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Genomes, free radicals and plant cell invasion: recent developments in plant pathogenic fungi

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This review describes current advances in our understanding of fungal–plant interactions. The widespread application of whole genome sequencing to a diverse range of fungal species has allowed new insight into the evolution of fungal pathogenesis and the definition of the gene inventories associated with important plant pathogens. This has also led to functional genomic approaches to carry out large-scale gene functional analysis. There has also been significant progress in understanding appressorium-mediated plant infection by fungi and its underlying genetic basis. The nature of biotrophic proliferation of fungal pathogens in host tissue has recently revealed new potential mechanisms for cell-to-cell movement by invading pathogens.

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Introduction

Fungi cause many of the world's most significant plant diseases and are unusual among the microbial pathogens because they have the capacity to breach the intact surfaces of plant hosts to gain entry to plant tissue. To do this, fungi have evolved a variety of morphogenetic strategies to enter plants that involve elaboration of infection structures such as appressoria. Having breached the plant surface, fungal pathogens can invade and occupy living plant cells, diverting nutrients to the growing fungus and suppressing plant defence mechanisms. The biotrophic development of fungal pathogens within plant hosts is still not well understood and identifying the effector molecules necessary to establish biotrophic infections and understanding the precise nature of the fungal–plant interface remain significant challenges. In this review we provide an evaluation of recent advances in genomic analysis and developmental biology of plant pathogenic fungi and describe how this knowledge has provided fresh insight into the evolution of fungal patho-

genicity and the molecular components necessary to establish plant disease.

Lessons from fungal genomics

The most striking recent advances in understanding the biology of plant pathogenic fungi have come from the rapid advances in fungal genome analysis (for review see [1]). There are now more than 40 completed fungal genome sequences and 312 fungal genome sequencing projects in progress (according to the Genomes Online database at www.genomesonline.org) and these have provided a first opportunity to assess the gene inventories associated with plant pathogenic fungi. The ability to infect plants is a very dispersed trait across the fungal kingdom and a comparison of 42 fungal species [2], predominantly ascomycetes, recently revealed that phytopathogenicity has arisen separately at least five times in the fungi [3•]. Furthermore, comparative analysis of fungi has shown numerous gene acquisitions, gene duplications and gene losses among ascomycete fungi [4•], including novel gene acquisitions and losses among phytopathogenic fungi such as *Fusarium graminearum* and *Magnaporthe grisea*. Recent analysis of 34 fungal genomes has also revealed gene sets associated specifically with either yeasts or filamentous fungi and also revealed the extent of genetic diversification, even in fundamental biochemical processes such as lipid metabolism, that exists between fungal species [5]. The genome sequences of phytopathogenic fungi have also revealed significant levels of diversity, especially with regard to G-protein-coupled receptor-encoding genes (GPCRs), which are abundant in fungi such as *F. graminearum*, which has 84 putative GPCRs, and *M. grisea*, with 61 GPCRs, although much less well represented in the wheat glume blotch pathogen *Stagonospora nodorum* or, in particular, in the biotrophic basidiomycete, corn smut pathogen *Ustilago maydis* which has only two pheromone receptor GPCRs [6•]. This highlights the extraordinary capacity that some phytopathogens possess to respond to changes in the environment and the diversity of ligands that they must encounter, while other species have alternative strategies that do not require such an elaborate repertoire of cell surface receptors.

Genome sequence analysis has also revealed large sets of genes encoding putatively secreted proteins in phytopathogenic species. This is most apparent in *U. maydis* where there are 12 gene clusters encoding 18.6% of the predicted secreted proteins of the fungus. These gene clusters show co-ordinated expression when the fungus invades plant tissue. The predicted proteins are of

unknown function and it is worth speculating that they undertake effector-like functions in plants, perhaps suppressing plant defence responses [6**]. Recent analysis of the related basidiomycete species *Laccaria bicolor*, which is an ectomycorrhizal fungus, showed a complex predicted secretome of 2931 proteins, including a set of mycorrhiza-induced small secreted proteins (MISSPs) that may play effector-type functions. Consistent with this, one of the putative effectors, MISSP7, was shown to localize to the plant–fungal interface [7**].

Another recent surprise from carrying out comparative genome analysis in pathogenic fungi has been the likely role of lateral gene transfer in the evolutionary biology of pathogenic microbes. Strong phylogenetic evidence has been presented which suggests that lateral gene transfers have occurred between fungi and the oomycetes [8*]. This is significant because these are both filamentous, osmotrophic eukaryotic groups which are major causal agents of plant disease. Four genes were analysed phylogenetically and shown to be conserved only in ascomycete fungi and oomycetes such as *Phytophthora ramorum*, which causes sudden oak death disease and *P. sojae*, the soybean stem rot pathogen, showing characteristics of lateral transfers to these oomycete pathogens from an ascomycete-like fungus. A much more recent lateral gene transfer has been reported, however, between *S. nodorum*

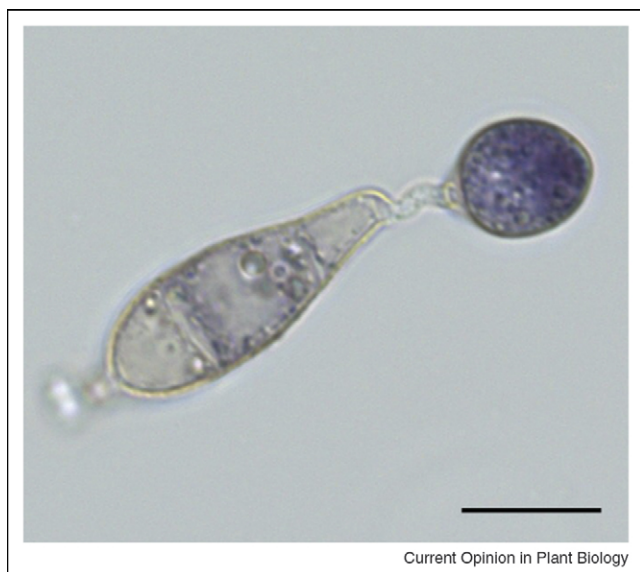
and the wheat tan spot fungus *Pyrenophora tritici-repentis*. The host-selective toxin-encoding gene, *ToxA*, allows infection of wheat cultivars which carry the *Tsn1* gene. Lateral transfer of *ToxA* from *S. nodorum* to *P. tritici-repentis* has therefore allowed spread of a much more damaging variant of tan spot disease to wheat cultivars carrying the *Tsn1* gene and around 80% of *P. tritici-repentis* isolates now carry the laterally acquired gene [9**].

Pre-penetration events

To enter the host and initiate infection, foliar fungal pathogens must first overcome the formidable barrier presented by the plant cuticle. While some fungi actively seek-out natural openings such as stomata to gain entry, many of the world's most devastating crop diseases are caused by fungi that mechanically breach the cuticle by means of specialised infection cells called appressoria. Recent evidence has shown that generation of reactive oxygen intermediates by fungal NADPH oxidase enzymes is essential for appressorium function in the rice blast pathogen *M. grisea* [10**] (Figure 1). Pharmacological scavenging of these oxygen radicals significantly delayed the development of appressoria and affected their morphology. Deletion of two NADPH oxidase encoding genes, *NOX1* and *NOX2*, independently rendered the fungus completely non-pathogenic owing to an inability to bring about appressorium-mediated cuticle penetration [10**]. By contrast, in the ergot pathogen of ryegrass *Claviceps purpurea*, the NADPH oxidase, *cpnox1*, was shown to be dispensable for host penetration, yet essential for subsequent colonization of plant ovarian tissue [11*]. It is likely that NADPH-oxidase-derived ROS regulate infection-related differentiation through the redox modification of regulatory proteins such as GTPases, and this may be a fundamental trait of plant-associated fungi including endophytic species (see review by Scott and colleagues in this issue). The small GTPase *MgRho3* is an essential regulator of pathogenicity in the rice blast fungus. *MgRho3* deletion mutants produced aberrant, narrow, ellipsoid conidia, which elaborated small non-functional appressoria [12**]. Interestingly, overexpression of *MgRho3* generates a hypervirulent phenotype, with enhanced appressorium formation and accelerated infectious growth [12**]. *Fusarium graminearum*, which does not produce specialised infection structures to enter plant cells, instead relies on the induction of a battery of cell-wall-degrading enzymes to establish infection [13], a process regulated by the mitogen-activated protein kinase *Gpmk1* [14]. Recently, the Ras GTPase, *Ras2*, has been shown to regulate virulence of the *F. graminearum* by acting upon *Gpmk1* [15]. Phosphorylation of *Gpmk1* and the expression of a secreted lipase (*FGL1*) required for infection, were significantly reduced in a *ras2* mutant [15].

A number of important plant–pathogenic fungi undergo morphological transitions during host invasion. The fungal

Figure 1



Photomicrograph showing superoxide generation during appressorium development by the rice blast fungus *Magnaporthe grisea*. Conidia were inoculated onto hydrophobic glass coverslips and incubated in a moist chamber at 26 °C for five hours. They were then stained with 0.5% nitroblue tetrazolium solution for 20 min and viewed by bright field microscopy. Bar = 10 μm. The rice blast fungus produces reactive oxygen species during plant infection and mutation of the NADPH oxidase-encoding genes *NOX1* or *NOX2* render the fungus unable to cause disease [10**].

wheat pathogen *Mycosphaerella graminicola*, for instance, switches from a yeast-like form to an infectious filamentous growth habit that penetrates the host foliage through stomata. This virulence-associated dimorphic switch has recently been shown to be regulated in part, by the mitogen-activated protein kinase MgHog1 [16]. *MgHog1* mutants show impaired initiation of infectious germ tubes and as a result are unable to infect wheat leaves [16]. The corn smut fungus *Ustilago maydis*, also exhibits a remarkable dimorphic switch from budding, yeast-like cells which fuse to form a filamentous dikaryon that penetrates and colonizes plant tissue. This sustained period of polarised growth necessary for *U. maydis* infection, is thought to be dependent on an essential cyclin-dependent kinase, Cdk5 [17]. The Rho-type GTPase Rac1 is similarly required for filament formation in the corn smut fungus. Furthermore, overexpression of Rac1 was sufficient to induce filamentation in haploid *U. maydis* cells [18].

Appressorium development by *M. grisea* has recently been shown to be dependent on completion of mitosis, nuclear migration, and fungal spore death [19^{••}]. An *M. grisea* strain expressing a histone H1 enhanced green fluorescent protein (eGFP) fusion protein was used to observe the pattern of nuclear division during infection-related development. During spore germination a fungal nucleus was shown to migrate into the germ tube, where mitosis occurred between four and six hours post inoculation. Following nuclear division, one of the daughter nuclei was shown to migrate back into the conidium, while the other enters the incipient appressorium [19^{••}]. When hydroxyurea was applied to conidia between zero and four hours, nuclear division within the germ tube is inhibited, and appressorium development significantly declines. This coupling of cell cycle regulation to appressorium development was tested genetically by generation of a thermo-sensitive allele of the *MgNIMA* gene, which encodes a protein kinase necessary for mitosis. When the thermosensitive *MgnimA^{E.37G}* allele was introduced into *M. grisea* by homologous gene replacement, appressorium development was inhibited at the non-permissive temperature [19^{••}]. Appressorium morphogenesis in *M. grisea* is also always accompanied by spore collapse and nuclear degeneration which appears to be an autophagic process. Mutants lacking the *MgATG8* gene, encoding a protein essential for autophagy, cannot undergo conidial collapse or nuclear degeneration [19^{••}]. Despite forming appressoria, Δ *Mgat8* mutants are completely non-pathogenic and unable to produce penetration hyphae. Consistent with this, targeted gene deletion of the *M. grisea* serine/threonine protein kinase gene *MgATG1*, which is also essential for autophagy, prevented plant infection [20]. In *M. grisea*, turgor generation requires mass transfer of lipid bodies to the incipient appressorium, followed by rapid lipolysis, a process requiring the orchestrated action of many triacylglycerol lipases [21]. While lipases display significant functional redundancy, enzymes involved in

subsequent fatty acid metabolism, such as the peroxisomal multi function β -oxidation protein Mfp1, are necessary for appressorium function in *M. grisea* [21]. Deletion of *MgPEX6*, a gene encoding a peroxin protein, essential for peroxisome biogenesis, completely prevents appressorium-mediated plant infection by the rice blast fungus [21]. Fatty acid β -oxidation yields acetyl CoA, a substrate for dihydroxynaphthalene melanin biosynthetic pathways. Interestingly, cell wall-associated melanin which is essential appressorium turgor generation, is depleted in Δ *mfp1*, Δ *pex6* and Δ *pth2* mutants, which also show other cell-wall associated mutant phenotypes [22,23,21]. The *PTH2* gene, originally identified in a genetic screen for non-pathogenic mutants, encodes a carnitine acetyl transferase, which catalyzes the traversal of acetyl CoA across the peroxisomal membrane [22,23] and is essential for appressorium function.

The role of cell wall degrading cutinases in fungi capable of turgor-driven cuticle rupture, is somewhat of a contentious issue. Previously, a cutin-degrading enzyme, encoded by the *CUT1* gene, was shown to be dispensible for pathogenicity in the rice blast fungus [24]. A recent analysis of the *M. grisea* genome has, however, revealed a further 16 putative cutinase/methyl esterase-encoding genes [25[•]]. Transcript profiling of several of these, selected following angular distribution decomposition of 13,666 ESTs, revealed one particular gene, termed *CUT2*, which was dramatically upregulated during penetration *in vivo* [25[•]]. Subsequent disruption of *CUT2* attenuated virulence owing to severe inhibition of penetration events. However, pathogenicity could be restored to wild-type levels by exogenous addition of pharmacological activators of the cAMP/protein kinase A and DAG/PKC signalling cascades which mediate appressorium formation [25[•]], indicating a morphogenetic role for cutinase-mediated attachment/penetration of the cuticle.

Plant tissue colonization by fungi

To successfully colonise host tissue, fungal pathogens must overcome the multilayered plant defence response that confronts them upon invasion. Generation of extracellular reactive oxygen species (ROS) surrounding infection sites is an early response to plant attack and a hallmark of successful recognition of plant pathogens by the host [26]. ROS are potentially toxic molecules and as a result fungal pathogens have evolved effective means of rapid ROS detoxification. The biotrophic maize pathogen, *U. maydis* regulates detoxification of host-derived H₂O₂ via the transcription factor Yap1 [27^{••}]. Deletion of *yap1* results in mutants that display increased sensitivity to H₂O₂ and are attenuated in virulence. During the early stages of maize infection, H₂O₂ accumulates in the hyphal tips of Δ *yap1* mutants, but is absent in wild-type infections. Subsequent microarray analysis has revealed a large set of Yap1-regulated genes including two peroxidase genes [27^{••}].

The ability of fungal plant–pathogens to evade detection and establish host infection could also be a consequence of their cell wall composition. A screen for pathogenicity mutants of the cucumber anthracnose fungus, *Colletotrichum lagenarium* led to identification of *ClasSD1*, an ortholog of the *Saccharomyces cerevisiae* regulator of cell wall assembly, *SSD1* [28*]. Following gene deletion, *classd1* mutants are defective in penetration owing to increased host papilla formation. Similarly, colonization of rice leaves by the *M. grisea*, Δ *Mgssd1* deletion mutant was severely reduced and accompanied by the accumulation of ROS within the host cells. Taken together, these results suggest that *SSD1*-mediated cell wall assembly might allow fungi to avoid induction of host defence responses [28*].

A recent cytological study has also revealed much about the nature of the hemibiotrophic colonization of plant tissue by *M. grisea* during blast infections. Intracellular invasive hyphae grow entirely within plant cells, and are sealed within the invaginated plant plasma membrane, which has been termed the extra-invasive hyphal membrane [29**]. Epidermal cells invaded by *M. grisea* are therefore completely viable and remain so during the initial stages of tissue colonization, before the onset of disease symptom expression. Time-lapse imaging revealed that *M. grisea* invasive hyphae actively scan plant cell walls for plasmodesmata, which they then co-opt for cell-to-cell movement, hence allowing biotrophic progression of the pathogen [29**]. This strategy raises a number of questions regarding the mechanisms of host manipulation by fungal pathogens, including their manipulation of the host cytoskeleton to allow fungal ingress, their perception mechanisms for pit field sites, the mechanism of fungal hypha constriction through plasmodesmata and the mechanism of fungal effector secretion that must be essential for development of the fungus in living host cells.

Fungal effectors

Pathogenic bacteria deliver effector proteins directly into plant cells by means of the type III secretion system, where they induce host cellular responses that suppress defence mechanisms and allow morphological changes to be elicited in host cells [30]. Similar secretion apparatus are absent in eukaryotes such as oomycetes and fungi. There is an increasing body of evidence that oomycete effector proteins contain a conserved host-cell-targeting motif, RXLR-EER, that is required for their translocation from biotrophic feeding structures called haustoria, to the plant cytoplasm [see [31**] and associated reviews in this issue]. By contrast, little is known about effector delivery in the plant pathogenic fungi [32], although genome sequence analysis in particular has identified numerous candidate effectors in fungi, as discussed previously. Evidence that fungi deliver effector proteins directly into plant cells comes largely from the percep-

tion of avirulence (Avr) proteins by intracellular host resistance (R) proteins, indicating that fungal effectors target intracellular host proteins. The immuno-localisation of the secreted broad bean rust (*Uromyces fabae*) protein, RTP1p, in the nucleus and cytoplasm of the host cell represents strong direct evidence of uptake of a fungal protein by plant cells [33], in addition to recent studies in flax rust and powdery mildew [34–36] discussed in other reviews in this issue, which also demonstrate the likely intracellular perception of fungal effector proteins. The *M. grisea* metalloprotease, Avr-Pi-ta, has been demonstrated to interact directly with a corresponding rice R-protein, Pi-ta, suggesting intracellular perception of the fungal effector. Biolistic transient expression of Avr-Pi-ta into the cytoplasm of rice cells with Pi-ta, triggers hypersensitive resistance [37]. Another *M. grisea* avirulence gene *ACE1*, however, differs from previously characterised Avr genes because it encodes a cytoplasmic enzyme involved in secondary metabolism, and is expressed only in appressoria [38]. It is likely that *ACE1* controls the biosynthesis of a secreted small molecular weight secondary metabolite recognised by cultivars carrying the *Pi33* R gene [38], highlighting the potential importance of secondary metabolites as effectors during fungal infection [1].

Recent characterisation of a P-type ATPase-encoding gene, *MgAPT2*, in *M. grisea*, may also provide an insight into the mechanism by which pathogenic fungi deliver effector proteins into plant cells [39*]. *MgAPT2* encodes a Golgi-localised protein required for exocytosis during plant infection. Δ *Mgapt2* mutants produce abnormal penetration hyphae and are non-pathogenic [39*]. Significantly, Δ *Mgapt2* mutants also fail to induce a hypersensitive reaction in the blast resistant cultivar IR-68, even after cuticle abrasion, suggesting that delivery of the Avr protein required for hypersensitive resistance in the Guy11/IR-68 interaction, was not secreted into the plant cell in the absence of the P-type ATPase [39*].

Future prospects

Recent advances in defining the developmental checkpoints necessary for appressorium morphogenesis by fungal pathogens and the signalling components necessary for plant infection have been achieved using the tools of molecular genetics, guided by the recent acquisition of genome sequence information for a variety of phytopathogenic species. Future progress will, however require much wider analysis of the genomes of pathogenic fungi and the deployment of high throughput methods for gene functional analysis. A recent report in which 20,000 insertional mutants were analysed in *M. grisea* demonstrates the utility of adopting a high throughput strategy for genetic analysis, as 200 novel pathogenicity loci were defined in this study [40**]. The use of strains of fungi in which the non-homologous DNA end-joining pathway has been removed by targeted deletion of either the Ku70

or Ku80-encoding genes also provides the opportunity to carry out gene replacement at much improved levels of efficiency [41,42]. Moreover, recently a high throughput method for RNA interference-mediated gene silencing has also been reported and was used to characterise the calcium signalling pathways of *M. grisea* [43^{**}]. When considered together these high throughput methods should allow rapid generation of collections of mutants of phytopathogenic fungus in which most genes in the genome have been systematically deleted or silenced. Generating such a resource will prove invaluable in determining the gene sets necessary for fungal pathogenicity and will allow greater focus on developing a systems level understanding of the establishment of plant disease.

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