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Cellular differentiation and host invasion by the rice blast fungus *Magnaporthe grisea*

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This review describes current advances in understanding the biology of plant infection by the rice blast fungus *Magnaporthe grisea*. Development of the specialized infection structure, the appressorium, in *M. grisea* has recently been shown to be controlled by cell cycle progression and initiation of autophagic, programmed cell death in the fungal spore. Re-cycling of the contents of the fungal spore and peroxisomal fatty acid β-oxidation are therefore important processes for appressorium function. Following entry to the host plant, new evidence suggests that *M. grisea* grows biotrophically within rice cells, bounded by the plant plasmalemma, and the fungus moves from cell-to-cell by means of plasmodesmata. Biotrophic proliferation of the fungus is likely to require secretion of effector proteins and suppression of host defences. Consistent with this, a component of the polarized exocytosis machinery of *M. grisea* is necessary for pathogenicity and also for induction of host defences in an incompatible interaction. Large-scale insertional mutagenesis is now allowing the rapid analysis of gene function in *M. grisea*, heralding a new approach to the study of this important fungal pathogen.

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Introduction

The filamentous ascomycete fungus *Magnaporthe grisea* is the causal agent of rice blast disease, one of the most devastating of all cereal diseases and a significant and persistent problem to rice cultivation [1]. Owing to its economic and social significance, rice blast disease has been extensively studied and *M. grisea* — which is easily grown in culture and is genetically tractable — has emerged as a model organism for studying fungal pathogenicity [1,2]. In this review, we provide an evaluation of recent progress towards understanding the biology of plant infection by the rice blast fungus and describe how these studies have provided new insight into the molecular mechanisms deployed by pathogenic fungi to cause plant disease.

The genome sequence of *M. grisea*

The complete genome sequence of *M. grisea* was recently generated [3••] and has provided the first indication of the gene inventory required by a pathogenic fungus to cause plant disease. *M. grisea* is predicted to have 11 109 genes, which is around 10% greater than its closest saprotrophic relative for which genome sequence information is available, *Neurospora crassa* (10 082). The *M. grisea* gene set is greater in number than the 6522 predicted genes of the basidiomycete plant pathogen *Usilago maydis* [4••], but similar to predicted gene numbers in other ascomycete plant pathogens such as *Stagonospora nodorum* (12 841) and *Fusarium graminearum* (11 641). These gene estimates are, however, moving numbers because gene identification techniques continually improve and large-scale empirical analysis of coding regions is underway. The *M. grisea* genome encodes a large and diverse set of secreted proteins (784 based on the most conservative estimate), including those defined by unusual carbohydrate-binding domains. The *M. grisea* genome also contains a large repertoire of putative G-protein-coupled receptor (GPCR)-encoding genes — including 61 GPCRs not previously described [3••]. One set of GPCRs contains an unusual fungal-specific extracellular membrane-spanning domain and has been termed the CFEM-GPCR group [5]. Two of the putative CFEM-GPCR-encoding genes are differentially expressed during infection-related development, consistent with a role in host/environmental perception before initial infection [3••]. The putative GPCR gene Pth11 has previously been shown to be essential for appressorium development, suggesting the importance of cell surface recognition, or perception of plant-associated signals for induction of infection structure formation [6]. The *M. grisea* genome also encodes large suites of enzymes involved in secondary metabolism, including 23 polyketide synthases, several non-ribosomal peptide synthases and cytochrome P450 mono-oxygenases, consistent with the fungus having a significant capacity for secondary metabolite production. The precise function of such metabolites in pathogenesis is not well established, but interestingly one of the PKS-encoding genes *ACE1*, has been identified as an avirulence gene [7], indicating that its product is recognized by a plant resistance protein during an incompatible interaction. It therefore seems likely that secondary metabolites produced by the fungus play significant roles within the plant during establishment of disease.

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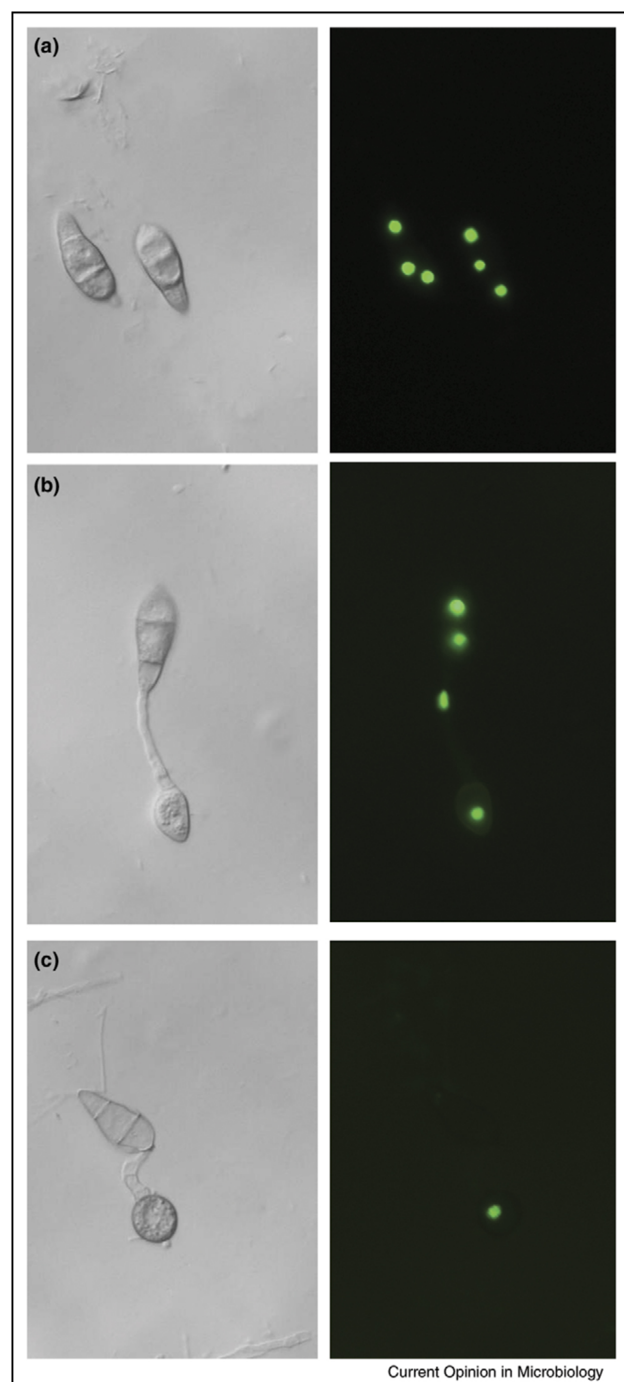
Figure 1



Photomicrographs showing *M. grisea* strain Guy11 expressing Histone H1-e-GFP in order to visualize nuclei during infection-related development. (**a**) Three-celled conidia containing one nucleus per cellular compartment. (**b**) After six-hour incubation on a hydrophobic surface, germination has occurred, followed by nuclear migration into the germ tube and mitosis. One daughter nucleus moves into the incipient appressorium while the other returns to the conidium. (**c**) After 24 hours, a single nucleus is present in the apprerssorium and the other nuclei have degenerated during autophagic cell death in the conidium. Blocking mitosis prevents appressorium formation, and blocking

(Figure 1 Legend Continued) autophagic cell death in the conidium prevents plant infection. Micrograph by Claire Veneault-Fourrey [12••].

Analysis of the genome sequence of *M. grisea* has also led to strong phylogenetic evidence for multiple horizontal gene transfers (HGTs) between filamentous ascomycete fungi and the distantly related oomycetes, which are morphologically similar, osmotrophic and filamentous eukaryotes, but part of the Chromalveolata along with photosynthetic algae. These putative gene transfer events were identified as instances when an individual *M. grisea* gene tree topology contradicted the known species relationship of the fungus [8••]. Four putative ascomycete-to-oomycete HGTs were suggested on the basis of the identification of oomycete gene sequences nested within a clade of ascomycete gene sequences with strong bootstrap support [8••]. The genes identified putatively encode proteins associated with a filamentous, osmotrophic lifestyle, such as an extracellular dioxygenase, an aldose epimerase, a purine permease and a sugar transporter. This is consistent with transfer of important gene functions between ascomycetes and oomycetes long after the divergence of the Kingdoms to which they belong.

The early events of plant infection by *M. grisea*

M. grisea causes plant infection by means of specialized infection structures called appressoria. These dome-shaped cells differentiate from the end of fungal germ tubes and generate mechanical force to bring about rupture of the plant cuticle and entry to internal tissues [9]. Turgor is generated by accumulation of compatible solutes, including glycerol, which is synthesized in large quantities in the appressorium [9,10]. The fungus then elaborates a narrow penetration hypha from the base of the appressorium, which breaches the plant cuticle and enters the host epidermis. It has also been shown that *M. grisea* can infect roots by development of distinct infection structures resembling hyphopodia [11]. Once inside roots, the fungus can invade the plant vascular system and spread to aerial parts of the plant where it produces disease lesions. These morphological adaptations are consistent with the close genetic relationship of *M. grisea* with root-infecting fungi, such as the take-all pathogen *Gaeumannomyces graminis*.

Appressorium development by *M. grisea* has recently been shown to be tightly coupled to cell cycle regulation. A *M. grisea* strain was generated expressing a Histone H1-enhanced Green Fluorescent Protein (eGFP) gene fusion, enabling nuclei to be visualized during appressorium formation. This demonstrated that the three-celled conidium germinates from a single terminal cell and one nucleus migrates into the developing germ tube where it undergoes mitosis [12••]. Following mitosis, one of the daughter nuclei migrates into the incipient appressorium, while the other returns to the cell of the conidium from which the mother nucleus originated (see Figure 1).

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When hydroxyurea or benomyl, two drugs that block cell cycle progression at S phase or G2-M phase, respectively, are applied to conidia between zero and four hours after inoculation, mitosis is inhibited in the germ tube and appressorium development does not occur. Genetic evidence for cell cycle mediated regulation of appressorium morphogenesis came from generating a temperature-sensitive allele of the *MgNIMA* gene, which encodes a protein kinase essential for mitotic entry [13]. When this conditional *Mgnima*^{E37G} allele was introduced into *M. grisea* by homologous gene replacement, appressorium development was inhibited when germinating conidia were placed at the non-permissive temperature preventing mitosis from occurring [12••]. Appressorium development by *M. grisea* is also always accompanied by collapse and death of the fungal conidium, which drains its contents into the germ tube and developing appressorium. Conidial cell death is an autophagic process because a mutant lacking the *MgATG8* gene, which encodes a protein essential for autophagy, cannot undergo conidial collapse and, as a consequence, is unable to cause rice blast disease [12••]. Appressoria formed by *ΔMgatg8* mutants are non-functional and unable to produce penetration hyphae. When considered together, these observations indicate that appressorium development by *M. grisea* is cell cycle regulated and requires autophagic, programmed cell death in order to re-cycle the contents of the fungal spore, before plant infection.

Appressorium physiology in *M. grisea*

The appressorium of *M. grisea* brings about plant infection by the application of mechanical force at the rice leaf surface, subsequent to the generation of enormous cellular turgor. Autophagic re-cycling of the contents of the conidium is clearly necessary for this process [12••] and consistent with this, trafficking of lipid bodies and glycogen to the developing appressorium can be readily observed [14]. Recent reports have shown that lipid breakdown in the appressorium is a complex process, requiring the orchestrated action of many triacylglycerol lipases [15•]. Subsequent fatty acid metabolism in the appressorium is, however, clearly pivotal to their ability to form penetration hyphae and bring about plant infection. Absence of the *MFP1* gene, encoding the multi-functional β-oxidation enzyme, leads to substantially reduced virulence, while deletion of the *PEX6* peroxisomal biogenesis gene completely prevents appressorium function and rice blast disease [15•,16•]. Consistent with this observation, analysis of the *PTH2* gene, originally identified in a genetic screen for non-pathogenic mutants, showed that it encodes a carnitine acetyl transferase, which converts acetyl-CoA to acetyl carnitine, for transport across the peroxisomal membrane for subsequent utilization. Pth2 contributes to lipid mobilization during appressorium maturation and is necessary for penetration-hypha formation [16•,17•] Peroxisome biogenesis is also, however, essential for the generation of Woronin bodies, which normally seal septal pores in fungi in response to cellular damage. Woronin body production is essential for proper development and function of appressoria [18], most likely because of the necessity in producing a sealed, high turgor cell necessary for plant infection.

MAPK signaling during infection-related development of *M. grisea*

Mitogen-activated protein kinases (MAPKs) co-ordinate diverse cellular programs in eukaryotic cells in response to environmental signals [19–21] and in *Saccharomyces cerevisiae*, for instance, five MAPK signal transduction pathways have been extensively studied [22,23]. In *M. grisea* three distinct MAPK pathways have been identified: *PMK1* (pathogenicity MAP kinase), *MPS1* (MAP kinase for penetration and sporulation) and *OSM1* (osmoregulation MAP kinase) that are homologous to *S. cerevisiae FUS3*/*KSS1*, *SLT2* and *HOG1*, respectively [24–26]. MAPK-mediated signalling pathways have now been directly implicated in regulating infection-related development in numerous phytopathogenic fungi, in which functional homologues of the *M. grisea PMK1* MAPK have been analysed [26–28,29•], highlighting the conservation of MAPK signalling as a regulatory component of fungal pathogenicity. In *M. grisea*, the Pmk1 MAPK pathway has been elucidated in considerably greater detail in the past two years (see Figure 2). Gene replacement mutants lacking *PMK1* are non pathogenic, do not form appressoria and fail to cause blast lesions on rice plants, even when inoculated directly into plant tissue. A MAPK kinase (MAPKK), Mst7, and a MAPKK kinase (MAPKKK), Mst11, activate Pmk1 in *M. grisea* [29•] and *Δmst11* and *Δmst7* mutants are defective in appressorium formation and are also non-pathogenic [29•]. Expression of a dominant active form of Mst7 restores appressorium formation to a *Δmst11* mutant, consistent with Mst7 acting downstream of Mst11 [29•,30••]. Mst7 has also been shown to be responsible for Pmk1 phosphorylation. Mst7 and Pmk1 appear to interact physically during appressorium development, on the basis of bi-molecular fluorescence complementation and co-immunoprecipitation studies. Deletion of a MAPK-docking site on Mst7 eliminates this interaction and also prevents appressorium formation [31•]. The Mst11 MAPKKK contains a sterile α-motif (SAM) domain for protein–protein interactions and associates with the SAM-containing Mst50 protein, which may function as the adaptor or scaffold protein for the Mst11-Mst7 signalling module. Deletion of *MST50* gene abolishes appressorium formation and pathogenicity [30••]. Mst50 also contains a Ras-association domain in its C-terminus and can interact with two *M. grisea* Ras proteins, encoded by *RAS1* and *RAS2*, in addition to Cdc42 and Mgb1, in a yeast two-hybrid assay. Each of these proteins may therefore transmit distinct environmental or developmental signals and interact with Mst50 to regulate appressorium development, acting upstream of the Mst11-Mst7-Pmk1 cascade [30••]. The thigmotropic response to the rice leaf

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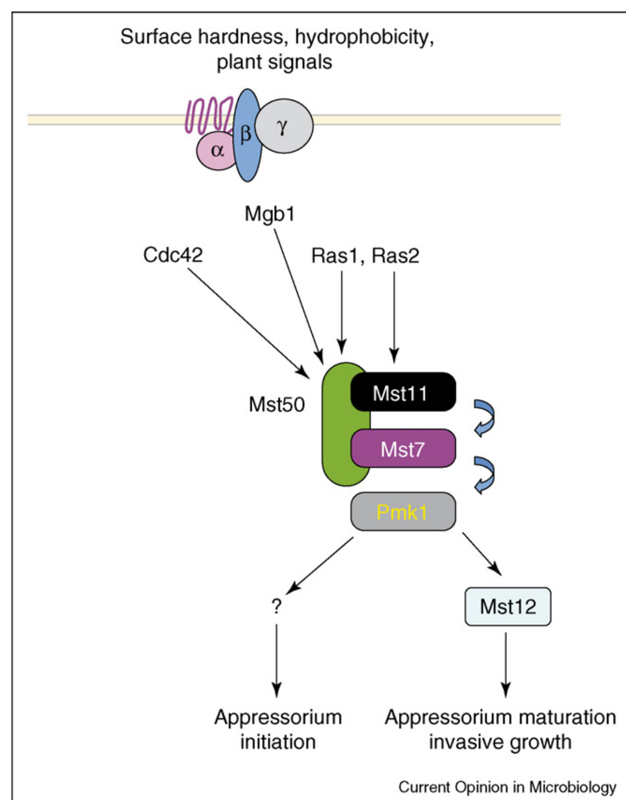
Figure 2



The Pmk1 MAP kinase pathway in *M. grisea*. The Pmk1 MAPK signalling module is regulated via interaction with Mst50, which acts as a scaffold or adaptor protein. Mst50 interacts with Ras1, Ras2, cdc42 and the gb sub0unit Mgb1 and likely transduces diverse signals from these proteins that stimulate appressorium development. Mst11 (MAPKKK) and Mst7 (MAPKK) also interact directly with Mst50. Mst7 is responsible for phosphorylating the Pmk1 MAPK and associates with the protein via a MAPK docking site. Activated Pmk1 can move to the nucleus and phosphorylate transcription factors. These include Mst12, which is responsible for regulating invasive growth by *M. grisea* (see [29•,30••,31•] for details).

surface may be mediated by heterotrimeric G-proteins and associated GPCRs, and a novel Rgs1 regulator of Ga subunits has recently been identified [32•].

The diversity of developmental phenotypes exhibited by *Δpmk1* mutants suggests that there are numerous downstream targets of the Pmk1 MAPK signalling pathway. One likely target is the Mst12 transcription factor [33], which is necessary for rice blast disease. Gene replacement mutants of *MST12* do not show obvious defects in vegetative growth, conidiation or appressorium development, but cannot bring about rice blast disease, even when the fungus is inoculated through wound sites [33]. The transcription factor may therefore regulate appressorium maturation and the development of invasive hyphae (Figure 2).

Plant tissue invasion by *M. grisea*

Studies of rice blast disease have until recently focused primarily on the pre-penetration phase of development. This has occurred largely because of the fact that non-pathogenic mutants, selected by forward genetic screens, normally exhibit defects in appressorium development [1,2]. In the past year, however, there have been significant advances in the study of *M. grisea* development in plant tissues, which has revealed that the fungus is capable of fascinating developmental biology during the colonization of rice leaf tissue. Appressorium maturation and turgor pressure lead initially to the production of a narrow (3–5 μm diameter) penetration peg, which breaks the surface of the rice leaf [1,9,34] and then forms bulbous (>5 μm diameter) invasive hyphae that grow within rice epidermal cells. Using a plasmolysis assay to detect the intactness and viability of host rice cells undergoing colonization by the fungus, it has recently been established that the fungus grows entirely within rice cells and is bounded by the rice cell plasmalemma [35••]. The membrane, termed the extra-invasive hyphal membrane, was visualized by staining with the lipophilic styryl dye FM4-64 and showed numerous connections to rice peripheral membranes. In an elegant cytological study, it was also demonstrated that *M. grisea* moves from cell-to-cell by means of plasmodesmata, appearing to seek out pit field sites as invasive hyphae move to adjacent cells [35••]. This remarkable means of tissue invasion suggests that the fungus has exquisite means of perceiving plant cell structures, altering membrane conformation and cytoskeletal organization, and also that it is capable of evading or suppressing plant defences during tissue colonization. How such plant defence suppression is achieved by *M. grisea* is not yet known but may involve delivery of effector proteins into plant cells during fungal growth *in planta*.

Bacterial pathogens have evolved type III secretion systems (TTSS) to deliver effector proteins directly into plant cells [36,37] and the functions of these effector proteins generally involve subverting the functions of the host cell to evade pathogen recognition and facilitate bacterial proliferation. This can, for instance, involve alteration of the host cytoskeleton, signal transduction or suppression of plant defence signalling [38]. By contrast, in plant pathogenic fungi delivery mechanisms for proteins during plant infection are currently unknown and only in oomycete pathogens are there emerging indications of how effector delivery might take place [39]. The best evidence that *M. grisea* are delivered to plant cells is the Avr-Pi-ta protein, a metalloprotease that acts as an avirulence protein and is recognized by the intracellular Pi-ta resistance protein. Yeast two-hybrid analysis has demonstrated a physical interaction between the host and pathogen proteins and biolistic transient expression of *Avr-Pi-ta* into a host plant carrying *Pi-ta* brings about a hypersensitive resistance response, consistent with an interaction between the

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proteins in plant cells [40]. The first clues to the means by which *M. grisea* might deliver effector proteins in rice has, however, come from analysis of a P-type ATPase family in the fungus. *M. grisea* has four putative members of the aminophospholipid translocase (APT) family of P-type ATPases, two of which, *PDE1* and *MgATP2*, are pathogenicity factors [41,42••]. APTs maintain the asymmetrical distribution of aminophospholipids in cellular membranes, which is important in both endocytosis and exocytosis. MgApt2 is a Golgi-localized protein that is required for exocytosis during plant infection [42••]. Mutants lacking *MgAPT2* make appressoria normally but are unable to progress beyond initial penetration hyphae development or to cause disease when inoculated directly into plant tissue. Interestingly, *ΔMgapt2* mutants also fail to induce a hypersensitive reaction in the resistant cultivar IR-68, even after wounding the leaf surface, suggesting that delivery of effector proteins, including the avirulence gene product necessary for HR induction in the Guy11/IR-68 interaction, was prevented by absence of the Golgi-localized P-type ATPase [42••].

Evasion of plant defence may also be brought about in *M. grisea* by expression of ABC transporters that play important roles as efflux pumps, providing resistance to a variety of metabolic poisons [43]. A previous study identified the Abc1 transporter in *M. grisea* and showed that it is required as an efflux pump during early stages of pathogenesis of rice [44]. In a recent study, the *ABC3* gene was characterized, which encodes a product with extensive similarity to several multi-drug resistance-like proteins. *ABC3* is required for *M. grisea* pathogenesis but its function is more likely to be crucial at the point of penetration peg generation by the appressorium during host surface infection [45•].

Functional genomics of *M. grisea*

The prospect for high throughput gene functional analysis in *M. grisea* appears closer after a recent study in which large mutant libraries have been generated by insertional mutagenesis. T-DNA tagging via agrobacterium-mediated transformation (ATMT) has been carried out to generate a set of >20 000 mutants of *M. grisea* [46••]. The T-DNA insertions show coverage of 61% of the genome and have so far led to identification of ∼200 novel gene loci that are necessary for rice blast disease. This industrial scale analysis of fungal pathogencity will be necessary to dissect the functions of the 10 000 or so genes that constitute the *M. grisea* genome [46••,47]. However, there are still serious obstacles to studying *M. grisea* that have yet to be overcome, including most notably the absence of saturated genetic maps to facilitate effective conventional forward genetics and map-based cloning, the absence of effective inducible promoters for conditional gene expression and a more reproducible way to carry out gene silencing. Bridging the technology gaps will significantly advance the study of this pathosystem in the forthcoming period [47].

Conclusions and future prospects

The study of pathogenicity of *M. grisea* has undergone very rapid progress in recent years and fundamental new information has been generated regarding the development of appressoria and their physiology. It will be important now to link the new information regarding the signal transduction pathways, such as the Pmk1 MAPK cascade, that are essential for appressorium morphogenesis, for the control of mitosis and the initiation of autophagic cell death in the conidium [12••,30••,31•]. The identification of downstream targets of Pmk1 will also prove pivotal in developing an understanding of appressorium physiology and morphogenesis [15•,16•,17•]. The most dramatic advances, however, have taken place in the study of plant tissue invasion by *M. grisea* and its interaction with the host plant [35••,42••]. These processes are now beginning to be investigated in much greater detail using the new techniques of live-cell imaging and reporter gene fusions to identify the individual genetic components necessary for tissue colonization by the fungus. The key questions emerging from these new studies are (1) How does the fungus deliver proteins into plant cells? (2) What are the functions of fungal effectors in rice blast disease and how is the biotrophic phase maintained during fungal proliferation? (3) What is the role of fungal secondary metabolism in plant defence modulation and do small molecules produced by the fungus play important effector-type functions, instead of (or in addition to) proteins? (4) What is the mechanism by which plasmodesmata are perceived and located by *M. grisea*, and how does the fungus then alter plant membrane organization to facilitate its movement through plasmodesmata? (5) What signals lead to disease symptom expression and eruption of conidiophores from disease lesions? Answering these questions will be the exciting challenge for the next few years and will be essential in developing a deeper understanding of this devastating disease and the fundamental nature of fungal pathogenicity.

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