*kpp2* mutants are reduced in virulence, but are still capable of causing disease symptoms in solopathogenic (*a1::mfa2*, *bE1*, *bW2*) haploid strains of the fungus that possess the *b*-encoded heterodimeric

transcription factor necessary for filamentous growth (98, 106). This indicates that *Kpp2* contributes to the regulation of pathogenesis, but is not absolutely required, perhaps owing to the presence of a second partially redundant MAPK. The MAPKK-encoding gene *Fuz7* may act upstream of *kpp2*, or in a parallel pathway, and has a similar mutant phenotype. The *kpp2/ubc3* MAPK pathway in *U. maydis* may be connected to a cAMP response pathway, which also regulates the transition from yeast-like sporidium to filamentous dikaryon. Mutations that lower the intracellular cAMP content of *U. maydis*, such as *uac1* adenylate cyclase mutations, or that prevent cAMP signaling (*adr1* protein kinase A mutants) cause filamentous growth of haploid cells. In contrast, a protein kinase A regulatory subunit mutation (*ubc1*), which results in cAMP-independent PKA signaling, causes a multiple budding phenotype (73, 81). The dimorphic switch in *U. maydis*, which is vital for pre-penetration phase development and subsequent plant infection, is therefore regulated by several of the same components that regulate apressorium formation in *M. grisea* and *C. lagenarium* and pathogenesis of *B. cinerea*, *P. teres*, and *F. oxysporum*. Thus, although the inductive signals being responded to are different in each of these species, for example, nonself recognition and pheromone responsiveness in *U. maydis* and surface perception in *M. grisea*, the mechanism by which signal transduction occurs is reasonably conserved, at least in the central MAPK module. Similarly, the output signals from these pathways, which result in highly specialized appressoria in *M. grisea* but polarized dikaryotic infection hyphae in *U. maydis*, are equally diverse.

## Appressorium Formation and Maturation

Once formed, appressoria adhere tightly to the leaf surface and then secrete extracellular enzymes, or generate physical force (or use a combination of both factors) to bring about cuticle penetration. The attachment of appressoria needs to be strong enough to withstand the invasive forces applied by fungi and to be resistant to the action of secreted fungal enzymes. Little is known about specific appressorial adhesives, although in *M. grisea* appressorial surface extracts are known to contain lipid, polysaccharide, and protein components (172). Fungi that produce melanin-pigmented appressoria such as *C. graminicola* and *M. grisea* use predominantly physical force to puncture the plant cuticle, producing a narrow penetration hypha from the base of the appressorium (60). The physical force applied has been measured in *C. graminicola* by allowing appressoria to form on optical waveguides (5). An optical waveguide is composed of a thin layer of polydimethylsiloxane sandwiched between two thin films of aluminum. To measure the invasive force, light was focused on the underside of the waveguide through a prism and the reflected light detected and quantified. The invasive force exerted by *C. graminicola* could be detected because the fungus caused the waveguide to deform as the penetration peg pushed against it. The alteration in thickness of the optical waveguide was then detected by an alteration in the intensity of the reflected light. *C. graminicola* appressoria were shown to exert an invasive force of $16.8 \pm 3.2\ \mu N$,

a considerable force that is certainly capable of breaching most plant cuticles (5, 104, 142). The invasive force generated by *C. graminicola* strongly implied that the appressorium was developing a high internal turgor pressure. In *M. grisea*, the turgor of appressoria has been measured using a cell collapse (cytorrhysis) assay and has shown that appressoria can develop pressures of up to 8 MPa (40 times that of a typical car tire). Such pressure allows the fungus to be able to penetrate inert plastic surfaces using physical force and turgor has been shown to be necessary for plant infection (67). Appressoria of *C. graminicola* and *M. grisea* develop in water droplets on plant surfaces and water is essential for development of turgor pressure. *M. grisea* accumulates enormous concentrations of glycerol in the appressorium, which draws water into the cell by osmosis to generate hydrostatic turgor. Glycerol accumulates to concentrations as high as 3.2 M in appressoria of *M. grisea* and is retained in the cell at such concentrations by the presence of the melanin cell wall layer (25). By using a cytorrhysis assay with glycerol as the external solute, it was shown that melanin-lined appressoria are relatively impermeable to glycerol whereas appressoria from mutants impaired in melanin biosynthesis allowed glycerol to pass freely across the cell wall (25).

How glycerol is accumulated in such vast quantities to generate appressorium turgor is an intriguing question. Glycerol generation in appressoria is genetically regulated in a manner distinct from the process in budding yeast where the high osmolarity glycerol MAP kinase cascade controls glycerol synthesis in response to hyperosmotic stress. In *M. grisea*, a MAPK gene called *OSM1*, functionally homologous to yeast *HOG1*, was found to be dispensable for appressorial turgor generation, although still regulating the response of the fungus to hyperosmotic stress (33). Recent evidence has implicated glycogen and lipid as the principal precursors for glycerol synthesis in appressoria of *M. grisea* (151). In this pathway, glycogen degradation would proceed via glycogen phosphorylase and amyloglycosidase activity, ultimately liberating glucose-phosphate for processing via the first steps of glycolysis. Enzymatic activities for glycerol biosynthesis from dihydroxyacetone-3-phosphate, dihydroxyacetone, and glyceraldehyde are all present in appressoria, indicating that glycerol-3-phosphate dehydrogenase and glycerol dehydrogenase enzymes are present in appressoria (151). These enzymatic activities are not, however, induced during appressorium maturation. In contrast, triacylglycerol lipase activity was found to be strongly induced during appressorium maturation in *M. grisea*, and both lipid bodies and glycogen granules have been shown to localize to the appressorium and to be degraded before the onset of turgor generation. Significantly, in nonpathogenic Δ*cpkA* mutants, lipid and glycogen degradation are severely delayed, suggesting that mobilization of conidial storage products to the developing appressorium and subsequent glycerol biosynthesis is regulated by the cAMP signaling pathway. Initial movement of lipid bodies and glycogen deposits to the developing appressorium was also found to be regulated by the *PMK1* MAP kinase, indicating that the maturation of appressoria and their specific biochemical activity is intimately associated with genetic control of initial appressorium development (151, 160).

Turgor and development of invasive forces are clearly important for penetration by *Colletotrichum* and *Magnaporthe*, but recent evidence has shown that *B. graminis* f. sp. *hordei*, which elaborates a non-melanin–pigmented appressorium, also generates substantial turgor pressure (116). Using incipient cytorrhysis and plasmolysis experiments, the internal turgor of appressoria was shown to rise to between 2 and 4 MPa during cuticle penetration. However, the presence of extracellular enzymes including cellobiohydrolase and cutinase has also been observed during appressorium penetration by *B. graminis*, indicating that primary infection may be due to a combination of physical force and enzymatic degradation of the cell wall (41, 116). The importance of extracellular enzymes in cuticle penetration has been systematically studied in the maize pathogen *C. carbonum* in a comprehensive series of experiments to produce loss-of-function mutations in genes encoding each of the major classes of extracellular enzymes (157). The principal conclusion from this series of experiments is that *C. carbonum* possesses extensive redundancy in extracellular enzyme production. By addressing the global regulation of extracellular enzyme production, however, it has been shown that production of these activities is important for pathogenicity and primary infection. The *C. carbonum SNF1* gene, homologous to the regulator of carbon catabolite repression in budding yeast, was shown to be essential for virulence on maize and to regulate extracellular enzyme production (153).

The production of penetration hyphae by appressoria, or directly from pre-penetration stage germ tubes, has not yet been studied in detail at the genetic level. In *M. grisea*, penetration peg production requires localization of actin to the hyphal apex and rapid cell wall biosynthesis as the hypha extends through the cuticle and epidermal cell wall layers (66). Production of penetration hyphae appears to be regulated in *M. grisea* by a MAP kinase pathway involving a MAPK encoded by the *MPS1* gene. *MPS1* encodes a MAPK that is 80% similar and 64% identical to the *S. cerevisiae* Slt2/Mpk1 and 65% identical to *Schizosaccharomyces pombe* MAPK Spm1/Pmk1 (152, 179). Phylogenetic analysis indicated that MPS1 clustered with kinases related to Slt2/Mpk1 and was less related to *S. cerevisiae* Hog1 or Fus3/Kss1 MAPKs (48, 61). Consistent with this, *MPS1* was able to complement Δ*slt2* mutant phenotypes including osmotically remedial autolytic growth characteristics (175). A targeted replacement of *MPS1* led to mutants that showed dramatically reduced aerial hyphal growth and conidiation, and that were also hypersensitive to the fungal cell-wall degrading enzymes. Δ*mps1* mutants were nonpathogenic when conidia were sprayed onto plants, but unlike Δ*pmk1* mutants, Δ*mps1* mutants were able to cause blast disease symptoms when conidia were injected directly into leaf tissue or introduced at wound sites. *MPS1* is thus essential for plant infection by *M. grisea* but this is not due to a role in appressorium development—appressoria form normally in Δ*mps1* mutants—but rather due to a function in regulating elaboration of the penetration hypha (175). Direct infection assays on onion epidermis revealed that appressoria of Δ*mps1* mutants were unable to penetrate cuticle layers, even though they generated turgor.

*MPS1* may have a similar role to that of the *SLT2* gene in *S. cerevisiae* where control of cell wall growth in response to membrane stress is mediated by a protein kinase C pathway that activates the MAPK, Slt2/Mpk1 (22, 23, 87). Elaboration of the penetration peg is likely to cause severe membrane stress, particularly in its early stages when an apparently wall-less appressorium pore, directly in contact with the plant surface, is distended prior to wall synthesis and hyphal elongation (66). *M. grisea* Mps1 may therefore be involved in the maintenance of cell wall integrity and in cell wall remodeling during this process and may also be necessary for polarization of the actin cytoskeleton to facilitate the formation of a penetration hypha. Consistent with severe membrane stress accompanying penetration peg formation in *M. grisea*, a protein implicated in regulating membrane integrity has been shown to be involved in penetration peg formation. The *PDE1* gene putatively encodes a P-type ATPase that is functionally related to an aminophospholipid translocase from yeast. These enzymes maintain aminophospholipid asymmetry in membrane bilayers, which is often required for regulating morphological transitions and determining the fluidity of membranes (4).

## CONCLUSIONS AND FUTURE STRATEGIES FOR INVESTIGATING PRE-PENETRATION PHASE DEVELOPMENT OF FUNGAL PATHOGENS

The most pressing objective in understanding infection-related development in phytopathogenic fungi is to integrate the knowledge gained by targeted mutation studies with cell biological and physiological investigations. Determining the interconnectedness and order of signaling pathways should prove possible in genetically tractable pathogens such as *M. grisea* and has already proceeded rapidly in *Ustilago maydis*, where clear epistatic relationships have been established allowing some ordering of the cAMP/MAP kinase pathways and mating-type control mechanisms (for reviews see 73, 81). One firm conclusion is that *PMK1*-related MAP kinases are central to infection-related development in all the pathogenic fungi so far tested, and it is therefore tempting to speculate that the central MAPK module of this pathway will be conserved in diverse phytopathogenic species, regardless of the input signals being perceived or the varied developmental biology exhibited by each species. This is a breakthrough for the study of fungal pathogens and means that a signaling framework can be established upon which to hang future studies of pre-penetration phase biology. Defining the input signals regulating signal response pathways is arguably a more difficult challenge because one cannot rely solely on the candidate gene approach that has proved so useful in defining signaling components in fungi. Definition of the signals being perceived by fungal pathogens on plant surfaces and corresponding receptors is much more likely to be achieved by a mutant approach—identifying mutants that fail to form appressoria, for example, as carried out to identify *PTH11* from *M. grisea*—or by a physiological and biochemical approach designed to identify and purify receptors. Similarly, identifying the output morphogenetic target genes regulated by

signaling pathways may require systematic study of the physiology, cell biology, and biochemistry of appressorium development in parallel to mutational studies.

## The Application of Functional Genomics Strategies

Whole genome sequences of a variety of filamentous fungi are currently being generated, including plant pathogenic fungi such as *M. grisea* and *Ustilago maydis*, and these promise to revolutionize the study of infection-related development. Significant numbers of EST sequences are also available for *B. graminis*, *Mycosphaerella graminicola*, *B. cinerea*, and other species (76). These resources make genome-level approaches to the study of infection-related development an exciting prospect. For the first time it will be possible to define all of the genetic components that are expressed specifically during spore adhesion, germination, and appressorium formation, and to identify all of the genes that are transcriptionally regulated in response to the cAMP and MAP kinase signaling pathways recognized as central to infection-related development. The genetic components of development may therefore be identified very rapidly. The generation of whole genomes also allows proteomic approaches to study development. Identification of the protein set involved in making a functional appressorium and a comparison with the proteome of a nonfunctional appressorium from a developmental mutant would offer a powerful means of determining the important effectors of appressorium function, including posttranslationally regulated proteins not recognized from gene expression studies. In combination, these genome-level approaches offer a rapid means of determining global responses to signaling pathways and to the external signals encountered by a pathogen on the leaf surface.

The most significant advance, however, may well be in technology aimed at rapidly generating mutants in phytopathogenic fungi. Generation of mutants is perhaps the most severe constraint on current progress and only by large-scale mutational studies and subsequent genetic analyses will a true understanding of pre-penetration phase development emerge. In this regard, the development of techniques for transposon-based tagging of genes, either *in vitro* using bacterial transposons to generate rapid gene disruption constructs or *in vivo* using native fungal transposons, is particularly significant and worthy of considerably more study (53). Similarly, large-scale antisense suppression of genes, as carried out recently in *Candida albicans*, offers a powerful strategy for functionally defining the components of a given developmental process (24). Taken together, the large-scale generation of mutants, coupled with genome-wide analysis of mRNAs and proteins, offers a revolutionary means of investigating phytopathogen development and suggests exciting times ahead.

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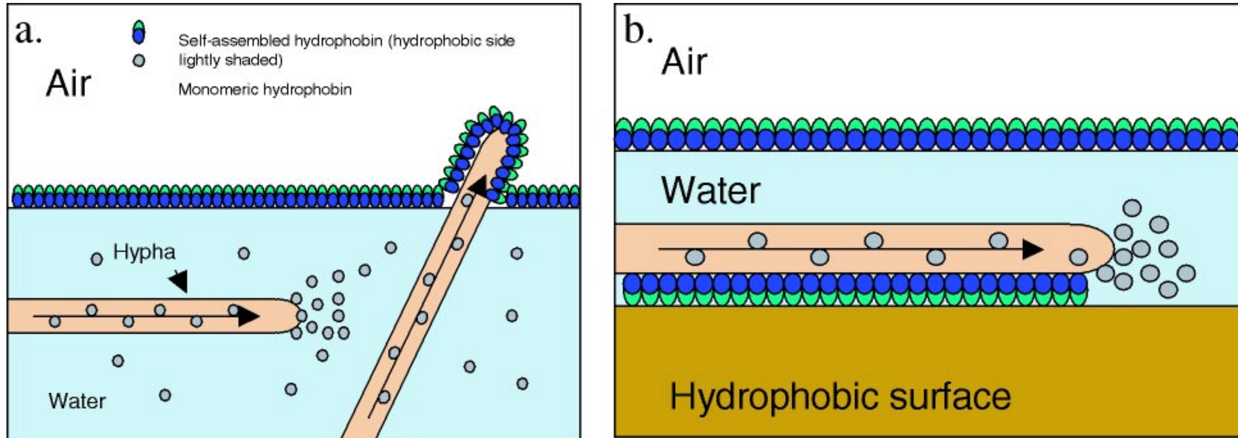


**Figure 1** (*a*) The role of hydrophobins in erection of aerial hyphae. Hydrophobins are secreted as monomeric proteins. These self-assemble when they encounter the air-water interface. Hydrophobin self-assembly decreases the surface tension allowing the hypha to emerge into the air. Continued secretion of hydrophobin produces a hydrophobic coating for the aerial structure. (Based on models presented in References 162, 164 based on data obtained in 169.) (*b*) Hydrophobin self-assembly mediates attachment of hyphae to hydrophobic surfaces. Hydrophobins are secreted as monomeric proteins and self-assemble upon encountering a hydrophobic surface. This provides a hydrophilic surface for the hyphal wall to adhere to, normally using accessory adhesive molecules such as mucilages. (Based on a model presented in Reference 168.)