Cellular Microbiology (2011) **13**(12), 1849–1857
doi:10.1111/j.1462-5822.2011.01665.x
First published online 14 September 2011

Microreview

The role of effectors of biotrophic and hemibiotrophic fungi in infection

Markus Koeck,[1,2] Adrienne R. Hardham[2] and Peter N. Dodds[1]*
[1]*CSIRO Plant Industry, Canberra, ACT 2601, Australia.*
[2]*Plant Science Division, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia.*

Summary

Biotrophic and hemibiotrophic fungi are successful groups of plant pathogens that require living plant tissue to survive and complete their life cycle. Members of these groups include the rust fungi and powdery mildews and species in the *Ustilago*, *Cladosporium* and *Magnaporthe* genera. Collectively, they represent some of the most destructive plant parasites, causing huge economic losses and threatening global food security. During plant infection, pathogens synthesize and secrete effector proteins, some of which are translocated into the plant cytosol where they can alter the host's response to the invading pathogen. In a successful infection, pathogen effectors facilitate suppression of the plant's immune system and orchestrate the reprogramming of the infected tissue so that it becomes a source of nutrients that are required by the pathogen to support its growth and development. This review summarizes our current understanding of the function of fungal effectors in infection.

Introduction

During the co-evolution of fungal plant pathogens and their hosts there has been a seesawing interplay between pathogen virulence and host resistance. To date, there are more than 10 000 fungal species known to cause diseases on plants, compared with roughly 50 species that cause disease in humans (Agrios, 2005). This diversity is reflected by a wide variety of phytopathogen infection strategies. The parasite must penetrate pre-formed and induced physical barriers, such as the waxy cuticle on the leaf surface and the plant cell wall, and withstand pre-formed and induced antimicrobial chemicals. It must induce release of plant nutrients and acquire them for its own use. Throughout infection, the pathogen must overcome or avoid triggering the host immune system. Biotrophic fungi establish a close association with the host through the development of specialized infection hyphae or haustoria within living plant cells from which nutrients are taken up (Mendgen and Hahn, 2002). On the other hand, necrotrophic fungi secrete toxins and enzymes that kill host cells and then take up nutrients released from the dead tissue (Horbach *et al*., 2011). This latter strategy may limit the capacity of the host plant to mount a defence response including production of antifungal molecules. Hemibiotrophic fungi combine both strategies (Horbach *et al*., 2011). An initial biotrophic phase, during which the host's immune system and cell death is actively suppressed, allows invasive hyphae to spread throughout the infected plant tissue. This is followed by a necrotrophic phase during which toxins are secreted by the pathogen to induce host cell death.

Until recently, the mechanisms biotrophic and hemibiotrophic fungi use to deal with the host immune system and to manipulate the living host cells were a mystery. However, in recent years it has become apparent that, like bacterial pathogens of plants and animals (Staskawicz *et al*., 2001; Hann *et al*., 2010), plant pathogenic fungi produce and secrete many so-called effector proteins that interact with the host and play an important role in virulence (Dodds *et al*., 2009; Kamoun, 2009). Since many of these fungal effectors were initially identified through their recognition by the plant immune system, in this review we will first briefly summarize plant immunity and then discuss the role of effectors from various model pathogenic fungi.

Multilayered plant immunity

Plants are able to recognize the presence of pathogens on different levels during infection (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Initially, recognition of

Received 30 June, 2011; revised 10 August, 2011; accepted 10 August, 2011. *For correspondence. E-mail Peter.Dodds@csiro.au; Tel. (+61) 2 6246 5039; Fax (+61) 2 62464864.

conserved pathogen-associated molecular patterns (PAMPs) leads to PAMP-triggered immunity (PTI). The second layer of defence is activated when the host recognizes specific pathogen effector proteins, which are produced to suppress PTI and facilitate infection, and is commonly referred to as effector triggered immunity (ETI).

Plants recognize common microbial patterns

Pathogen-associated molecular patterns recognized by the plant immune system are generally molecules essential to the pathogen and cannot be modified without significant loss of viability. They include the abundant bacterial proteins, elongation factor Tu (EF-Tu) and flagellin, and the fungal cell wall polysaccharide, chitin (Zipfel, 2009). During infection, PAMPs present in the extracellular space (known as the apoplast) are recognized by transmembrane pattern recognition receptors (PRRs) present on the plant plasma membrane, a process that induces PTI (Zipfel, 2009). For instance, the *Arabidopsis thaliana* flagellin receptor, FLS2, recognizes the highly conserved N-terminus of bacterial flagellin (flg22) and activates a MAP kinase pathway to induce expression of defence response genes (Gomez-Gomez and Boller, 2002). In addition, antimicrobial compounds and reactive oxygen species are produced and callose is deposited at the site of infection to strengthen the cell wall. Similarly the Arabidopsis CERK1 (Petutschnig *et al*., 2010) and rice CeBip receptors (Kaku *et al*., 2006) recognize chitin during fungal infection.

Plant pathogen effectors

In order to facilitate infection, plant pathogens secrete numerous effector proteins into the plant apoplast or cytosol. Most of our understanding of plant pathogen effectors comes from studies of prokaryotic pathogens, such as the bacterium *Pseudomonas syringae* which uses a type three secretion system (T3SS) to deliver about 30 effectors directly into the plant cytosol (Chang *et al*., 2005; Schechter *et al*., 2006). Many of these proteins target PRRs or their signalling partners, thereby interfering with defence signal transduction (Axtell and Staskawicz, 2003; Gohre and Robatzek, 2008; Hann *et al*., 2010; Xiang *et al*., 2011). One of the best characterized is *P. syringae* AvrPto. This small secreted protein inhibits PTI directly by interfering with flg22-induced FLS2 signalling inside the plant cell, allowing the bacteria to grow on *A. thaliana* (Xiang *et al*., 2008). Similar to a bacterial T3SS, plant pathogenic nematodes secrete effectors through their stylet, either into the apoplast or via feeding tubes directly into the cytosol of plant cells (Vanholme *et al*., 2004; Vieira *et al*., 2011). Less is known about effectors of filamentous pathogens, such as fungi and oomycetes, but it is now clear that these pathogens also secrete effectors into the plant apoplast or deliver them into host cells, where they may act to suppress defence responses or alter host metabolism (Ellis *et al*., 2009; Kamoun, 2009; Panstruga and Dodds, 2009).

Effector recognition by plant immune receptors

To counteract effector molecules, plants have developed an additional layer of immune recognition based on intracellular NB-LRR (nucleotide binding – leucine-rich repeats) receptor proteins that can detect individual effectors either directly or indirectly. These receptors are often referred to as resistance (R) proteins and the effectors they recognize as avirulence (Avr) proteins (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Generally, the plant ETI response to R-protein-mediated recognition is more severe than PTI and frequently results in localized plant cell death, also known as the hypersensitive response (HR). This response is particularly effective against biotrophic pathogens which depend on living host cells for nutrition.

Effector function in biotrophic and hemibiotrophic fungi

The first fungal effectors were identified in attempts to clone Avr proteins recognized by host R proteins, and it became apparent that many were recognized within the host cell cytoplasm (Jia *et al*., 2000; Dodds *et al*., 2004). This implies delivery of the effectors into the plant cell during infection, and subsequent studies have directly visualized fungal effectors inside host cells (Kemen *et al*., 2005; Khang *et al*., 2010; Rafiqi *et al*., 2010). How do they get there? No system analogous to the bacterial T3SS has been identified in fungi. The fungal effectors contain canonical secretion signals and appear to be secreted through the standard endomembrane pathway. Some insight into the process of movement into the plant cell has come from the finding that oomycete pathogens, which have similar infection strategies to fungi but belong to the Stramenopile kingdom, secrete effector proteins with an N-terminal RxLR (Arg–x–Leu–Arg) host targeting signal (HTS) (Morgan and Kamoun, 2007). This signal, together with a downstream dEER (Asp–Glu–Glu–Arg) motif, is required for host cell entry of the *Phytophthora infestans* effector Avr3a (Whisson *et al*., 2007). Internalization appears to be pathogen-independent (Dou *et al*., 2008). Kale *et al*. (2010) suggested that the RxLR motif of several oomycete effectors binds to phosphatidyl inositol phosphates (PIPs) on the outer surface of the plant plasma membrane to mediate endocytosis. In contrast, Yaeno *et al*. (2011) found that PIP binding by these effec-

tors was mediated by the C-terminal domain and not the RxLR region, arguing against a role in effector delivery. Similar pathogen-independent uptake occurs with effectors from the biotrophic flax rust fungus, *Melampsora lini* (Rafiqi *et al*., 2010), but does not correlate with PIP binding (Gan *et al*., 2010a). However, unlike oomycete effectors, fungal effector proteins do not share a conserved HTS and it is not yet known how they are targeted to and enter the plant cell.

In contrast to the relatively small set of effectors produced by bacteria, recent advances through next-generation sequencing and large-scale proteome analysis have now identified hundreds of proteins that are secreted by biotrophic and hemibiotrophic fungi (Dean *et al*., 2005; Kamper *et al*., 2006; Yoshida *et al*., 2009; Dodds, 2010; Spanu *et al*., 2010; Duplessis *et al*., 2011). Typically, these secreted proteins have low sequence homology to any known protein and there is little understanding of their function. Quite often all that is known is that they are recognized by cognate host R proteins, i.e. that they are Avr proteins (Stergiopoulos and de Wit, 2009; Gan *et al*., 2010b). Clarification of their role as effectors is hampered by apparent functional redundancy which often masks any obvious phenotype when individual genes are deleted or silenced (Muller *et al*., 2008; Mosquera *et al*., 2009; Lawrence *et al*., 2010). Nevertheless, recent research is beginning to reveal the function of increasing numbers of fungal effectors and the remainder of this review will focus on a selection of recently characterized effectors from biotrophic and hemibiotrophic fungi.

Cladosporium fulvum – a colonizer of the apoplast

Cladosporium fulvum is a biotrophic fungal pathogen which is the causal agent of leaf mould of tomato (Thomma *et al*., 2005). *C. fulvum* colonizes host leaves, where it grows in the extracellular spaces without penetrating host cells (Fig. 1). The first characterized fungal effector was Avr9 from *C. fulvum* (Vandenackerveken *et al*., 1992). Avr9 is a small (28 amino acid), cysteine-rich secreted protein with yet unknown function that triggers HR in tomatoes containing the Cf-9 resistance gene. To avoid initial host recognition, *C. fulvum* employs a dual strategy to suppress PTI induced by chitin in its cell wall. The plant apoplast contains various plant-derived cell wall-degrading enzymes, including chitinases. These can digest chitin in the fungal cell wall, which not only has a direct antifungal effect, but also releases chitin oligomers that are detected by PRRs and act as potent elicitors of PTI. During infection, *C. fulvum* secretes the effector, Ecp6, which contains a LysM chitin-binding domain and which binds selectively to the chitin oligosaccharides, preventing their recognition by host PRRs (de Jonge *et al*., 2010). To limit the release of chitin oligosaccharides, *C. fulvum* also secretes the Avr4 effector which binds to chitin in the intact fungal cell wall, preventing its hydrolysis by host chitinases (van den Burg *et al*., 2006; van Esse *et al*., 2007).

In addition to chitin-binding proteins, *C. fulvum* secretes the effector Avr2 which binds to and inhibits plant extracellular cysteine proteases required for basal defence (van Esse *et al*., 2008). Transgenic *A. thaliana* plants expressing Avr2 show enhanced susceptibility to the necrotrophic fungal pathogens of Arabidopsis, *Botrytis cinerea* and *Plectosphaerella cucumerina*. However, the transgenic Arabidopsis plants were not more susceptible to another necrotrophic fungus, *Alternaria brassicicola* nor to the biotrophic haustorium-forming oomycetes *Phytophthora brassicae* and *Hyaloperonospora parasitica* (van Esse *et al*., 2008).

The smut fungus, *Ustilago maydis* – clustering of putative effector genes

The maize pathogen *Ustilago maydis* is a biotrophic fungus that penetrates the plant cell wall and forms invasive hyphae that are surrounded by the plant plasma membrane and are thought to be the main interface for nutrient uptake and signal exchange (Brefort *et al*., 2009). The relatively small genome (20.5 Mb) of *U. maydis* compared with other plant pathogenic fungi, encodes 429 predicted secreted proteins, 70% of which are of unknown function (Kamper *et al*., 2006). About 20% of these genes are organized into 12 clusters, most of which exhibit increased expression during infection. Targeted deletion of individual clusters of these putative effector genes revealed that four clusters contribute to virulence. In contrast, deletion of one of the other eight clusters led to hypervirulence, suggesting that fungal effectors may also limit pathogen growth. Despite their general upregulation during infection, deletion of the other seven clusters did not alter virulence, possibly because of functional overlap with proteins outside these domains.

A more recent study compared the genomes of *U. maydis* and *Sporisorium reilianum*, two closely related smut fungi that infect maize (Schirawski *et al*., 2010). The two genomes show extensive synteny; however, 43 regions in the *U. maydis* genome, including seven of the 12 clusters discussed above, displayed low sequence conservation. In these divergent clusters more than 60% of the genes are predicted to encode secreted proteins, more than five times higher than in the rest of the genome (Schirawski *et al*., 2010). Deletion of six randomly picked *U. maydis* gene clusters out of the 43 regions with low sequence homology resulted in attenuated virulence in three cases, increased virulence in one case and wild-type virulence in two cases.

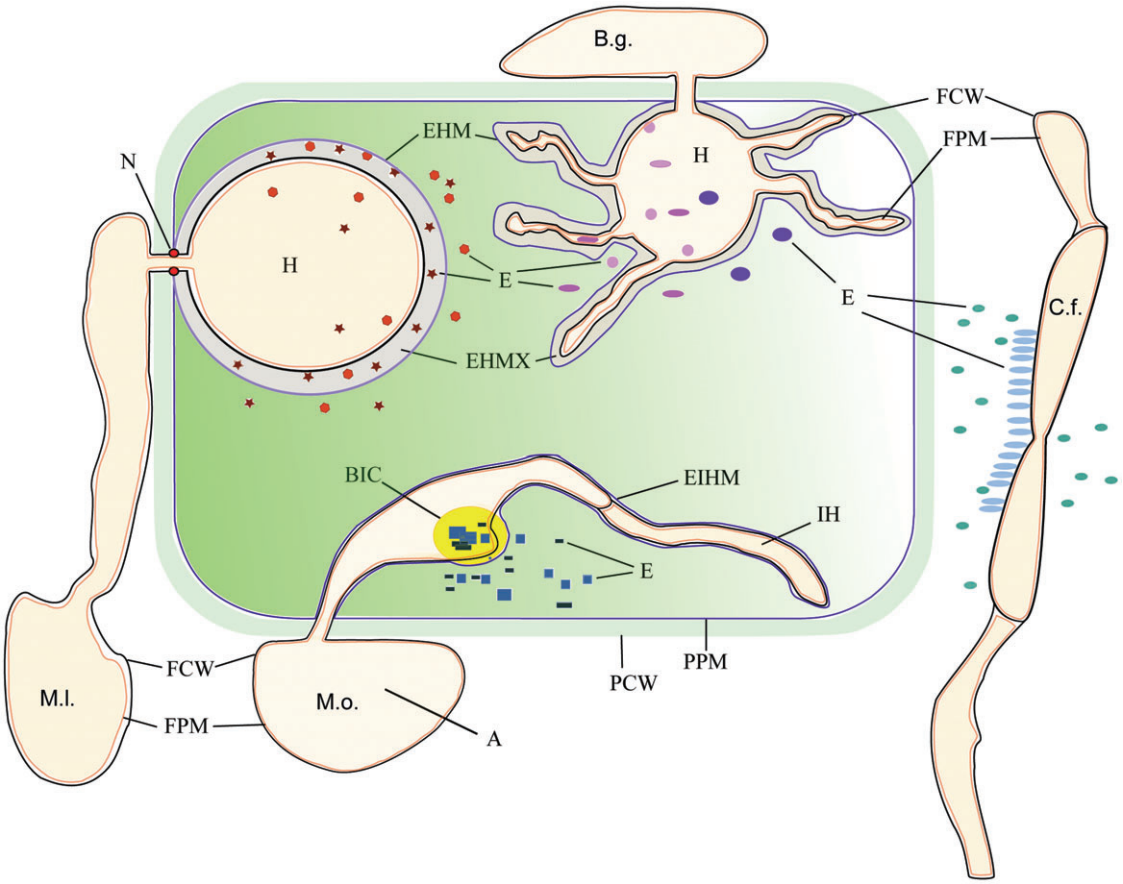

Fig. 1. Schematic diagram of fungal infection structures in and around an infected leaf cell. Four different fungal pathogens have different infection strategies, either invading the intercellular space (*Cladosporium fulvum*, C.f.), propagating hyphae within cells (*Magnaporthe oryzae*, M.o.) or penetrating host cells via haustoria (*Blumeria graminis*, B.g and *Melampsora lini*, M.l.) (see text for details). Effectors of the different pathogens (coloured shapes) are secreted from the fungal structures and either act in the apoplast or are delivered into the host cell. A, appressorium; BIC, biotrophic interfacial complex; E, effector; EHM, extrahaustorial membrane; EHMX, extrahaustorial matrix; EIHM, extrainvasive hyphal membrane; FCW, fungal cell wall; FPM, fungal plasma membrane; H, haustorium; IH, invasive hypha; N, neckband; PCW, plant cell wall; PPM, plant plasma membrane.

Although the precise roles of most of the predicted *U. maydis* effectors are unknown, the Pep1 effector has been characterized (Doehlemann *et al*., 2009). Pep1 is a small (178 amino acids) secreted protein that is required for successful invasion of epidermal cells of *Zea mays*. *In vivo* immunolocalization studies have revealed the presence of Pep1 in apoplastic spaces in leaves as well as its accumulation at sites of cell-to-cell passage of *U. maydis*. Inoculation of leaves with deletion mutants of *U. maydis* lacking Pep1 (SG200Δpep1) led to the development of large necrotic patches and failure of the pathogen to establish a compatible interaction with the host. SG200Δpep1 mutants were still able to penetrate the plant cell wall and grow within the first invaded cell, but were unable to penetrate a second cell and plant cell death occurred soon after. Expression of Pep1 in this mutant strain under the control of an early infection stage-specific promoter, allowed infection of the second cell, but further infection was still inhibited, suggesting that this protein is required for cell-to-cell passage throughout infection.

Studies of *U. maydis* proteins involved in cell adhesion and surface coating, have identified two other putative effectors that play a role in infection, namely the secreted hydrophobin, Hum3, and the hydrophobic repetitive and secreted protein, Rsp1 (Muller *et al*., 2008). Although individual mutations in these genes did not alter *U. maydis* virulence, double mutants were unable to infect *Z. mays* successfully. The double mutants showed normal morphology *in vitro*, and development, mating and aerial hyphal growth were identical to the wild type strains. However, hyphal growth in maize leaves stopped soon after the first few plant cells were invaded and the infected leaves displayed local chlorosis and necrosis (Muller *et al*., 2008). Thus, these two proteins are specifically required for the infection process allowing the fungus to successfully proliferate *in planta*.

The rice blast fungus, *Magnaporthe oryzae* – the biotrophic interfacial complex

Magnaporthe oryzae (previously *M. grisae*), the rice blast fungus, is a hemibiotrophic filamentous ascomycete and the major fungal pathogen of rice (*Oryza sativa*) (Couch and Kohn, 2002). *M. oryzae* has a genome approximately 38 Mb in size (Dean *et al*., 2005) and, depending on the threshold used for protein minimum length, encodes between 739 (Dean *et al*., 2005) and 2470 (Choi *et al*., 2010) putative secreted proteins.

After penetrating the leaf cuticle and outer epidermal cell wall, the *M. oryzae* appressorial penetration peg forms a filamentous primary hypha surrounded by the plant plasma membrane within the epidermal cell (Fig. 1). As the primary hypha develops into a bulbous, specialized invasive hypha (IH), a lobed structure called the biotrophic interfacial complex (BIC) forms at the hyphal tip (Khang *et al*., 2010). Before being translocated into the plant cytosol, *M. oryzae* effector proteins accumulate in the BIC. During the first 12 h after leaf inoculation, IH proliferate within the initially penetrated epidermal cell, but subsequently IH grow into neighbouring cells (Kankanala *et al*., 2007). Upon entering the adjacent cell, a new BIC is formed and secretes effectors. Fluorescent tagging of two BIC-associated secreted effectors, BAS1 and PWL2, showed that these proteins move from the infected cell into neighbouring, uninfected cells prior to their invasion by IH. These effectors may thus function to somehow 'prime' neighbouring cells for fungal invasion. Two other BIC-associated proteins, BAS2 and BAS3, are highly upregulated in IH, and are present at the sites at which hyphae cross the cell wall into an adjacent cell (Mosquera *et al*., 2009). In contrast, the BAS4 effector accumulates throughout the matrix between the IH and the extrainvasive hyphal membrane but not in the BIC, and was not detected in host cells, suggesting that that the BIC represents a specific interface for delivery of *M. oryzae* effectors. Individual knockout mutants of these genes did not display any phenotypic changes and their function remains to be determined.

AvrPiz-t, another *M. oryzae*-secreted fungal effector, is recognized in the plant cytosol by the cognate R protein, Piz-t, and can suppress BAX-induced cell death in tobacco (Li *et al*., 2009) suggesting that AvrPiz-t is translocated into the plant cytosol where it interferes with induction of host cell death. The fungal metalloprotease, AvrPita1, which causes R-mediated cell death in rice cultivars containing the Pi-ta R gene (Jia *et al*., 2000), is a member of the AvrPita family present in most *Magnaporthe* species (Khang *et al*., 2008). AvrPita1 is mainly localized to the BIC and in the plant cytosol where it is thought to exert its function (Khang *et al*., 2010).

The powdery mildew pathogen, *Blumeria graminis* – massive amplification of effector genes

Blumeria graminis is an obligate biotrophic fungus and the causal agent of powdery mildew disease on important crops such as barley (*B. graminis* f.sp. *hordei*) and wheat (*B. graminis* f.sp. *tritici*). *B. graminis* grows as filamentous hyphae on the leaf surface, but after penetrating the wall of the underlying epidermal cells, forms specialized feeding structures known as haustoria surrounded by an intact plant plasma membrane (Fig. 1). Because it is an obligate biotroph and cannot be grown in culture, the use of molecular genetic tools such as targeted gene knockouts to study effector function has been limited. The genome of the haploid *B. graminis* f.sp. *hordei* is estimated to be 120 Mb, about four times larger than that of other ascomycetes due to massive proliferation of retrotransposon-derived repetitive DNA (Spanu *et al*., 2010). Despite its size, the genome lacks key enzymes for primary and secondary metabolite production, transporters, enzymes for anaerobic fermentation as well as enzymes for inorganic nitrate and sulfate incorporation. The absence of these genes is likely to be associated with the pathogen's inability to grow *in vitro*.

Spanu *et al*. (2010) found 248 candidate-secreted effector proteins (CSEPs) in the *B. graminis* f.sp. *hordei* genome, defined as secreted proteins that lacked homologues outside the mildew family. In contrast to the situation in *U. maydis*, there was no obvious clustering of CSEP genes. Of the 248 CSEPs, 80% contained the N-terminal tripeptide motif Y/F/WxC, which was recently recognized in putative powdery mildew effector proteins, and predominantly occurs four amino acids after the signal peptide cleavage site (Godfrey *et al*., 2010). The conservation of this motif in powdery mildew effectors suggests a common functional role, possibly in effector delivery into the plant cytoplasm as observed for the oomycete RxLR motif. The CSEPs with an N-terminal Y/F/WxC motif are generally devoid of other cysteine residues except for a single conserved C-terminal cysteine, which may therefore constitute part of this motif, possibly forming a disulfide bond with the N-terminal cysteine.

These new CSEPs differ from the previously identified *B. graminis* effector candidates, AVR_{a10} and AVR_{k1}, which do not contain a secretion signal peptide (Ridout *et al*., 2006). Transient expression of AVR_{a10} and AVR_{k1} in susceptible plant varieties enhanced infection while their expression in resistant plant varieties carrying the cognate, cytosolic R genes Mla10 or Mlk1, respectively, caused cell death (Ridout *et al*., 2006). These experiments suggest that although AVR_{a10} and AVR_{k1} lack a recognizable signal peptide, they are secreted by the fungus and are delivered into the host plant cytoplasm. AVR_{a10} and AVR_{k1} appear to be have evolved as fusions to

TE1 retrotransposons and there are over a thousand paralogues in the *B. graminis* f.sp. *hordei* genome (Spanu *et al*., 2010). This linkage to retrotransposons could provide a mechanism for diversification and amplification during effector evolution. Evidence for the rapid evolution of effectors as compared with other genes also comes from comparison of the *B. graminis* genome with those of two related powdery mildew fungi, *Erisyphe pisi* and *Golovinomyces orontii* (Spanu *et al*., 2010). The effector complement in these three pathogens is highly diverse despite an otherwise well-conserved genome.

The rust fungi – the role of haustoria in effector secretion

Rust fungi are a diverse group of plant pathogens with over 7000 species known to cause diseases in plants (Maier *et al*., 2003). Similar to the powdery mildews, rust infection involves formation of haustoria that penetrate host cells, but rust hyphae proliferate within the leaf rather than on the leaf surface (Fig. 1). The flax rust fungus (*M. lini*) and its host plant flax (*Linum usitatissiumum*) have served as an important model for plant immunity since Flor's early analysis of resistance and virulence provided the first genetic description (the 'gene-for-gene' model) of ETI (Flor, 1955). Numerous R genes have been cloned from flax and encode cytosolic receptors while the corresponding effectors recognized by several of these receptors encode secreted proteins that are expressed in haustoria (Lawrence *et al*., 2007). The first flax rust effector identified was AvrL567, a protein recognized by the L5, L6 and L7 R proteins (Dodds *et al*., 2004). AvrL567 has undergone diversifying selection, resulting in at least 12 variants, some of which have now escaped recognition by the cognate R proteins by altering surface-exposed amino acid residues (Dodds *et al*., 2006; Wang *et al*., 2007). Screening of a haustorium-specific cDNA library, led to the identification of three other secreted rust effector proteins, AvrM, AvrP123 and AvrP4 (Catanzariti *et al*., 2006). Of these effectors, only AvrP123 shows homology to known proteins, since it contains a Kazal-like serine protease inhibitor motif, and their roles in infection are unknown. Silencing of AvrL567 in transgenic flax rust did not significantly affect rust infection (Lawrence *et al*., 2010), suggesting there may be redundancy of its effector function in *M. lini*. All of these secreted effectors are recognized inside the host cell by cytosolic R proteins, indicating that they are translocated into the host cytoplasm after their secretion from the pathogen. This was confirmed by immunolocalization of AvrM, which was detected inside host cells containing haustoria (Rafiqi *et al*., 2010), as was also observed for the bean rust effector RTP1 (Kemen *et al*., 2005). AvrL567 and AvrM both appear to translocate autonomously into host cells as a result of cellular uptake signals located in the N-terminal regions of the proteins (Rafiqi *et al*., 2010), although the transport mechanism is not yet defined.

Melampsora larici-populina, the causal agent of poplar leaf rust, is a close relative of the flax rust fungus. As in *B. graminis*, massive proliferation of transposable elements has led to the expansion of the *M. larici-populina* genome (101.1 Mb); however, in poplar rust, this proliferation has not coincided with a dramatic reduction in gene content (Duplessis *et al*., 2011). Of the 16 841 predicted proteins, about 1200 encode small secreted proteins (SSPs) and about 40% of these are expressed in *M. larici-populina*-infected leaves 96 h post inoculation. This corresponds to the stage of infection at which haustoria are formed in the infected leaves. The SSPs expressed at this time include homologues of flax rust AvrM, AvrL567, AvrP123 and AvrP4 effectors. Two-thirds of the 1200 SSPs can be organized in 169 families, each containing two to 111 members. The largest of these families contains SSPs that are cysteine-rich, a characteristic of many extracellular effectors (Dean *et al*., 2005; Mueller *et al*., 2008).

Genome expansion has also occurred in *Puccinia graminis* f.sp. *tritici*, the causal agent of wheat stem rust (Duplessis *et al*., 2011). Of 1105 predicted SSPs in *P. graminis* f.sp. *tritici*, 29 are among the most highly transcriptionally upregulated genes during infection. Recently, putative pathogenicity-related proteins were identified by proteome analysis of haustorium-enriched samples from infected plant tissue (Song *et al*., 2011) but no clear function in infection has been assigned.

Conclusion, future directions and challenges

Since Flor suggested the gene-for-gene model of plant immunity (Flor, 1955), we have come a long way in understanding the interplay between plant immunity and pathogen virulence mechanisms. However, important questions remain to be answered. The majority of fungal effectors that apparently function in the plant cytosol lack obvious translocation signals within their primary sequence, and it is not clear how the effectors are targeted to and taken across the plasma membrane into the host cell. The high level of diversity among fungal effectors and the low sequence homology to other proteins makes it difficult to predict their functions in disease. Although roles have been defined for some fungal effectors in suppressing immune responses, given the relatively long-lasting biotrophic nature of some of these interactions, it is likely that other effectors may have roles in manipulating the host metabolism to increase nutrient availability to sustain the infection. With next-generation sequencing we now have the tools to readily obtain whole-genome information from most biotrophic and hemibiotrophic fungi, even

though many are difficult to study *in vitro*. In many cases, this analysis has revealed that fungal genomes contain a large number of small secreted putative effectors that are rapidly evolving compared with the rest of the genome. This amplification and rapid evolution may be due to the strong selection pressure exerted by the plant's immune recognition system and/or to host species-specific adaptation to different pathogenicity targets. To what extent have effectors in different fungal pathogens evolved to target the same proteins in different host plants? Many of the fungal pathogens that are currently the focus of scientific investigation are pathogens of crop plants, and much of their evolution may have been influenced by domestication of the crop and creation of new pathogenic niches. Have host–pathogen interactions on native hosts evolved differently to those on crop plants? Interestingly, two symbiotic fungi have recently been shown to also direct secreted effectors into host cells (Kloppholz *et al*., 2011; Plett *et al*., 2011). To what extent do mutualistic and pathogenic fungi share common host manipulating processes? To answer these and many other questions, we need to have a much better understanding of the functions of the diverse array of effector proteins in plant pathogens. Given the large numbers of putative effectors, this will be a challenging task. Nevertheless, the rapid development of genomic tools and advanced microscopic techniques for studies of protein localizations and interactions are providing unprecedented avenues for exploring the roles of pathogen effectors in the interplay between plants and their fungal pathogens.

Acknowledgements

This work was supported by the Australian Research Council (DP1093850 and DP0771374), the Australian Grains Research and Development Corporation (CSP00099) and the US National Institutes of Health (GM074265-01A2). M.K. is supported by an Australian National University higher degree in research scholarship.

References

Agrios, G.N. (2005) *Plant Pathology*, 5th edn. San Francisco, CA: Elsevier Academic Press.

Axtell, M.J., and Staskawicz, B.J. (2003) Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. *Cell* **112:** 369–377.

Brefort, T., Doehlemann, G., Mendoza-Mendoza, A., Reissmann, S., Djamei, A., and Kahmann, R. (2009) *Ustilago maydis* as a pathogen. *Annu Rev Phytopathol* **47:** 423–445.

van den Burg, H.A., Harrison, S.J., Joosten, M., Vervoort, J., and de Wit, P. (2006) *Cladosporium fulvum* Avr4 protects fungal cell walls against hydrolysis by plant chitinases accumulating during infection. *Mol Plant Microbe Interact* **19:** 1420–1430.

Catanzariti, A.M., Dodds, P.N., Lawrence, G.J., Ayliffe, M.A., and Ellis, J.G. (2006) Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. *Plant Cell* **18:** 243–256.

Chang, J.H., Urbach, J.M., Law, T.F., Arnold, L.W., Hu, A., Gombar, S., *et al*. (2005) A high-throughput, near-saturating screen for type III effector genes from *Pseudomonas syringae*. *Proc Natl Acad Sci USA* **102:** 2549–2554.

Choi, J., Park, J., Kim, D., Jung, K., Kang, S., and Lee, Y.H. (2010) Fungal Secretome database: integrated platform for annotation of fungal secretomes. *BMC Genomics* **11:** 105.

Couch, B.C., and Kohn, L.M. (2002) A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M-grisea*. *Mycologia* **94:** 683–693.

Dean, R.A., Talbot, N.J., Ebbole, D.J., Farman, M.L., Mitchell, T.K., Orbach, M.J., *et al*. (2005) The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* **434:** 980–986.

Dodds, P.N. (2010) Genome evolution in plant pathogens. *Science* **330:** 1486–1487.

Dodds, P.N., and Rathjen, J.P. (2010) Plant immunity: towards an integrated view of plant–pathogen interactions. *Nat Rev Genet* **11:** 539–548.

Dodds, P.N., Lawrence, G.J., Catanzariti, A.-M., Ayliffe, M.A., and Ellis, J.G. (2004) The *Melampsora lini AvrL567* avirulence genes are expressed in haustoria and their products are recognized inside plant cells. *Plant Cell* **16:** 755–768.

Dodds, P.N., Lawrence, G.J., Catanzariti, A.M., Teh, T., Wang, C.I., Ayliffe, M.A., *et al*. (2006) Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proc Natl Acad Sci USA* **103:** 8888–8893.

Dodds, P.N., Rafiqi, M., Gan, P.H.P., Hardham, A.R., Jones, D.A., and Ellis, J.G. (2009) Effectors of biotrophic fungi and oomycetes – pathogenicity factors and triggers of host resistance. *New Phytol* **183:** 993–1000.

Doehlemann, G., van der Linde, K., Amann, D., Schwammbach, D., Hof, A., Mohanty, A., *et al*. (2009) Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. *PLoS Pathog* **5:** e1000290.

Dou, D., Kale, S.D., Wang, X., Jiang, R.H., Bruce, N.A., Arredondo, F.D., *et al*. (2008) RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* **20:** 1930–1947.

Duplessis, S., Cuomo, C.A., Lin, Y.-C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., *et al*. (2011) Obligate biotrophy features unraveled by the genomic analysis of rust fungi. *Proc Natl Acad Sci USA* **108:** 9166–9171.

Ellis, J.G., Rafiqi, M., Gan, P., Chakrabarti, A., and Dodds, P.N. (2009) Recent progress in discovery and functional analysis of effector proteins of fungal and oomycete plant pathogens. *Curr Opin Plant Biol* **12:** 399–405.

van Esse, H.P., Bolton, M.D., Stergiopoulos, L., de Wit, P., and Thomma, B. (2007) The chitin-binding *Cladosporium fulvum* effector protein Avr4 is a virulence factor. *Mol Plant Microbe Interact* **20:** 1092–1101.

van Esse, H.P., van't Klooster, J.W., Bolton, M.D., Yadeta, K.A., van Baarlen, P., Boeren, S., *et al*. (2008) The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense. *Plant Cell* **20:** 1948–1963.

Flor, H.H. (1955) Host–parasite interaction in flax rust – its genetics and other implications. *Phytopathology* **45:** 680–685.

Gan, P.H.P., Rafiqi, M., Ellis, J.G., Jones, D.A., Hardham, A.R., and Dodds, P.N. (2010a) Lipid binding activities of flax rust AvrM and AvrL567 effectors. *Plant Signal Behav* **5:** 1272–1775.

Gan, P.H.P., Rafiqi, M., Hardham, A.R., and Dodds, P.N. (2010b) Effectors of biotrophic fungal plant pathogens. *Funct Plant Biol* **37:** 913–918.

Godfrey, D., Bohlenius, H., Pedersen, C., Zhang, Z., Emmersen, J., and Thordal-Christensen, H. (2010) Powdery mildew fungal effector candidates share N-terminal Y/F/WxC-motif. *BMC Genomics* **11:** 317.

Gohre, V., and Robatzek, S. (2008) Breaking the barriers: microbial effector molecules subvert plant immunity. *Annu Rev Phytopathol* **46:** 189–215.

Gomez-Gomez, L., and Boller, T. (2002) Flagellin perception: a paradigm for innate immunity. *Trends Plant Sci* **7:** 251–256.

Hann, D.R., Gimenez-Ibanez, S., and Rathjen, J.P. (2010) Bacterial virulence effectors and their activities. *Curr Opin Plant Biol* **13:** 388–393.

Horbach, R., Navarro-Quesada, A.R., Knogge, W., and Deising, H.B. (2011) When and how to kill a plant cell: infection strategies of plant pathogenic fungi. *J Plant Physiol* **168:** 51–62.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B. (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J* **19:** 4004–4014.

Jones, J.D., and Dangl, J.L. (2006) The plant immune system. *Nature* **444:** 323–329.

de Jonge, R., van Esse, H.P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., *et al*. (2010) Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science* **329:** 953–955.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., *et al*. (2006) Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc Natl Acad Sci USA* **103:** 11086–11091.

Kale, S.D., Gu, B., Capelluto, D.G.S., Dou, D., Feldman, E., Rumore, A., *et al*. (2010) External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell* **142:** 284–295.

Kamoun, S. (2009) The secretome of plant-associated fungi and oomycetes. In *The Mycota*. Deising, H.B. (ed.). Berlin, Heidelberg: Springer, pp. 173–180.

Kamper, J., Kahmann, R., Bolker, M., Ma, L.J., Brefort, T., Saville, B.J., *et al*. (2006) Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature* **444:** 97–101.

Kankanala, P., Czymmek, K., and Valent, B. (2007) Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell* **19:** 706–724.

Kemen, E., Kemen, A.C., Rafiqi, M., Hempel, U., Mendgen, K., Hahn, M., and Voegele, R.T. (2005) Identification of a protein from rust fungi transferred from haustoria into infected plant cells. *Mol Plant Microbe Interact* **18:** 1130–1139.

Khang, C.H., Park, S.Y., Lee, Y.H., Valent, B., and Kang, S. (2008) Genome organization and evolution of the AVR-Pita avirulence gene family in the *Magnaporthe grisea* species complex. *Mol Plant Microbe Interact* **21:** 658–670.

Khang, C.H., Berruyer, R., Giraldo, M.C., Kankanala, P., Park, S.Y., Czymmek, K., *et al*. (2010) Translocation of *Magnaporther oryzae* effectors into rice cells and their subsequent cell-to-cell movement. *Plant Cell* **22:** 1388–1403.

Kloppholz, S., Kuhn, H., and Requena, N. (2011) A secreted fungal effector of *Glomus intraradices* promotes symbiotic biotrophy. *Curr Biol* **21:** 1204–1209.

Lawrence, G.J., Dodds, P.N., and Ellis, J.G. (2007) Rust of flax and linseed caused by *Melampsora lini*. *Mol Plant Pathol* **8:** 349–364.

Lawrence, G.J., Dodds, P.N., and Ellis, J.G. (2010) Transformation of the flax rust fungus, *Melampsora lini*: selection via silencing of an avirulence gene. *Plant J* **61:** 364–369.

Li, W., Wang, B., Wu, J., Lu, G., Hu, Y., Zhang, X., *et al*. (2009) The *Magnaporthe oryzae* avirulence gene AvrPiz-t encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene *Piz-t*. *Mol Plant Microbe Interact* **22:** 411–420.

Maier, W., Begerow, D., Weiss, M., and Oberwinkler, F. (2003) Phylogeny of the rust fungi: an approach using nuclear large subunit ribosomal DNA sequences. *Can J Bot* **81:** 12–23.

Mendgen, K., and Hahn, M. (2002) Plant infection and the establishment of fungal biotrophy. *Trends Plant Sci* **7:** 352–356.

Morgan, W., and Kamoun, S. (2007) RXLR effectors of plant pathogenic oomycetes. *Curr Opin Microbiol* **10:** 332–338.

Mosquera, G., Giraldo, M.C., Khang, C.H., Coughlan, S., and Valent, B. (2009) Interaction transcriptome analysis identifies *Magnaporthe oryzae* BAS1-4 as biotrophy-associated secreted proteins in rice blast disease. *Plant Cell* **21:** 1273–1290.

Mueller, O., Kahmann, R., Aguilar, G., Trejo-Aguilar, B., Wu, A., and de Vries, R.P. (2008) The secretome of the maize pathogen *Ustilago maydis*. *Fungal Genet Biol* **45:** S63–S70.

Muller, O., Schreier, P.H., and Uhrig, J.F. (2008) Identification and characterization of secreted and pathogenesis-related proteins in *Ustilago maydis*. *Mol Genet Genomics* **279:** 27–39.

Panstruga, R., and Dodds, P.N. (2009) Terrific protein traffic: the mystery of effector protein delivery by filamentous plant pathogens. *Science* **324:** 748–750.

Petutschnig, E.K., Jones, A.M.E., Serazetdinova, L., Lipka, U., and Lipka, V. (2010) The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. *J Biol Chem* **285:** 28902–28911.

Plett, J.M., Kemppainen, M., Kale, S.D., Kohler, A., Legue, V., Brun, A., *et al*. (2011) A secreted effector protein of *Lac-*

caria bicolor is required for symbiosis development. *Curr Biol* **21:** 1197–1203.

Rafiqi, M., Gan, P.H.P., Ravensdale, M., Lawrence, G.J., Ellis, J.G., Jones, D.A., *et al*. (2010) Internalization of flax rust avirulence proteins into flax and tobacco cells can occur in the absence of the pathogen. *Plant Cell* **22:** 2017–2032.

Ridout, C.J., Skamnioti, P., Porritt, O., Sacristan, S., Jones, J.D.G., and Brown, J.K.M. (2006) Multiple avirulence paralogues in cereal powdery mildew fungi may contribute to parasite fitness and defeat of plant resistance. *Plant Cell* **18:** 2402–2414.

Schechter, L.M., Vencato, M., Jordan, K.L., Schneider, S.E., Schneider, D.J., and Collmer, A. (2006) Multiple approaches to a complete inventory of *Pseudomonas syringae* pv. *tomato* DC3000 type III secretion system effector proteins. *Mol Plant Microbe Interact* **19:** 1180–1192.

Schirawski, J., Mannhaupt, G., Munch, K., Brefort, T., Schipper, K., Doehleman, G., *et al*. (2010) Pathogenicity determinants in smut fungi revealed by genome comparison. *Science* **330:** 1546–1548.

Song, X., Rampitsch, C., Soltani, B., Mauthe, W., Linning, R., Banks, T., *et al*. (2011) Proteome analysis of wheat leaf rust fungus, *Puccinia triticina*, infection structures enriched for haustoria. *Proteomics* **11:** 944–963.

Spanu, P.D., Abott, J.C., Burgis, T.A., Ahmedinejad, N., Ametx, C., Amselem, J., *et al*. (2010) Genome expansion and gene loss in powdery mildew fungi reveal functional tradoffs in extreme parasitism. *Science* **330:** 1543–1546.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L., and Galan, J.E. (2001) Common and contrasting themes of plant and animal diseases. *Science* **292:** 2285–2289.

Stergiopoulos, I., and de Wit, P. (2009) Fungal effector proteins. *Annu Rev Phytopathol* **47:** 233–263.

Thomma, B., Van Esse, H.P., Crous, P.W., and De Wit, P. (2005) *Cladosporium fulvum* (syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. *Mol Plant Pathol* **6:** 379–393.

Vandenackerveken, G., Vankan, J.A.L., and Dewit, P. (1992) Molecular analysis of the avirulence gene AVR9 of the fungal tomato pathogen *Cladosporium-fulvum* fully supports the gene-for-gene hypothesis. *Plant J* **2:** 359–366.

Vanholme, B., De Meutter, J., Tytgat, T., Van Montagu, M., Coomans, A., and Gheysen, G. (2004) Secretions of plant-parasitic nematodes: a molecular update. *Gene* **332:** 13–27.

Vieira, P., Danchin, E.G.J., Neveu, C., Crozat, C., Jaubert, S., Hussey, R.S., *et al*. (2011) The plant apoplasm is an important recipient compartment for nematode secreted proteins. *J Exp Bot* **62:** 1241–1253.

Wang, C.I., Guncar, G., Forwood, J.K., Teh, T., Catanzariti, A.M., Lawrence, G.J., *et al*. (2007) Crystal structures of flax rust avirulence proteins AvrL567-A and -D reveal details of the structural basis for flax disease resistance specificity. *Plant Cell* **19:** 2898–2912.

Whisson, S.C., Boevink, P.C., Moleleki, L., Avrova, A.O., Morales, J.G., Gilroy, E.M., *et al*. (2007) A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* **450:** 115–118.

Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., *et al*. (2008) *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. *Curr Biol* **18:** 74–80.

Xiang, T.T., Zong, N., Zhang, J., Chen, J.F., Chen, M.S., and Zhou, J.M. (2011) BAK1 is not a target of the *Pseudomonas syringae* effector AvrPto. *Mol Plant Microbe Interact* **24:** 100–107.

Yaeno, T., Li, H., Chaparro-Garcia, A., Schornack, S., Koshiba, S., Watanabe, S., *et al*. (2011) Phosphatidylinositol monophosphate-binding interface in the oomycete RXLR effector AVR3a is required for its stability in host cells to modulate plant immunity. *Proc Natl Acad Sci USA* (in press). doi: 10.1073/pnas.1106002108

Yoshida, K., Saitoh, H., Fujisawa, S., Kanzaki, H., Matsumura, H., Tosa, Y., *et al*. (2009) Association genetics reveals three novel avirulence genes from the rice blast fungal pathogen *Magnaporthe oryzae*. *Plant Cell* **21:** 1573–1591.

Zipfel, C. (2009) Early molecular events in PAMP-triggered immunity. *Curr Opin Plant Biol* **12:** 414–420.