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Under Pressure: Investigating the Biology of Plant Infection by *Magnaporthe oryza*

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Reviews
Under Pressure: Investigating the Biology of Plant Infection by *Magnaporthe oryza*

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Abstract

The filamentous fungus *Magnaporthe oryzae* causes rice blast, the most serious disease of cultivated rice. Cellular differentiation of *M. oryzae* forms an infection structure called the appressorium, which generates enormous cellular turgor that is sufficient to rupture the plant cuticle. Here, we show how functional genomics approaches are providing new insight into the genetic control of plant infection by *M. oryzae*. We also look ahead to the key questions that need to be addressed to provide a better understanding of the molecular processes that lead to plant disease and the prospects for sustainable control of rice blast.

Rice blast is the most important disease that affects global rice production. Its importance to food security is underlined by the fact that rice contributes 23% of the calories consumed by the global human population andis the most important food product in Asia, where 55% of the world's population lives and 92% of rice is grown and consumed. Population growth has been rapid in therice-growing regions of the world, creating an increase in demand for rice of 3% per year[1]. Indeed, a recent analysis by the International Food Policy Research Institute indicates that rice production will need to increase 38% by 2030 to feed the expanding human population and willneed to be cultivated on less ground as more arable landis lost to housing and industry.

In recent years, rice blast epidemics have occurred in China—where 5.7 million hectares of rice were destroyed between 2001 and 2005—Korea, Japan, Vietnam and the United States. The need for a better understanding of this disease becomes clear if we consider the poor durability of many blast-resistant cultivars of rice, which have atypical field life of only 2-3 growing seasons before disease resistance is overcome, and increasing energy costs, which affect fungicide and fertilizer prices[2]. Rice blast control strategies that can be deployed as part of an environmentally sustainable plan for increasing the efficiency of cereal cultivation are therefore urgently required.

Rice blast disease is caused by the filamentous ascomycete fungus *Magnaporthe oryzae*, which was recently defined as a new species, separate from *Magnaporthe grisea*, based on multilocus genealogy and mating experiments[3]. Phylogenetic analysis divides *Magnaporthe* isolates, which are morphologically indistinguishable, into two distinct clades, one that is associated with *Digitaria* (crabgrass)-infecting isolates of the fungus (*M. grisea*) and one that is associated with isolates capable of infecting rice, millets and other grasses (species of *Oryza*, *Setaria*, *Lolium*, *Eragrostis* and *Eleusine*), which was named *M. oryzae*[3].

It seems that rice-infecting strains of *M. oryzae* originated from a single point and then spread throughout the world as rice cultivation (which originated in the Middle Yangtze Valley of China around 7,000 years ago) expanded[4]. *M. oryzae* has recently emerged as a model organism for the investigation of plant diseases caused by fungi, largely because of its economic importance, but also owing to the experimental tractability of the fungus[5-7] (Box 1). Importantly, *M. oryzae* shares many characteristics associated with other important cereal pathogens, such as appressorium formation and intracellular tissue invasion[6-8]. This opens up the possibility of finding generic processes and disease determinants, which can be targeted for broad-spectrum crop disease intervention.

The Life Cycle of *M. oryzae*

The rice blast fungus attacks rice plants at all stages of development and can infect leaves, stems, nodes and panicles (Figure 1a-c). Foliar infection is initiated by attachment of a three-celled conidium of *M. oryzae* to the rice leaf cuticle. The teardrop-shaped conidium (Figure 1d) sticks tightly to the leaf surface by means of an adhesive, which is released from an apical compartment in the spore tip during hydration[9]. This adhesive provides the spore with a way to anchor itself tightly to the hydrophobic (non-stick) rice surface and allows germination to occur[9,10]. The life cycle of *M. oryzae* is shown in Figure 2. A single, polarized germ tube emerges from the spore, normally from its tapering end, andgrows across the leaf surface, before differentiating into the dome-shaped appressorium[5], as illustrated in Figure 1e. Formation of appressoria requires a hard, hydrophobic surface and can be induced in the laboratory using hydrophobic plastic surfaces, such as polytetrafluoroethylene (Teflon). On the leaf surface, the fungus may also respond to cutin monomers, ascis-9,10-epoxy-18-hydroxyoctadecanoic acid or lipidmonomers, such as 1,16-hexadecanediol, are powerful inducers of appressorium development[5,6]. Once formed, the appressorium matures and generates turgor by accumulating high concentrations of compatible solutes, such as glycerol[11]. The appressorium has a differentiated cell wall that is chitin rich and contains a distinct layer of melanin between the cell wall and the cell membrane, which is essential for turgor generation and acts as a barrier to the efflux of solute from the appressorium that occurs as pressure is generated[12]. Cellular turgor is translated into mechanical force, which is exerted by the emerging penetration peg, forcing it through the leaf cuticle. Fungal hyphae ramify through the plant tissue, resulting in the disease lesions that are symptomatic of rice blast disease. The fungus sporulates profusely from disease lesions under conditions of high humidity, allowing the disease to spread rapidly to adjacent rice plants by wind and dewdrop splash[2,5]. *M. oryzae*

Box 1. Gene Functional Analysis in *Magnaporthe oryzae*

The emergence of *M. oryzae* as a highly tractable experimental model organism for the study of plant diseases has been facilitated by the development of new methods thatallow rapid gene functional analysis. The availability of complete genome sequences forboth *M. oryzae* and rice has made gene identification, genome-wide expression profiling and proteomics straightforward[8,79,85]. These procedures have provided unparalleled insight into the genes expressed during appressorium morphogenesis and during each stage of rice blast disease[8,79,85]. In addition, the fact that rice-infecting strains of *M. oryzae*, such as Guy-11, can infect barley and the model grass species *Brachypodium distachyon*, has enabled a comparison of infection processes in each grass species[80]. Although DNA-mediated transformation is possible in filamentous fungi, the frequency of DNA uptake is normally low, which has largely precluded forward genetics and complementation cloning as a means of investigating fungal pathogenesis. These limitations have been overcome in *M. oryzae* by the application of insertional mutagenesis to generate large mutant collections[86,87] and development of methods for producing targeted gene deletions and gene silencing. Large-scale transferred DNA (T-DNA) tagging using *Agrobacterium*-mediated transformation of *M. oryzae* conidia, for example, has generated a collection of 20,000 mutants[88]. TheT-DNA insertions in this mutant collection show coverage of 61% of the *M. oryzae*genome, and this has allowed the identification of more than 200 novel gene loci thatare required for rice blast disease[88]. Similarly, targeted gene deletion in *M. oryzae* has been improved by using mutants that lack either the Ku70 or Ku80-encoding genes, which are involved in the non-homologous DNA end-joining pathway[89]. Generation of temperature-sensitive alleles, targeted insertion of reporter gene constructs and creation of other point mutations by gene replacement is also feasible, greatly extending the types of reverse genetic studies that can be carried out in the fungus. A high-throughput method for RNA interference-mediated gene silencing has also been reported recently[90]. This method uses a novel vector, pSILENT-Dual, which contains twofungal promoters arranged in a 'head-to-head' (3' to 3') orientation, one on each side of a multiple cloning site. This allows a fragment of any gene of interest to be cloned intothe vector and then introduced into the fungus, in which a double-stranded RNA molecule is transcribed, leading to RNA-mediated gene silencing[90]. This high-throughput method recently provided a rapid means of investigating thefunctions of 37 individual *M. oryzae* genes that are involved in Ca2+-mediated signaling[90]. RNA-mediated gene silencing has some disadvantages, including the occurrence of off-target effects on the expression of closely related genes and the variability of the process in *M. oryzae*. However, its potential for the study of essential genes means that it is likely to become a routine method for rapidly assessing the role of a gene while a corresponding targeted deletion mutant is being generated. *M. oryzae* is, of course, also amenable to classical genetic techniques, unlike many plant pathogenic fungi[91]. Fertile laboratory strains of the fungus allow random ascospore analysis and gene mapping, and one of the future challenges will be to use the *M. oryzae* genome sequence to facilitate map-based cloning of novel gene loci identified by classical genetic studies[91]. Finally, *M. oryzae* is increasingly being studied by live-cell imagingusing a wide range of reporters[92], and protocols for immunolocalization, ultrastructural analysis and biochemical analysis of the fungus are all available[92,93].

also has the capacity to infect roots and can form specialized infection structures produced by related root-infecting fungi, such as the take-all fungus *Gaeumannomyces graminis*[13]. Moreover, deletion of an *M. oryzae* homolog of *FOW1*, a *Fusarium oxysporum* gene that encodes a putative mitochondrial carrier protein essential for root infection[14], rendered *M. oryzae* unable to penetrate beyond the surface of the root tissue[13]. Invasive growth by *M. oryzae* involves a prolonged biotrophic stage in which the fungus grows within host plant cells, surrounded by the invaginated plant plasma membrane. An extra-invasive hyphal membrane has been visualized by staining infected rice with the lipophilic styryl dye FM4-64 and shows many connections to rice peripheral membranes[15]. The bulbous, branched cellsformed by *M. oryzae* during early tissue invasion are specialized fungal feeding structures adapted to deriving nutrition from living plant cells, and may be analogous to the haustoria produced by obligate biotrophic fungi, such as powdery mildews and rusts[5,6]. The biotrophic nature of early rice blast infections is also suggested by recent evidence that movement of the fungus from cell to cell may occur by means of plasmodesmata; the fungus appears to seek out pit field sites when invasive hyphae move to adjacent epidermal cells. Furthermore, the fungus appears to be excluded from entering stomatal guard cells, which lack plasmodesmata, consistent withthis mode of cell-to-cell spread[15]. Only later, as lesions become apparent, does the fungus become more necrotrophic, and for this reason *M. oryzae* has been classified a hemibiotroph[2,5,6].

The *M. oryzae* Genome Sequence

The availability of the *M. oryzae* genome sequence has radically altered the manner in which the biology of rice blast disease can be explored[8]. Several features of the *M. oryzae* genome provide insight into its ability to cause disease in plants. These include an elaborate predicted secretome of up to 1,546 proteins (estimates vary depending on which signal peptide prediction software is used), which is significantly higher than that observed for closely related saprophytic species, such as *Neurospora crassa*[8,16,17]. The secretome includes gene families that encode extracellular enzymes, suchas xylanases, glucanases, cutinases and other plant cell wall-degrading enzymes[16,18]. The *M. oryzae* genome also encodes putative effector proteins that could modulate plant defense mechanisms, including three families of cysteine-rich polypeptides and a protein family with similarity to the necrosis-inducing peptides of *Phytophthora infestans*[19].

The *M. oryzae* genome displays a high capacity for secondary metabolite production, and contains genes that encode 22 polyketide synthases (PKSs), 8 non-ribosomal peptide synthases (nRPSs) and 10 PKS-nRPS hybrid enzymes[19], which are particularly interesting, because they probably represent a family of enzymes that are unique to ascomycetes[19]. Hybrid PKS-nRPS enzymes are composed of a type I iterative PKS and a single nRPS module that can be truncated. They have probably arisen from agene fusion event between a PKS and an nRPS-encoding gene, followed by paralogous duplication and diversification among ascomycetes[19]. These hybrid enzymes produce a number of interesting metabolites, including the cholesterol-lowering drug lovastatin, produced by *Aspergillus terreus*, and the anti-viral drug equi-
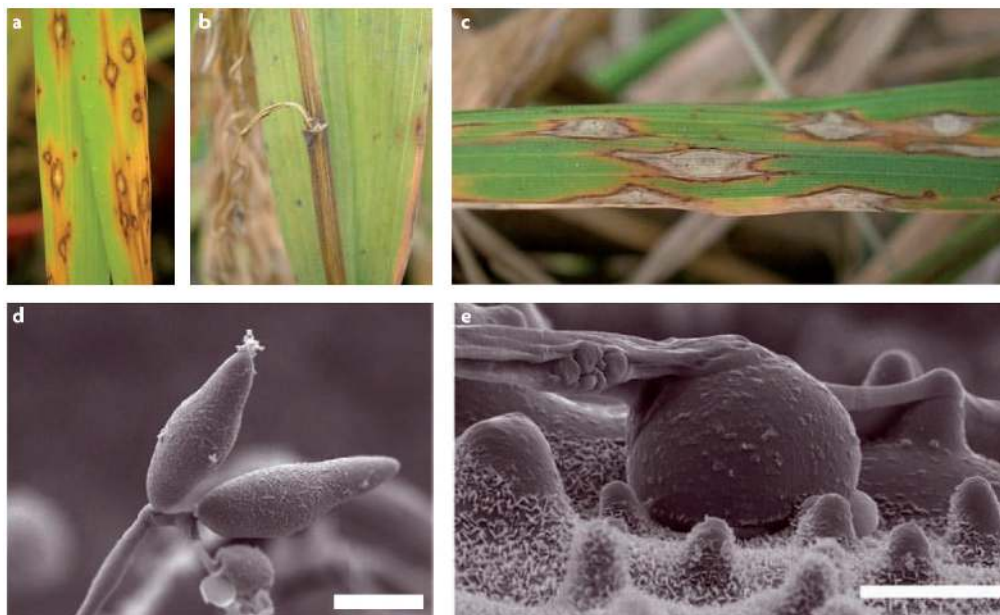
Figure 1. *Magnaporthe oryzae* causes rice blast disease. a) Rice blast affects seedlings, causing a leaf spot disease characterized by large, spreading lesions with a necrotic centre and a chlorotic margin. Under laboratory conditions, disease lesions appear 72-96 hours after inoculation of seedlings with a suspension of conidia. b) In the field, neck and panicle blast are the major causes of rice yield losses. The fungus sporulates profusely at nodes on the rice stem and rots the neck of the mature rice plant, either causing the panicle to be lost or preventing grain filling and maturation. c) Large rice blast lesions, which can be more than 1 cm in length, on a mature rice plant. *M. oryzae* sporulates from lesions, and spores are dispersed by dewdrop splash. Images a-c are from a rice blast outbreak in Hunan Province, China, in October 2007. d) Scanning electron micrograph of *M. oryzae* conidia, which initiate infection. The teardrop-shaped conidia produce an adhesive from an apical compartment that is released when the spores are wetted and attaches them to the hydrophobic leaf surface. e) Scanning electron micrograph of a dome-shaped appressorium on the rice leaf surface. The single-celled appressorium generates enormous turgor of up to 8 MPa to rupture the rice leaf cuticle. The scale bar in d represents 10 μm, whereas the scale bar in e represents 5 μm.

setin, produced by *Fusarium heterosporum*. *M. oryzae* contains more of these novel hybrid enzymes than any of the fungal species sequenced to date, and the toxin tenuazonic acid, which is a photosystem II inhibitor[20] produced by *M. oryzae* during plant infection, may be synthesized by one of these novel hybrid enzymes[19,20]. In addition, most secondary metabolic enzymes are encoded within clusters of genes that also encode enzymes; for example, cytochrome P450 monooxygenases modify nascent polyketides to produce functional secondary metabolites. Secondary metabolites produced by *M. oryzae* during the plant pathogenic process could suppress defenses and perturb host metabolism to the benefit of the invading pathogen, thereby carrying out functions often associated with protein effectors in plant pathogenic bacteria[18,19]. The avirulence gene *ACE1*, for example, encodes a polyketide synthase that is necessary for the induction of resistance in rice cultivars containing the major *Pi-33* resistance gene[21]. Loss of *ACE1* function leads to virulence, suggesting that effector-triggered immunity in rice can be caused by the biosynthesis of a secondary metabolite by the rice blast fungus[21,22]. This, together with the wide range of secondary metabolites that are likely to beproduced by *M. oryzae* and the extraordinary functional diversity of fungal metabolites that modulate cell biology in other eukaryotes[18,19], indicates that secondary metabolite production by the fungus is important

to modulation of the host response during blast infection.

The genome of *M. oryzae* also encodes a large repertoire of predicted G protein-coupled receptors (GPCRs)[8]. Of the 61 previously unidentified GPCRs that have been found in *M. oryzae*, 12 form a subfamily that contains a conserved fungi-specific extracellular membrane-spanning domain (the CFeM domain) at the amino terminus[8,23]. One member of this family, Pth11, had already been identified as an *M. oryzae* virulence factor[24] that was essential for appressorium development on hydrophobic surfaces, and all CfeM GPCRs were expressed during infection-related development; two genes were specifically expressed during appressorium formation[8]. By contrast, only one CFeM GPCR gene has been identified in the genomes of the ascomycete, saprophytic species *N. crassa* and *Aspergillus nidulans*, and interestingly, CFeM GPCRs are completely absent from the hemi-ascomycete yeasts and the basidiomycetes. Proliferation of G protein-coupled extracellular receptors in filamentous fungi, including several pathogenic fungal species, is consistent with an enhanced ability of fungi such as *M. oryzae* to respond to environmental signals as the fungus progresses through the different stages of the disease process. A systematic, comparative genome analysis of phytopathogenic and saprotrophic fungal species has recently been published and has further defined the gene family expansions and unique genes associated with phytopathogenic fungi[16].
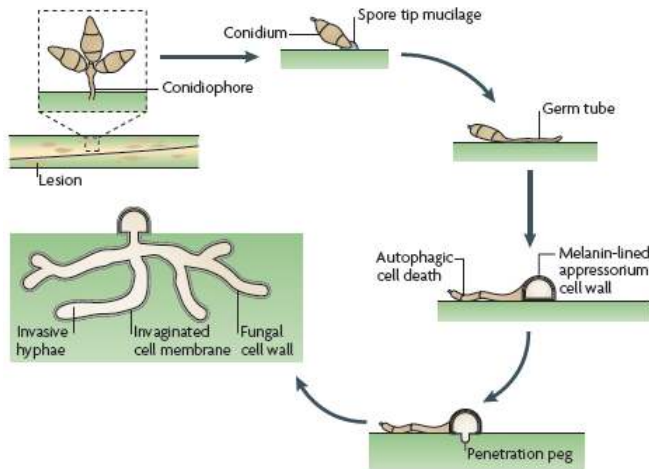
Figure 2. Life cycle of the rice blast fungus *Magnaporthe oryzae*. The rice blast fungus starts its infection cycle when a three-celled conidium lands on the rice leaf surface. The spore attaches to the hydrophobic cuticle and germinates, producing a narrow germ tube, which subsequently flattens and hooks at its tip before differentiating into an appressorium. The single-celled appressorium matures and the three-celled conidium collapses and dies in a programmed process that requires autophagy. The appressorium becomes melanized and develops substantial turgor. This translates into physical force and a narrow penetration peg forms at the base, puncturing the cuticle and allowing entry into the rice epidermis. Plant tissue invasion occurs by means of bulbous, invasive hyphae that invaginate the rice plasma membrane and invade epidermal cells. Cell-to-cell movement can initially occur by plasmodesmata. Disease lesions occur between 72 and 96 hours after infection and sporulation occurs under humid conditions; aerial conidiophores with sympodially arrayed spores are carried to new host plants by dewdrop splash.

Appressorium Development by *M. oryzae*

Appressorium formation in *M. oryzae* is regulated by cell cycle progression[25]. Emergence of the fungal germ tube from the three-celled conidium is followed by migration of one nucleus into the developing germ tube, where it undergoes mitosis 4-6 hours after germination. Following mitosis, a daughter nucleus enters the developing appressorium, while the other returns to the conidium. The three nuclei in the conidium are then degraded together with the rest of the spore contents, leaving a single nucleus in the mature appressorium. The appressorium nucleus later migrates into the penetration peg, where it undergoes further rounds of mitosis as invasive hyphae develop (Figure 2). The nucleus in the appressorium is therefore the source of all genetic material for subsequent *in planta* colonization. Appressorium development was shown to be cell cycle regulated in *M. oryzae*, as a temperature-sensitive mutation in the *MgNIMA* gene, which encodes a protein kinase that is necessary for mitosis[26], prevents appressoria from forming at non-permissive temperatures[25]. Interestingly, blocking mitosis also prevented degeneration of the conidial nuclei and collapse of the conidium. Targeted deletion of the *M. oryzae ATG8* gene, which is functionally related to the *ATG8* (also known as *AUT7*) gene in budding yeast (*Saccharomyces cerevisiae*) and is required for the generation of autophagosomes, or the *ATG1* kinase-encoding genes, resulted in mutants that were unable to undergo autophagy and could not infect plants. This showed that the mechanism of conidial cell death is autophagic[25,27,28].

The Role of Cyclic AMP Signaling in *M. oryzae*

Deletion of the *MAC1* gene, which encodes adenylate cyclase, results in mutants that cannot form appressoria, which indicates that the cyclic AMP (cAMP) response pathway also regulates appressorium morphogenesis[29]. Moreover, addition of exogenous cAMP to *Δmac1* deletion mutants remediates appressorium development and restores pathogenicity, and addition of exogenous cAMP to wild-type *M. oryzae* strains can induce appressorium development on normally non-inductive hydrophilic surfaces. cAMP generated by adenylate cyclase interacts with the regulatory subunit of cAMP-dependent protein kinase A (PKA), resulting in detachment and activation of the catalytic subunit, which in *M. oryzae* is encoded by *CPKA*[29]. Mutations in the PKA regulatory subunit lead to constitutive activation of CpkA and can override the cAMP signaling requirement such that appressorium development is restored to a *Δmac1* mutant[30]. However, deletion of *CPKA* still results in a small, albeit non-functional, appressorium. Interestingly, a second gene that encodes a PKA catalytic subunit exists in the *M. oryzae* genome, although its role in infection-related development is not yet known. Taken together, the results reported to date suggest that PKA activation is required for the generation of infection-competent appressoria.

The Pmk1 MAPK Signaling Pathway

The *M. oryzae PMK1* gene encodes a homolog of the mitogen-activated protein kinases Fus3 and Kss1 from *S. cerevisiae*, and is essential for appressorium formation and infectious hyphal growth[31]. *Δpmk1* mutants still recognize, and respond to, hydrophobic surfaces or exogenous cAMP, by producing swollen germ tube tips, but are unable to develop appressoria. Moreover, *Δpmk1* mutants cannot grow invasively in plants or form necrotic lesions, even when spores are inoculated directly into wounded leaf tissue[31]. Interestingly, *PMK1* gene homologs are also essential for disease progression in a wide range of phytopathogenic fungi, indicating that the requirement for mitogen-activated protein kinase (MAPK) signaling during plant pathogenicity is widely conserved[32].

Activation of the Pmk1 MAPK occurs through the Mst7 and Mst11 proteins (Figure 3), which are homologs of the *S. cerevisiae* Ste7 MAPK/eRK kinase (MeK) and the Ste11 MeK kinase, respectively[33]. *M. oryzae* deletion mutants that lack these genes fail to form appressoria on hydrophobic surfaces. In a *Δmst11* background, expression of a dominant active allele of *MST7* restored appressorium formation on hydrophobic surfaces. However, although appressoria were fully melanized, they were smaller compared with the wild type[33]. These results positioned the Mst7 kinase downstream of Mst11 in the MAPK cascade. Pmk1 is present in an inactive form in vegetative hyphae, and is phosphorylated in appressoria[33]. In transformants that express a dominant active allele of *MST7*, phosphorylated Pmk1 is present in both appressoria and mycelia of all strains that carry this allele, except for *Δpmk1*. This indicates that constitutive activation occurs during all stages of development owing to phosphorylation of Pmk1 by the dominant active *MST7* allele and confirms that Pmk1 is the immediate downstream target of Mst7. No direct interaction occurred between Pmk1 and either Mst11 or Mst7, and only a weak interaction was detected between Mst11

and Mst7. Mst11, however, contains an N-terminal sterile α-motif(SAM) domain, which is known to interact with other proteins that carry SAM domains[34]. In *M. oryzae*, a SAM-containing protein, Mst50, was identified that is homologous to Ste50 from *S. cerevisiae*. Ste50 is not required for the pheromone response pathway in yeast, but has been shown to interact with, and regulate the function of, Ste11. Yeast two-hybrid analysis suggested that there is a strong interaction between Mst50 and Mst11, and a weaker interaction between Mst50 and Mst7 (Reference 34). However, no Mst50-Pmk1 interaction could be detected, indicating that Mst50 might associate with upstream components of the *MST11-MST7-PMK1* cascade and might function as an adaptor or scaffold protein to stabilize the Mst11-Mst7 interactions. Using bimolecular fluorescence complementation and co-immunoprecipitation, a direct interaction was revealed between Mst7 and Pmk1 and, importantly, this occurred only during appressorium formation andonly in the presence of the putative MAPK docking site in Mst7 (Reference 35). Consistent with the scaffold role for *MST50* in the Pmk1 MAPK module, *Δmst50* mutants are defective for appressorium formation and are non-pathogenic. Removal of the SAM domain from the Mst50 protein also results in loss of appressorium formation and an inability to interact with Mst11. Taken together, these results suggest that the interaction between Mst50 and Mst11 is mediated by the SAM domain and, because Pmk1 is not phosphorylated in *Δmst50* strains, that Mst50 operates upstream of Mst7 (References 32-35). In addition to the SAM domain, Mst50 carries a carboxy-terminal RAS association domain, and can interact with two *M. oryzae* Ras homologs, Ras1 and Ras2 (Reference 35). Expression of a dominant active *RAS2* allele results in abnormal appressorium formation, suggesting that Ras might play a part in regulation of the Pmk1 MAPK cascade. However, *Δmst50*, *Δmst11*, *Δmst7* and *Δpmk1* mutants that carry a dominant active allele of *RAS2* remain non-pathogenic and are unable to form appressoria, providing evidence that, although Ras proteins might interact with Mst50 to regulate appressorium development, they must function upstream of the Mst11-Mst7-Pmk1 MAPK cascade. Mst50 also interacts with Cdc42, providing a direct link to polarized growth control[32-35].

G Protein Signaling

Upstream activation ofthe Pmk1 MAPK is mediated by heterotrimeric G protein signaling in response to physical characteristics of the leaf surface, such as hardness and hydrophobicity. Furthermore, the G protein b-subunit-encoding gene, *MGB1*, is essential for appressorium development and physically interacts with Mst50 (Reference 36). Linkage of the Pmk1 MAPK pathway and the cAMP response pathway is also apparent and *Δpmk1* mutants still respond to cAMP[31]. A recent report has shown that the regulator of G protein signaling, Rgs1, interacts with all three of the Gα subunit proteins in *M. oryzae*, MagA, MagB and MagC[37]. Rgs1 interacts with the activated GTP-bound form of MagA during appressorium initiation, suggesting that Rgs1 negatively regulates GTP-MagA-dependent adenylate cyclase activity. Consistent with this, *Δrgs1* and the Rgs-insensitive magA[G187S] and magA[Q208L] strains accumulate elevated cAMP levels. MagB may benecessary to suppress appressorium formation on non-inductive surfaces and may even be a negative regula-
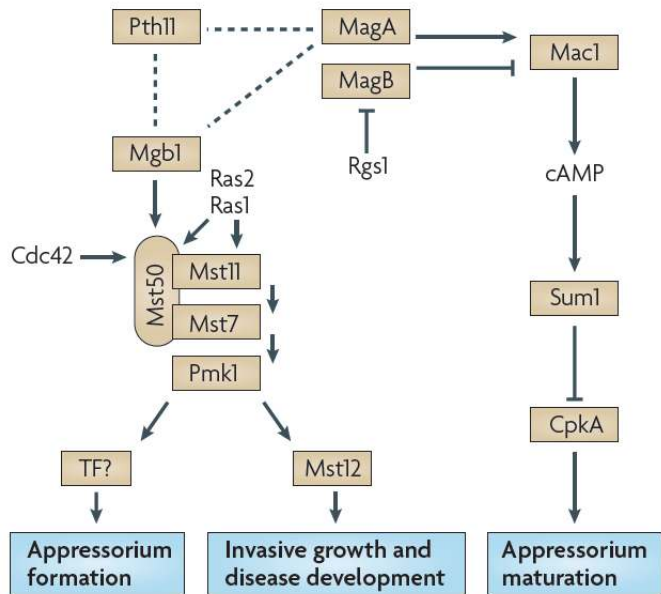

Figure 3. Signal transduction pathways required for infection-related development by *Magnaporthe oryzae*. Schematic of the Pmk1 mitogen-activated proteinkinase (MAPK) pathway and the cyclic AMP (cAMP) response pathway in the rice blast fungus. The fungus perceives the hard, hydrophobic rice leaf surface, which induces appressorium formation. The Pmk1 MAPK module is tethered by the Mst50 scaffold protein, and constitutes a phosphorelay that culminates in movement of the phosphorylated Pmk1 MAPK to the nucleus to activate transcription factors (TFs), such as Mst12. Activation of the Pmk1 pathway involves Ras proteins, Cdc42 and the Gβ-subunit protein Mgb1. Crosstalk with the cAMP pathway may occur through the Gα-subunitprotein MagB. The cAMP response pathway seems to be regulated by the G proteins MagA and MagB, which potentially interact with the Pth11 G protein-coupled receptor. Adenylate cyclase Mac1 causes the accumulation of cAMP, which binds to the regulatory protein kinase A subunit Sum1, allowing detachment of the catalytic subunit CpkA. Solid lines denote physical or genetic interactions that are supportedby experimental evidence. Dotted lines denote tentative interactions that require further experimental testing.

tor of adenylate cyclase under these conditions, as suggested by its classification to the inhibitory Gai subfamily[37]. However, *ΔmagB* mutants fail to make appressoria and can be remediated by adding cAMP[38,39]. More work is therefore required to determine its exact function during appressorium formation. The G protein-coupled receptor-encoding gene *PTH11* is necessary for appressorium development on hydrophobic surfaces, consistent with its role as the receptor for the cAMP response pathway and perhaps also the Pmk1 MAPK cascade[24]. Interestingly, the distinct functions of the two p21-activated kinase (PAK) kinase-encoding genes, *CHM1* and *MST20* (Reference 40), and the distinct biological functions of Rgs1 compared withits counterpart in *A. nidulans*, *flbA*, a regulator of conidiogenesis[41], show that upstream activation of the Pmk1and cAMP response pathways is fundamentally different in *M. oryzae* compared with other fungal model systems[42]. Determining the precise physical interactions that occur between Pth11, MagA, MagB, MagC, Mgb1, Rgs1 and Mac1 is now urgently required and will be necessary to test the model presented in Figure 3 and define how appressorium morphogenesis is genetically controlled.
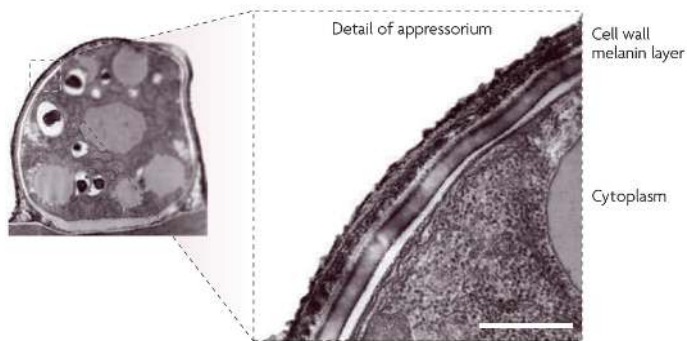
Figure 4. The appressorium cell wall. Transmission electron micrograph of a transverse section of an appressorium. The inset shows the melanin layer in the matureappressorium cell wall. The scale barrepresents 200 nm. Figure reproduced from Reference 48. Figure copyright 2007, American Phytopathological Society. Used by permission.

Physiology of Plant Infection

Maturation of the appressorium in *M. oryzae* involves deposition of melanin and chitin within the inner side of the cell wall[5,6,43] (Figure 4). Melanin plays a structural part in strengthening the appressorium and forms an impermeable layer to prevent leakage of osmolytes, which generate the substantial internal turgor (up to 8.0 MPa) required for cuticle penetration[11,12,43]. Melanin biosynthesis mutants cannot generate sufficient internal turgor owing to leakage of the appressorial contents and are consequently non-pathogenic[11,12]. Cell wall differentiation of the appressorium also involvesan oxidative burst in the developing infection cell. Prevention of superoxide generation by pharmacological intervention prevented appressorium formation and deletion of two NADPH oxidase-encoding genes, *NOX1* and *NOX2*, independently affecting appressorium function and preventing plant infection[44]. Generation of reactive oxygen species (RoS) maybe associated with cross-linking of proteins into the cell wall during appressorium maturation. Consistent with this, a cell wall-associated metallothione in encoded by the *MMT1* gene is required for appressorium function and could be necessary to promote cross-linking by preventing hydroxyl radical formation through the Fenton reaction[45]. The small GTPase MgRho3 is a potential candidate regulator of superoxide formation in *M. oryzae*. *MgRho3* deletion mutants produce small non-functional appressoria, whereas over-expression of *MgRho3* causes a hypervirulent phenotype characterized by enhanced appressorium formation and accelerated infectious growth[46]. The genome of *M. oryzae* also contains a putative p67phox (also known as NoxR) regulator gene, which may well play a part in the control of RoS generation, perhaps interacting with Rac and/or Rho3 GTPases[44,46].

The *M. oryzae* appressorium is well known for its generation of enough turgor and physical force to breach the cuticle[5,6,11]. Understanding how this isachieved in the absence of external nutrients is a fascinating physiological question. Hydrostatic turgor in appressoria is generated by the accumulation of glycerol[11], which is derived from storage compounds in the spore, such as mannitol, glycogen, lipid and trehalose. Lipid bodies are mobilized during appressorium

formation and taken up into appressorial vacuoles, where they undergo rapid lipolysis[47]. Trafficking of lipid bodies depends on Pmk1 MAPK, and lipolysis occurs by the action of up to seven intracellular triacylglycerol lipases[47,48]. Lipase activity liberates glycerol directly from the fatty acid moiety, but catabolism of fatty acids through β-oxidation is also essential for appressorium function, indicating that peroxisomal fatty acid β-oxidation, which yields acetyl-CoA, is vital to appressorium function. During the fungal infection process, fatty acid-derived acetyl-CoA can be used, for example, in secondary metabolic pathways, such as melanin biosynthesis and polyketide biosynthesis[48]. Mutants that lack isocitrate lyase show significant delays in plant infection and disease symptom generation, indicating that the glyoxylate cycle and subsequent gluconeogenesis could also fuel chitin and glucan synthesis, or osmolyte generation[49]. Deletion of *PEX6*, which encodes a peroxin, causes loss of pathogenicity owing to non-functional appressoria that are melanin-deficient and do not elaborate penetration pegs, and peroxisome biogenesis is therefore also essential for appressorium formation[49,50]. The importance of the appressorial acetyl-CoA pool is also highlighted by the observation that mutants lacking the PTH2-encoded carnitine acetyl transferase (CAT) are also non-pathogenic[50,51]. CAT activity is necessary to allow acetyl-CoA to traverse the peroxisome and mitochondrial membranes by forming acetyl carnitine. The role of Pth2 in appressorium physiology is linked to its ability to make acetyl CoA available in the cell for the generation of melanin, and probably glucan and chitin. The addition of glucose to Δ*pth2* appressoria, for example, partially restored virulence, and Δ*pth2* infection hyphae showed a marked reduction in cell wall chitin compared with the wild type[51]. Peroxisome function is also necessary for the formation of woronin bodies, which are essential for appressorium function in *M. oryzae*[52]. The importance of peroxisomal biogenesis and lipid metabolism to disease processes has also been observed in other pathogenic fungi. Peroxisome biogenesis is necessary for appressorium function in the anthracnose fungus *Colletotrichum lagenarium*[53,54], and isocitrate lyase is required for full virulence in fungal pathogens such as *Candida albicans* and the brassica pathogen *Leptosphaeria maculans*, whereas malate synthase is important for virulence in the wheat pathogen *Stagonospora nodorum*[54-57].

Trehalose is abundant in conidia and rapidly degraded during spore germination. Deletion mutants of *TPS1*, which encodes the major trehalose biosynthetic enzyme trehalose-6-phosphate synthase, do not produce trehalose and, despite germinating and forming appressoria normally, are unable to infect plants[58]. Recent work has suggested that the Tps1 protein itself is necessary for pathogenicity of *M. oryzae* rather than the production of trehalose[59]. Trehalose is synthesized from glucose-6-phosphate and uridine diphosphate-glucose by Tps1, and an associated phosphatase encoded by *TPS2*. A third subunit encoded by *TPS3* has a regulatory role in trehalose biosynthesis, and both Tps1 and Tps3 are essential for pathogenicity[59]. Interestingly, Δ*tps1* mutants cannot growon nitrate, owing to their inability to regulate the oxidative pentose phosphate pathway, which leads to a reduction in the cellular NADPH pool and loss of nitrate reductase activity. However, Tps1 also seems to regulate nitrogen-source utilization genes at the level of transcription. The role of Tps1 in
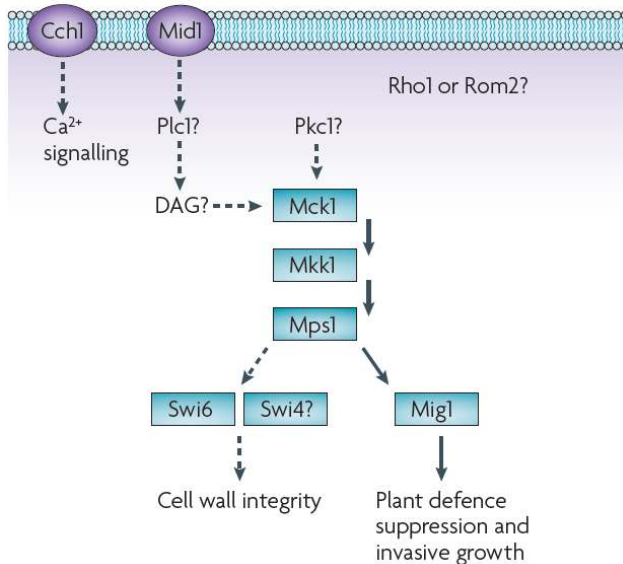
Figure 5. Schematic of the Mps1 mitogen-activated protein kinase pathway. Mps1 is necessary for penetration peg formation and plant tissue colonization. The transcription factor Mig1 is activated by Mps1 and is necessary for invasive growth and plant defense suppression. Activation of the Mps1 pathway occurs through calcium channel proteins (Cch1 or Mid1) or protein kinase C. Solid lines denote physical or genetic interactions that are supported by experimental evidence. Dotted lines denote tentative interactions that require further experimental testing. DAG, diacyl glycerol.

pathogenicity is associated with its ability to bind glucose-6-phosphate, rather than its catalytic activity[59], and it may therefore act as a nutritional sensor that is pivotal to the control of appressorium physiology. This is consistent with the emerging view that trehalose-6-phosphate synthesis is important for the integration of metabolic control and developmental gene expression in eukaryotes (reviewed in Paul et al. 2008)).

Invasive Growth Control

Formation of the penetration peg at the base of the appressorium requires the Mps1 MAPK signaling pathway, which is comparable to the cell integrity pathway in *S. cerevisiae*[61]. Mps1 is a functional homolog of the yeast protein Slt2 and is necessary for appressorium function[61]. Recent analysis of the MAPK kinase kinase Mck1, which is functionally related to Bck1 from *S. cerevisiae*, shows a similar mutant phenotype[62]. Both Δ*mps1* and Δ*mck1* mutants also show an autolytic phenotype and severe reductions in conidiogenesis, consistent with their inability to appropriately regulate cell wall biogenesis. A MADS-box transcription factor encoded by the *MIG1* gene that might act downstream of the Mps1 MAPK pathway has been characterized[63]. *MIG1* is a homolog of the *S. cerevisiae* *RLM1* MADS-box transcription factor-encoding gene, which is a downstream target of the *SLT2* MAPK. Deletion mutants of *MIG1* failed to infect plants, even when inoculated into wounded plant tissue. Furthermore, they failed to form infectious hyphae, but were able to elaborate these structures in heat-killed plant cells or after penetration of cellophane membranes. Interestingly, unlike Δ*mps1* mutants, Δ*mig1* mutants

are not autolytic and do not display overt cell wall defects. This suggests that Mig1 might be involved in the regulation of genes associated with plant defense suppression and functions associated with the colonization of living plant tissue, as shown in Figure 5. Yeast two-hybrid analysis has provided evidence of an interaction between Mig1 and Mps1, and a Mig1-GFP fusion protein was found to localize to nuclei of conidia, appressoria and invasive hyphae, but not vegetative hyphae[63]. Other likely downstream targets of Mps1 include the Swi4 and Swi6 homologs, which are necessary in *S. cerevisiae* to link cell wall biogenesis to cell cycle control (Figure 5). Homologs of *SWI4* and *SWI6* are present in *M. oryzae*, and characterizing their function in relation to plant infection is an important objective for the future.

Penetration hyphae of the rice blast fungus can breach inert plastic surfaces, consistent with a turgor-driven infection mechanism. However, the action of cutinases in plant infection may be greater than previously realized. A cutin-degrading enzyme, encoded by the *CUT1* gene, was previously shown to be dispensable for pathogenicity[64], but *M. oryzae* genome analysis has revealed a further 16 putative methyl esterase-encoding genes. One putative cutinase gene, *CUT2*, is upregulated during infection, and Δ*cut2* mutants were reported to show attenuated virulence, which could be restored to wild-type levels by adding pharmacological activators of the cAMP-protein kinase A and protein kinase C signaling cascades[65]. This suggests a developmental role for cutinase-mediated attachment to the cuticle and a potential role in cuticle rupture.

Evasion of Plant Defenses

The mechanical nature of fungal entry into the plant host contradicts the fact that, once the leaf cuticle has been breached, *M. oryzae* develops an intimate association with living plant cells and proliferates in a symptomless, biotrophic manner for the first 72 hours of infection[15]. How *M. oryzae* is able to elude or suppress plant defenses is unknown, although the process probably involves active modulation of the plant defense response by fungal-derived molecules[18]. The *M. oryzae* genome sequence predicts a large number of secreted proteins that could be used in this capacity as effectors, but whether they are secreted into the apoplastic space between the fungal cell wall and the invaginated plant plasma lemma, or delivered directly into the plant cytoplasm, is not clear[15,16]. Evidence for delivery of proteins into the cytoplasm of rice cells comes from an analysis of the Avr-Pi-ta gene product, which is perceived by a resistance protein, Pi-ta, that has a cytoplasmic leucine-rich repeat domain[66]. As a direct interaction has been detected between Pi-ta and Avr-Pi-ta[66], it seems likely that Avr-Pi-ta has an intracellular function in rice cells. Identification of such effector proteins has been attempted based on a bioinformatics analysis of the *M. oryzae* genome[16,17], to reveal secreted protein families that are over-represented in pathogenic fungal species compared with closely related saprotrophic species. The acquisition of new genome sequences from multiple isolates of the fungus will also make it possible to identify gene sets showing evidence of diversifying selection that is consistent with the need to evade recognition by host defense mechanisms. Such an approach has been used productively

to analyse effector families from oomycete pathogens, such as *P. infestans*[67,68]. Sequence alignment of the oomycete avirulence (AVR) proteins also revealed a conserved motif within 32 amino acids of the signal peptide. This motif, RXLR, resembles the RXLX (E/D/Q) motif, which is required for the translocation of proteins from the malaria parasite, *Plasmodium falciparum*, into host erythrocytes[69-71]. Three out of four AVR proteins also have an EER motif less than 25 residues downstream of the RXLR motif. In *P. infestans*, it has been shown that the RXLR-EER motifis required for delivery of AVR proteins into plant cells; translocation of an Avr3a-β-glucuronidase (GUS) fusion protein to the cytoplasm of plant cells was dependent on the presence of the RXLR-EER motif[70,71]. Such a motif is absent from proteins encoded by the *M. oryzae* genome[16,17], but the identification of a distinct, but conserved, delivery signal in effectors encoded by pathogenic fungi is an exciting prospect.

A forward genetic screen to identify genes involved in plant tissue colonization by *M. oryzae* has led to identification of a component of the secretory apparatus that might be necessary for effector function[72]. The *M. oryzae* genome has four putative type IV amino phospholipid translocases, which are integral membrane proteins of the P-type ATPase family that are required for the maintenance of membrane phospholipid asymmetry[72-76]. The *M. oryzae* genes *PDE1* and *MgAPT2* are closely related to the Drs2 family of amino phospholipid translocases, which are involved in Golgi function, endocytosis and exocytosis[73]. *PDE1* was identified by insertional mutagenesis, and formed truncated, misshapen infection hyphae that failed to ramify throughout host tissue[74]. Targeted gene replacement of *MgAPT2* resulted in a similar phenotype and loss of pathogenicity, but secretion assays that involved α-amylase showed that *MgAPT2* was required for exocytosis of a subset of extracellularproteins[72]. To test whether *MgAPT2* might be necessary for delivery of effector proteins during infection, *Δmgapt2* strains were used to challenge a resistant cultivar of rice, IR-68, and failed to elicit a hypersensitive response even when the leaf surface was abraded and spores of *Δmgapt2* were inoculated directly into plant tissue. These results suggest that secretion of the fungal protein (or proteins) recognized by the rice plant during an incompatible interaction requires a functional MgApt2 protein[72]. Characterizing this mutant further might therefore identify the repertoire of effector proteins in *M. oryzae*. Genetic regulation of effector function might involve the Mir1 gene product, a nuclear-localized protein that is specifically expressed in infected plant tissue[77]. Recent cell biological analysis of rice blast infections has also revealed the presence of membrane-rich regions at the host-pathogen interface within infected plant cells, which may be associated with effector protein delivery[15]. Investigating the role of these structures, and whether exocytotic mechanisms are deployed in these areas by the fungus and correlate with corresponding plant endocytosis of fungal proteins, will be important in the future.

Future Directions

Rapid progress has recently been made in the identification of new virulence determinants in the rice blast fungus. The availability of high-throughput methods for gene function-

al analysis (Box 1) means that obtaining mutants either by targeted deletion or gene silencing is now a trivial task. By contrast, forward genetics is still problematic and, although there has been some progress in this area, the true power of this model system will not be fully realized until saturated genetic maps are available to allow rapid map-based cloning. Once this is achieved, the use of high-throughput genetic screens will allow greater insight into the processes required for appressorium-mediated plant infection. Some of the key questions that need to be addressed in these future studies areasked in Box 2. A more integrated understanding of appressorium morphogenesis and plant infection is certainly required. The Pmk1 MAPK pathway, fore xample, is of central importance to the development of appressoria, but its relationship to initial perception of the leaf surface by the fungus, cell cycle control and infection-associated autophagy needs further exploration. We also remain ignorant of the molecular mechanisms that underpin colonization of rice tissue by *M. oryzae*, the cell-to-cell spread of the fungus and its ability to evade plant defense responses. Identification of effector proteins that could mediate this biotrophic proliferation of *M. oryzae* will most likely be achieved by comparative and evolutionary genomics approaches, coupled with transcriptional profiling of infected plant tissues using next-generation sequencing methods[78] or microarray analyses[79]. However, it is not yet clear whether there will be large sets of such proteins and whether they will have overlapping functions, or whether the fungus has a more limited repertoire of key effectors that are deployed to cause disease. The considerable secondary metabolic potential of *M. oryzae* must also not be overlooked and it is plausible, and perhaps even probable,that many effector-type functions are fulfilled by secreted fungal metabolites[18,19]. Metabolomic approaches have been developed to identify such compounds in plant tissue for rice blast infections and show considerable potential[80].

Finally, what is the prospect for durable controlof rice blast disease based on the advances in ourunderstanding of the biology of *M. oryzae* (Box 2)? New targets for fungicides may become apparent from studies of appressorium function and invasive growth. For example, the P-type ATPases that are necessary for plant infection are members of a class of protein that is known to be amenable to chemical inhibition. Similarly, conserved signaling molecules, such as components of the MAPK pathways (especially those for which mammalian and plant homologs are absent), could be effective targets for disease intervention. Plant-breeding strategies that can integrate multigene traits for disease tolerance with pyramiding of several major resistance genes could produce resistant rice cultivars thatare more durable. Integration of resistance genes incultivars such as Oryzica Llanos 5 from Columbia and the Texas cultivar Jefferson show the potential of this approach[81-84]. Biotechnology solutions to rice blast could also introduce broad-spectrum resistance genes, or pyramids of several genes, directly into high-cropping commercial rice cultivars, or could allow the intervention and rapid deployment of plant defense signaling within the plant using rice-expressed transgenes[84]. A number of these approaches have led to enhanced blast resistance in laboratory tests, but require evaluation at the field scale[84]. Thus, the prospect for durable control of rice blast still represents a substantial challenge, but certainly not an insurmountable one.

Box 2. Frontiers in Rice Blast Research

Pre-penetration stage
• How are surface cues perceived by the fungus to induce appressorium development and activation of the cyclic AMP response pathway?
• How is surface perception linked to operation of the Pmk1 mitogen-activated proteinkinase cascade and how is this integrated with cell cycle control and induction ofinfection-associated autophagy?

Plant infection
• How does the *Magnaporthe oryzae* penetration peg rupture the cuticle and enter the epidermis?
• What is the repertoire of the protein effectors produced by *M. oryzae* during invasion and how do these proteins suppress basal plant defenses in rice?
• Does *M. oryzae* deliver effector proteins into the cytoplasm of host cells and, if so, how is this accomplished?
• Do fungal secondary metabolites also act as effectors that suppress host defenses andallow *M. oryzae* to evade recognition?

Symptom development
• How is lesion formation induced by *M. oryzae*?
• What are the biological functions of the known repertoire of fungal toxins produced by *M. oryzae*?
• How does the fungus switch from biotrophic to necrotrophic growth and how is sporulation genetically regulated during disease progression?
• Do the different pathologies caused by *M. oryzae* (neck, node, panicle and leaf blast) require distinct morphogenetic programmes and different patterns of pathogen gene expression?

Host range
• What are the key genomic differences associated with host-limited forms of *M. oryzae*?
• Do effector-like proteins mediate all forms of host specificity and what is their precise biological function?

Evolution of pathogenicity
• What is the ancestral relationship of *M. oryzae* to taxonomically-related saprotrophic and mutualistic fungal species?
• How did rice pathogenic forms of *M. oryzae* first arise and how genetically distinct are they from grass pathogenic *M. oryzae* and *Magnaporthe grisea* isolates?

Disease control
• Are any key genetic determinants of rice blast disease conserved among cereal pathogens, and, if so, could they provide targets for new anti-fungal drugs?
• Can the operation of effector-triggered immunity pathways in rice be engineered to allow durable disease resistance?
• Can *M. oryzae* host-range determinants be used to identify novel resistance loci from wild grasses for introgression into commercial rice cultivars?

Glossary

Appressorium
A specialized infection cell that is used by plant pathogenic fungi to penetrate the host plant surface using either mechanical force and/or enzymatic action to breach the cuticle.

Ascomycete
A fungus that reproduces sexually and produces a structure called an ascus. An ascus is a bag that carries four or eight ascospores, which represent the products of meiosis.

Biotrophic
Refers to a plant pathogen that proliferates within living plant tissue and derives its nutrition from living plant cells. Biotrophs evade or suppress plant defense mechanisms during infection.

Compatible solute
A solute that can accumulate inside a cell to high concentrations in response to hyperosmotic conditions. Filamentous fungi often use polyols, such as glycerol, mannitol or arabitol, as compatible solutes.

Conidium
An asexual spore produced by filamentous fungi.

Fenton reaction
A reaction caused by the presence of metals, such as copper and iron, in the presence of hydrogen peroxide, causing formation of highly reactive hydroxyl radicals.

Haustorium
A specialized fungal feeding structure that occupies living plant cells by invagination of the plant plasma membrane. Haustoria are commonly produced by biotrophic fungi.

Hemibiotroph
A plant pathogen that grows initially as a biotroph but later causes severe plant disease symptoms, including host cell destruction.

Hypha
A cylindrical cell produced by filamentous fungi that extends by tip growth and forms a branched network called a mycelium.

Map-based cloning
The use of genetic mapping and molecular markers to isolate a gene based on its chromosomal position.

Metallothionein
A small, cysteine-rich protein that binds metals such as copper, zinc or iron.

Necrotrophic
Refers to a plant pathogen that kills plant cells and derives nutrition from dead or dying tissue.

Panicle
The branched inflorescence that carries rice grain on a mature rice plant.

Acknowledgements

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Databases