Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2009) **10**(6), 735–747 DOI: 10.1111/J.1364-3703.2009.00591.X

Review

Fungal effector proteins: past, present and future

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SUMMARY

The pioneering research of Harold Flor on flax and the flax rust fungus culminated in his gene-for-gene hypothesis. It took nearly 50 years before the first fungal avirulence (*Avr*) gene in support of his hypothesis was cloned. Initially, fungal *Avr* genes were identified by reverse genetics and map-based cloning from model organisms, but, currently, the availability of many sequenced fungal genomes allows their cloning from additional fungi by a combination of comparative and functional genomics. It is believed that most *Avr* genes encode effectors that facilitate virulence by suppressing pathogen-associated molecular pattern-triggered immunity and induce effector-triggered immunity in plants containing cognate resistance proteins. In resistant plants, effectors are directly or indirectly recognized by cognate resistance proteins that reside either on the plasma membrane or inside the plant cell. Indirect recognition of an effector (also known as the guard model) implies that the virulence target of an effector in the host (the guardee) is guarded by the resistance protein (the guard) that senses manipulation of the guardee, leading to activation of effector-triggered immunity. In this article, we review the literature on fungal effectors and some pathogen-associated molecular patterns, including those of some fungi for which no gene-for-gene relationship has been established.

INTRODUCTION

The gene-for-gene hypothesis proposed by Flor states that, for every dominant avirulence (*Avr*) gene in the pathogen, there is a cognate resistance (*R*) gene in the host, and the interaction between their gene products leads to the activation of host defence responses, such as the hypersensitive response (HR), that arrests the growth of biotrophic fungi (Flor, 1942). Since the pioneering genetic research of Flor, many plant pathologists have searched for molecular and biochemical evidence of the gene-for-gene concept. The molecular cloning of the first bacterial *Avr* gene was reported in 1984 (Staskawicz *et al*., 1984), the first fungal *Avr* gene in 1991 (van Kan *et al*., 1991) and the first oomycete *Avr* gene in 2004 (Shan *et al*., 2004). Over the last two decades, numerous novel *Avr* genes and cognate *R* genes have been identified, and have increased our molecular understanding of plant–microbe interactions considerably. In response to pathogen attacks, plants have evolved at least two lines of active defence. The first line provides basal defence against all potential pathogens and is based on the recognition of conserved pathogen-associated molecular patterns (PAMPs), by so-called PAMP recognition receptors (PRRs) that activate PAMP-triggered immunity (PTI) and prevent further colonization of the host (De Wit, 2007; Jones and Dangl, 2006). One of the best-known fungal PAMPs is chitin, a major structural component of fungal cell walls, for which two LysM-type receptors have been characterized in rice and Arabidopsis (Kaku *et al*., 2006; Miya *et al*., 2007). There is now evidence that some *Avr* genes encode effectors that suppress PTI, enabling a pathogen to infect a plant and cause disease. Once the basal defence system of plants had been overcome by pathogens, during evolution plants responded by the development of a second recognition system based on effector perception by R proteins and subsequent activation of effector-triggered immunity (ETI), which leads to rapid and enhanced defence responses in plants, including HR. This, in turn, triggered a second wave of co-evolutionary arms race between pathogens and plants, during which pathogens responded by mutating effectors or developing novel effectors that could avoid or suppress ETI, whereas plants developed novel R proteins mediating the recognition of these novel effectors (De Wit, 2007; Jones and Dangl, 2006). One intriguing question relates to the molecular interaction between necrotrophic fungal pathogens and their host plants. It is assumed that necrotrophic pathogens do not comply with the gene-for-gene model. However, recently, several necrotrophic fungal pathogens have been reported to produce ribosomal proteins that might function as effectors that induce necrosis in plants with responsive genes to facilitate disease (Friesen *et al*., 2008a). The same could be true for the necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) that are produced by several organisms, including fungi (Ottmann *et al*., 2009).

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Many reviews on bacterial, fungal and oomycete *Avr* genes have been written in recent years (Bent and Mackey, 2007; Gohre and Robatzek, 2008; Kamoun, 2007; Stergiopoulos and de Wit, 2009). In this review, we also briefly discuss some fungal PAMPs and provide an update on fungal effectors, also including those of fungi for which no gene-for-gene type of interaction has been established with their hosts.

PAMPS OF FUNGAL PLANT AND HUMAN PATHOGENS

The first layer of plant defence is triggered after recognition of PAMPs by PRRs, leading to the activation of basal defence. Chitin and β-glucan represent major fungal and oomycete PAMPs for which PRRs have been identified (Kaku *et al*., 2006; Miya *et al*., 2007). Several other cell wall components have been reported previously to be nonspecific elicitors, such as the galactoglucomannans of *Cladosporium fulvum* (De Wit and Kodde, 1981).

Likewise, several PAMPs have been described for the fungal human pathogen *Candida albicans* (Jouault *et al*., 2009). In general, the core structure of the fungal cell wall is composed of a skeleton of polysaccharide fibrils containing β-(1,3)-glucan, which is covalently linked to β-(1,6)-glucan and chitin. The outer layer also consists of proteins that are mainly glycosylated through N-linked (Cutler, 2001) or O-linked (Ernst and Prill, 2001) mannosylation. Although this basic model of the fungal cell wall is shared by many fungi, at the molecular level these structures differ between fungal species. In *Aspergillus* species, an important component of the cell wall is galactomannan, whereas the outermost cell wall layer is composed of hydrophobic proteins (hydrophobins) that contribute to the shielding properties of the cell wall (Bernard and Latge, 2001). In *Cryptococcus neoformans*, a capsule of mannoproteins, galactoxylomannan and glucoronoxylomannan masks the recognition and activation of host defence mechanisms (McFadden *et al*., 2006). This diversity in cell wall components will result in different qualities of PRR–ligand interaction and the activation of different sets of PRRs, leading to specific host responses. In addition to the recognition of PAMPs (infectious nonself molecules), plants have evolved the ability to sense their own degraded polymer molecules (infectious self molecules). These self-released molecules, termed danger-associated molecular patterns (DAMPs), often result from the degradation of plant cell walls by secreted fungal hydrolytic enzymes, such as xylanases, during host invasion (Matzinger, 2007). For example, plants can recognize oligo-α-galacturonides released from their damaged cell walls by fungal degrading enzymes, and subsequently activate the defence response (Denoux *et al*., 2008). It is anticipated that additional fungal PAMPs and DAMPs will be discovered in the near future that are recognized by novel plant and human PRRs.

EFFECTORS OF FUNGAL PLANT PATHOGENS

Cladosporium fulvum

To date, four *Avr* genes have been cloned from *C. fulvum* that all encode small cysteine-rich proteins that are secreted during infection, including Avr2, Avr4, Avr4E and Avr9, whose recognition in tomato is mediated by the cognate Cf (for *C. fulvum*) proteins Cf-2, Cf-4, Cf-4E and Cf-9, respectively (De Wit *et al*., 1997; Joosten and De Wit, 1999; Thomma *et al*., 2005). Also, additional extracellular proteins (Ecps), namely Ecp1, Ecp2, Ecp4 and Ecp5, have been characterized from this fungus that invoke an HR in tomato accessions that carry a cognate *Cf-Ecp* gene (De Kock *et al*., 2005; Laugé *et al*., 2000). Recently, Ecp6 and Ecp7 have been identified but, for these two effectors, no HR-responding tomato accessions have been detected to date (Bolton *et al*., 2008). All Avrs and Ecps are assumed to be virulence factors (Bolton *et al*., 2008; van Esse *et al*., 2007, 2008; Thomma *et al*., 2005).

The Avr2 effector inhibits tomato cysteine proteases that are presumed to be important in basal host defence, including Rcr3, Pip1, aleurain and TDI-65 (Doehlemann *et al*., 2009; van Esse *et al*., 2008; Kruger *et al*., 2002; Muller *et al*., 2008; Rooney *et al*., 2005; R. Kahmann, unpublished; Shabab *et al*., 2008; Yoshida *et al*., 2009). Avr2 also facilitates the virulence of other fungal tomato pathogens, such as *Botrytis cinerea* and *Verticillium dahliae*, which are more virulent on *Arabidopsis thaliana* transgenic for *Avr2* (van Esse *et al*., 2008). In the presence of Cf-2, Avr2 behaves as an avirulence factor and its recognition is mediated by $Rcr3^{pimp}$ (required for *C. fulvum* resistance), a cysteine protease originating from *Lycopersicon pimpinellifolium* (Kruger *et al*., 2002; Rooney *et al*., 2005). Structural modification of Rcr3 by Avr2, rather than Rcr3 inhibition, is the most likely cause of the triggering of Cf-2-mediated defence signalling, as a natural variant of Rcr3 occurs in *Lycopersicon esculentum* ($Rcr3^{esc}$) that is still an active enzyme but causes spontaneous HR in the presence of Cf-2 in an Avr2-independent manner. The $Rcr3^{esc}$ protein probably has a modified tertiary structure when compared with the $Rcr3^{pimp}$ protein (Kruger *et al*., 2002). Circumvention of Avr2-triggered Cf-2-mediated HR can be achieved by point mutations, deletions or transposon insertions in the *Avr2* gene (Luderer *et al*., 2002b; Stergiopoulos *et al*., 2007).

The Avr4 effector contains a functional chitin-binding domain that protects chitinous fungi against plant chitinases, including *C. fulvum* (van den Burg *et al*., 2003, 2004, 2006; van Esse *et al*., 2007). In the presence of Cf-4, Avr4 induces an HR, but natural isoforms of this effector occur that no longer trigger Cf-4-mediated HR but can still bind chitin (van den Burg *et al*., 2003; Joosten *et al*., 1997; Stergiopoulos *et al*., 2007).

The virulence function of Avr4E is not yet known but, in the presence of Cf-4E, Avr4E triggers Cf-4E-mediated resistance

(Westerink *et al*., 2004). Evasion of Cf-4E-mediated recognition is achieved by point mutations in the *Avr4E* gene or jettison of the *Avr4E* gene, suggesting that the fitness penalty associated with the loss of this gene is not very high (Stergiopoulos *et al*., 2007). However, *Avr4E*-expressing tomato plants are more susceptible than control plants to natural *C. fulvum* strains that lack *Avr4E*, suggesting that Avr4E is a virulence factor (H. P. van Esse and B. P. H. J. Thomma, Wageningen, Laboratory of Phytopathology, Wageningen University).

The Avr9 effector contains a cystine knot with structural but, so far, no functional homology to carboxypeptidase inhibitors (van den Ackerveken *et al*., 1993; van den Hooven *et al*., 2001; van Kan *et al*., 1991; Vervoort *et al*., 1997). Disruption of *Avr9* in *C. fulvum* did not affect the virulence on tomato plants, suggesting that *Avr9* is not required for full virulence (Marmeisse *et al*., 1993). However, *Avr9*-expressing tomato plants appear to be more susceptible than control plants to natural *C. fulvum* strains that lack *Avr9*, suggesting that Avr9 is a virulence factor with redundant activity (H. P. van Esse and B. P. H. J. Thomma, personal communication). Expression of *Avr9 in vitro* is induced under nitrogen-limiting conditions (van den Ackerveken *et al*., 1994; Perez-Garcia *et al*., 2001; Thomma *et al*., 2006) and an *Nrf1* gene (for nitrogen-responsive factor) has been identified in *C. fulvum*. *Nrf1* deletion mutants no longer express *Avr9* under nitrogen-limiting conditions *in vitro* and are compromised in their virulence on Cf-0 tomato plants. However, these strains are still avirulent on Cf-9 tomato plants, suggesting that Nrf1 is a major, but not only, positive regulator of *Avr9* expression (Perez-Garcia *et al*., 2001). The expression of all other *Avr* and *Ecp* effector genes of *C. fulvum* is not induced under nitrogen-limiting conditions (Bolton and Thomma, 2008; Thomma *et al*., 2006), indicating that nitrogen starvation is not a general environmental condition that induces effectors of *C. fulvum*.

In addition, effector genes coding for Ecps have been cloned from *C. fulvum*, including *Ecp1*, *Ecp2*, *Ecp4*, *Ecp5*, *Ecp6* and *Ecp7* (van den Ackerveken *et al*., 1993; Bolton *et al*., 2008; Laugé *et al*., 2000). Ecps are abundantly secreted by all strains of *C. fulvum* during infection, and possess an even number of cysteine residues that are most probably involved in intramolecular disulphide bridges (Luderer *et al*., 2002a). *Ecp6*, *Ecp1* and *Ecp2* are all virulence genes, as silencing or disruption of these genes compromises the virulence of *C. fulvum* on tomato (Bolton *et al*., 2008; Laugé *et al*., 1997). For *Ecp1*, *Ecp2*, *Ecp4* and *Ecp5*, tomato accessions have been identified that carry single cognate dominant *Cf-Ecp* genes mediating Ecp-triggered HR, which, however, have not yet been cloned (Laugé *et al*., 1998, 2000; Soumpourou *et al*., 2007).

Until recently, no homologues of Avr and Ecp effectors could be found in public databases because of the small numbers of fungal plant pathogens sequenced, except for Avr4 and Ecp6 which contain orthologues in many other fungal species. This is mainly a result of the presence of CBM14 and LysM domains in these proteins, which are implicated in carbohydrate binding, including chitin, suggesting that Ecp6 might be a functional homologue of Avr4 (Bolton *et al*., 2008). However, Ecp6 most probably does not protect chitinous fungi against plant chitinases, but is most likely to be involved in the scavenging of chitin fragments that are released from fungal cell walls during infection, thus preventing them from acting as PAMPs that trigger PTI (Bolton *et al*., 2008).

Rhynchosporium secalis

Rhynchosporium secalis secretes three low-molecular-weight peptides, designated Nip1–Nip3. Nip1 triggers specific (non-HR) defence responses in barley cultivars carrying the as yet uncloned *Rrs1* resistance gene (Hahn *et al*., 1993). Mature Nip1 (also known as AvrRrs1) contains 10 cysteine residues (Rohe *et al*., 1995) that are all involved in intramolecular disulphide bonds (Van't Slot *et al*., 2003). Strains of *R. secalis* virulent on *Rrs1* plants either lack *Nip1* or contain alleles with point mutations that translate into single amino acid substitutions (Rohe *et al*., 1995). It has been found that Nip1 interacts with a single plasma membrane receptor that is involved in both the mediation of virulence and the triggering of defence, but the exact receptor has not yet been characterized (Van't Slot *et al*., 2007). A study of field populations of the pathogen showed clear evidence of positive diversifying selection operating on the *Nip1* locus (Schurch *et al*., 2004). In total, 14 Nip1 isoforms were identified, at least three of which were correlated with gain of virulence on Rrs1 plants, whereas a high deletion frequency of *Nip1* was also observed that was much higher than that seen for *Nip2* and *Nip3*. As single amino acid substitutions in Nip1 that correlated with gain of virulence on *Rrs1* plants were observed at much lower frequencies than gene deletions, the fitness cost associated with the loss of this gene is probably not high.

Recently, the *Nip2* and *Nip3* genes have also been cloned (W. Knogge, Halle, Leibniz Institute for Plant Biochemistry). *Nip2* encodes a 109-amino-acid protein with a predicted signal peptide of 16 amino acids, whereas *Nip3* encodes a 115-amino-acid protein with a predicted signal peptide of 17 amino acids. Mature Nip2 and Nip3 presumably carry six and eight cysteines, respectively (W. Knogge, Halle, Leibniz Institute for Plant Biochemistry).

Fusarium oxysporum f. sp. *lycopersici*

The so-called Six effectors (for secreted in xylem) are produced by *F. oxysporum* f. sp. *lycopersici* during infection of tomato. Six1 (renamed Avr3) is required for full virulence on tomato (Rep *et al*., 2004, 2005), but also triggers ETI in the presence of the cognate *I-3* resistance gene (Huang and Lindhout, 1997; Rep *et al*., 2004). In addition, Six3 (renamed Avr2) contributes to virulence, but also

triggers ETI in the presence of the cognate *I-2* resistance gene (Houterman *et al*., 2009). *Avr3* resides on a small chromosome that contains additional effector genes, including *Six2* and *Six3*. This chromosome is not found outside the *F. oxysporum* f. sp. *lycopersici* lineage, nor in nonpathogenic *Fusarium* species, suggesting that it confers virulence specifically on tomato (van der Does *et al*., 2008). This suggests that the ability to cause disease has probably arisen once during the evolution of *F. oxysporum* f. sp. *lycopersici* by the acquisition or emergence of the genomic region harbouring the effector genes necessary for the infection of tomato. Subsequently, this region might have spread to other clonal *F. oxysporum* f. sp. *lycopersici* strains by horizontal gene transfer (van der Does *et al*., 2008). *Six4* (renamed *Avr1*) confers avirulence to *F. oxysporum* f. sp. *lycopersici* strains on tomato lines carrying the *I* or *I-1* resistance gene. However, Avr1 is not required for full virulence of *F. oxysporum* f. sp. *lycopersici* strains on tomato plants that lack the cognate *I* or *I-1* gene. This suggests that Avr1 has evolved as an effector that suppresses ETI rather than PTI. Indeed, *Avr1* functions as a suppressor of *I-2*- and *I-3*-mediated resistance (Houterman *et al*., 2008) and is always present in *F. oxysporum* f. sp. *lycopersici* strains virulent on these lines. When *F. oxysporum* f. sp. *lycopersici* strains avirulent on *I-2* and/or *I-3* lines were transformed with *Avr1*, they gained virulence on these lines, indicating that *Avr1* suppresses both *I-2*- and *I-3*-mediated resistance. *Avr1* might have been acquired by *F. oxysporum* f. sp. *lycopersici* in order to avoid the fitness penalty associated with the loss of *Avr3* and probably also *Avr2* in overcoming *I-2*- and *I-3*-mediated resistance. This could explain why all *F. oxysporum* f. sp. *lycopersici* strains analysed to date contain *Avr3*, whereas *Avr1* is only present in strains that are virulent on *I-2* and/or *I-3* lines. Possibly, the I-3 protein fits the guard model, where not the Avr3 protein itself, but the modulation of its virulence target, is recognized by I-3, whereas *F. oxysporum* f. sp. *lycopersici* strains can regain virulence towards *I-3*-containing lines by the acquisition of *Avr1*. During evolution, tomato responded to this adaptation with the development of the *I* or unlinked *I-1* resistance gene that specifically mediates the recognition of Avr1 (Houterman *et al*., 2008). Interestingly, the Avr2 protein is secreted in the xylem when *F. oxysporum* f. sp. *lycopersici* colonizes tomato and is recognized intracellularly by I-2, implying uptake by the host (Houterman *et al*., 2009). Point mutations resulting in single amino acid changes are the mechanism for gaining virulence on *I-2* genotypes.

Leptosphaeria maculans

At least nine distinct *Avr* genes, designated *AvrLm1–AvrLm9*, have been genetically mapped in *L. maculans* on unlinked genomic regions (Balesdent *et al*., 2002). *AvrLm1* and *AvrLm6* (Fudal *et al*., 2007; Gout *et al*., 2006) are in relatively close proximity at a locus that also harbours *AvrLm2* (Balesdent *et al*., 2002). *AvrLm1* and *AvrLm6* reside in a gene-poor heterochromatin-like region surrounded by GC-rich isochors that comprises long-terminal repeat (LTR) retrotransposons, whereas both genes show a low GC content. *AvrLm1* and *AvrLm6* are single-copy genes, encoding small secreted proteins (SSPs) that lack any characteristic signatures. In contrast with AvrLm1, AvrLm6 contains six cysteine residues and might be secreted in the apoplast, resembling the cysteine-rich effectors of *C. fulvum* and *F. oxysporum* f. sp. *lycopersici* (Gout *et al*., 2006). Races virulent on *Rlm1* cultivars mostly lack the *AvrLm1* gene (Balesdent *et al*., 2005, 2006; Rouxel *et al*., 2003).

The *AvrLm4-7* gene shows the same characteristics as the *AvrLm1-2-6* locus, including the presence of multiple LTR retrotransposons (Parlange *et al*., 2009). *AvrLm7* confers avirulence on both *Rlm7* and *Rlm4* genotypes, and has been renamed *AvrLm4-7*. *AvrLm4-7* encodes a putatively secreted protein with eight cysteine residues with no homology to proteins currently present in public databases. Complete or partial deletion of the *AvrmL4-7* gene is the main mechanism for gaining virulence on both *Rlm4* and *Rlm7* genotypes, whereas most isolates virulent on *Rlm4* genotypes alone show a single point mutation in the *AvrLm4-7* gene. Strains with the wild-type *AvrLm4-7* allele appear to be more virulent than those lacking it, suggesting that AvrLm4-7 is a virulence factor (Parlange *et al*., 2009).

Magnaporthe oryzae

Several *Avr* genes have been cloned and characterized from *M. oryzae*, including *Avr–Pita* (Orbach *et al*., 2000; Valent *et al*., 1991), *Avr1–CO39* (Farman and Leong, 1998), *Ace1* (Bohnert *et al*., 2004; Collemare *et al*., 2008) and the Pwl effectors (Kang *et al*., 1995; Sweigard *et al*., 1995). The Avr-Pita effector shows homology to fungal zinc-dependent metalloproteases and is dispensable for virulence on rice (Jia *et al*., 2000; Orbach *et al*., 2000). Avr-Pita interacts with the cognate Pi-ta resistance protein (Jia *et al*., 2000). Recognition specificity for Avr-Pita is determined by a difference in one amino acid residue (Ala-918) of the Pi-ta protein present in resistant vs. susceptible rice varieties. An array of mutations has been described at the *Avr-Pita* locus causing virulence of the fungus, including various size deletions, point mutations and a transposon insertion (Bryan *et al*., 2000; Jia *et al*., 2003; Kang *et al*., 2001; Orbach *et al*., 2000; Zhou *et al*., 2007). Recently, it has been shown that *Avr-Pita* (currently renamed *Avr-Pita1*) belongs to a gene family with at least two additional members, *Avr-Pita2* and *Avr-Pita3* (Khang *et al*., 2008). Avr-Pita2 acts as an elicitor of defence responses mediated by Pi-ta, but Avr-Pita3 does not. Members of the *Avr-Pita* family are widely distributed among strains of *M. grisea* isolated from diverse hosts, including isolates that are not pathogenic on rice. However, although *Avr-Pita1* and *Avr-Pita2* are present in both *M. oryzae* and *M. grisea*, *Avr-Pita3* is only present in *M. oryzae*

isolates, suggesting that *Avr-Pita1* and *Avr-Pita3* are derived from a gene duplication event that must have occurred after the separation of *M. oryzae* from *M. grisea* (Khang *et al*., 2008).

The Ace1 effector is a putative cytoplasmic fusion polypeptide containing a polyketide synthase (PKS) and a nonribosomal peptide synthetase (NRPS), two distinct classes of enzymes that are involved in the production of microbial secondary metabolites (Bohnert *et al*., 2004; Collemare *et al*., 2008). Ace1 seems to mediate avirulence indirectly by producing a secondary metabolite that activates Pi33. *Ace1* is exclusively expressed in appressoria, suggesting that the secondary metabolite produced might have a role in virulence, although mutants in which *Ace1* was deleted were not compromised in virulence (Bohnert *et al*., 2004; Fudal *et al*., 2005).

The Pwl effectors, encoded by the *Pwl* (pathogenicity towards weeping lovegrass) gene family, are rapidly evolving, small, glycine-rich secreted proteins that are generally found in rice pathogens. At least four members of this family, designated *Pwl1–Pwl4*, are present in *M. oryza*, confer species-specific avirulence on weeping lovegrass and finger millet, but have no effect on rice (Kang *et al*., 1995; Sweigard *et al*., 1995). *Pwl2* is located on a highly unstable locus, where frequent genetic rearrangements associated with large deletions have led to the emergence of spontaneous mutants virulent on weeping lovegrass. Pwl1, the allelic Pwl3 and Pwl4 were identified on the basis of homology to Pwl2, but only Pwl1 is a functional homologue of Pwl2, conferring avirulence on weeping lovegrass. However, *Pwl4* could become functional when expressed under the control of the *Pwl2* promoter, which was not the case for *Pwl3*.

To isolate additional *Avr* genes from *M. grisea*, Yoshida *et al*. (2009) recently retrieved 1032 putative secreted protein genes from the genomic sequence of isolate 70-15 (an experimental isolate obtained from a sexual cross between a rice and grass isolate), examined their DNA polymorphisms among 46 isolates and looked for association with Avr function on a set of differential rice cultivars harbouring different *R* genes. However, no association was found with Avr activity, indicating that isolate 70-15 may have lost several functional *Avr* genes through sexual recombination. Indeed, sequencing of the genome of another natural isolate, Ina 168, revealed that isolate 70-15 lacked a total of 1.68 Mb of regions comprising 316 candidate effector genes. Association analyses of these 316 genes revealed three novel *Avr* genes, *AVR-Pia*, *AVR-Pii* and *AVR-Pik/km/kp*. *AVR-Pia* and *AVR-Pii* have probably evolved by gene gain/loss processes, whereas *AVR-Pik/km/kp* has evolved by a combination of gene gain/loss processes and nucleotide substitutions (Yoshida *et al*., 2009).

Blumeria graminis f. sp. *hordei*

Powdery mildews are a large group of ascomycete obligate biotrophic fungi that produce haustoria in the epidermis of their host plants (Glawe, 2008). *Blumeria graminis* f. sp. *hordei* causes powdery mildew on barley and interacts with its host in a gene-for-gene manner (Zhang *et al*., 2005). At least 85 dominant or semi-dominant mildew *R* genes (*Ml*) have been characterized in barley, including *Mlk* genes and 28 highly homologous genes that all map to the *Mla* (for mildew A) locus of barley chromosome 5 (Jensen *et al*., 1980; Jorgensen, 1994). Six of the genes present at this locus, including *Mla1*, *Mla6*, *Mla7*, *Mla10*, *Mla12* and *Mla13*, have been cloned, and they all encode highly related intracellular coiled-coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR)-type R proteins that all recognize isolate-specific effectors of *Blumeria graminis* f. sp. *hordei* (Halterman and Wise, 2004; Halterman *et al*., 2001; Shen *et al*., 2003). Two *Avr* genes, designated *Avrk1* and *Avra10*, have been cloned that induce defence responses in barley varieties containing the cognate Mlk1 and Mla10 proteins, respectively. Both genes belong to a large multigene family of more than 30 paralogues in *Blumeria graminis* f. sp. *hordei*, whereas homologues are present in formae speciales that are pathogenic on other grasses. The predicted proteins encoded by *Avra10* and *Avrk1* both lack an N-terminal signal sequence or a signature for uptake by host cells. However, recently, it has been shown by fluorescence microscopy that the majority of the Mla10 protein is localized in the cytoplasm and approximately 5% in the nucleus (Bieri *et al*., 2004; Shen *et al*., 2007). Perturbation of nucleocytoplasmic Mla10 partitioning by the expression of an Mla10 fusion protein containing a nuclear export signal (NES), that enhances nuclear export over import, decreased Mla10-specified disease resistance (Shen *et al*., 2007). In the nucleus, Mla10 showed an Avr10-dependent physical association with two WRKY transcription factors (*Hv*WRKY1 and *Hv*WRKY2 transcription factors), suggesting that these transcription factors serve as immediate downstream targets of the activated receptor.

Melampsora lini

At least 30 *Avr* genes corresponding to cognate flax *R* genes have been identified in genetic analyses of *Melampsora lini* (Ellis *et al*., 1997). They have been cloned from four different loci, including *AvrL567*, *AvrM*, *AvrP123* and *AvrP4*, that code for haustorially expressed secreted proteins (HESPs) and elicit HR in flax plants that carry the cognate *R* genes (Catanzariti *et al*., 2006).

The *AvrL567A*, *AvrL567B* and *AvrL567C* genes cluster at the *AvrL567* locus and trigger HR in flax lines that carry the *L5*, *L6* and *L7* resistance genes, respectively. Virulent variants that no longer trigger HR have been identified (Dodds *et al*., 2006), which exhibit substitutions in amino acid residues that are exposed to the surface of the protein and interact directly with the cognate R proteins (Ellis *et al*., 2007; Wang *et al*., 2007).

AvrM is recognized by the M resistance protein, AvrP4 by P4 and the complex AvrP123 proteins are variously recognized by P, P1, P2 and/or P3 resistance proteins (Catanzariti *et al*., 2006). At least five different paralogues (*AvrMA–AvrME*) have been detected at the *AvrM* locus of an avirulent strain, whereas one paralogue encodes an effector that is not recognized by any known flax R protein. The six AvrM proteins have no known homologues in the public databases and show significant sequence and size variations caused by DNA insertions, deletions or polymorphisms in the location of stop codons. AvrP123 proteins contain 10 cysteine residues, including the characteristic CX7CX6YX3CX2-3C signature present in the Kazal family of serine protease inhibitors, suggesting that host proteases might be a target of these effectors. *AvrP4* encodes a protein with six cysteine residues at the C-terminal part of the mature protein which show a spacing (CX3–7CX4–6CX0–5CX1–4CX4–10C) typical for cystine-knotted peptides (Pallaghy *et al*., 1994). Both *AvrM* and *AvrP4* are expressed *in planta*. Transient intracellular expression of *AvrM* and *AvrP4* in flax plants carrying the cognate *R* genes triggers an HR, suggesting that effector translocation into the host cells occurs during infection, which is consistent with the predicted cytoplasmic location of M and P resistance proteins (Anderson *et al*., 1997; Lawrence *et al*., 1995). However, both effectors also induce an HR in flax when targeted into the apoplast, suggesting re-entry from the apoplast into host cells after secretion (Catanzariti *et al*., 2006; Dodds *et al*., 2004). A role in virulence for the *Avr* genes of *M. lini* has not been shown to date.

EFFECTORS OF FUNGAL PATHOGENS FOR WHICH NO GENE-FOR-GENE RELATIONSHIP HAS BEEN ESTABLISHED

Ustilago maydis

For the corn smut fungus *Ustilago maydis*, no gene-for-gene interaction has been established, but several effector genes from this fungus have been analysed for a function in virulence (Kahmann and Kamper, 2004). Several hydrophobins or repellent genes that encode secreted proteins were examined for their roles in virulence. Single knock-outs of these genes did not affect virulence, but a double knock-out of *Hum3* (a gene encoding a protein containing both hydrophobin repellent domains) and the repellent-encoding gene *Rsp1* were arrested at an early stage of penetration. This indicates that Hum3 and Rsp1 are effectors with a partly redundant virulence function during the early stages of infection (Muller *et al*., 2008).

Pep1 is a novel effector protein from *U. maydis* that is also essential during penetration. Pep1 is secreted into the apoplast and accumulated at sites of cell-to-cell passages. Disruption mutants of *pep1* are not affected in saprophytic growth and develop normal infection structures, but are arrested during the penetration of epidermal cells and elicit a strong plant defence response. In addition, two of the four cysteine residues in Pep1 are essential for its function. *Ustilago hordei* contains an orthologue of *Pep1* which is also required for the penetration of barley and is able to complement the *U. maydis ΔPep1* mutant (Doehlemann *et al*., 2009).

Stp1 encodes an effector that is secreted by *U. maydis* into the apoplast (R. Kahmann, unpublished), and *stp1* deletion mutants are avirulent as a result of growth arrest directly after the penetration of plant cells. This coincides with induction of strong plant defence responses. However, transient expression of Stp1 lacking the N-terminal signal peptide in *Nicotiana benthamiana* and *Zea mays* revealed that the protein localizes specifically to subcompartments of the nucleus. This suggests that Stp1 is transferred from the apoplast to the plant cell after infection (R. Kahmann, unpublished).

It would be interesting to determine whether some of the effectors of *U. maydis* could give differential responses on accessions of wild cereal and corn species, providing evidence that they could also act as Avr factors.

Necrotrophic fungi

Obligate and biotrophic fungi retrieve their nutrients from living host cells, whereas necrotrophic pathogens thrive on killed host cells. As necrotrophic pathogens produce proteinaceous effectors (some also known as host-selective toxins) that promote disease, and the host produces receptors that are required for susceptibility, these systems are often seen as a mirror image of the classical gene-for-gene systems found in biotrophic pathosystems, where matching of dominant host and pathogen gene products triggers resistance (Friesen *et al*., 2008a).

Stagonospora nodorum and *Pyrenophora tritici-repentis* are two necrotrophic fungal pathogens that produce several necrogenic host-specific peptide effectors which can be recognized by host susceptibility genes to cause disease. *Stagonospora nodorum* is a fungal pathogen of wheat, causing *Stagonospora nodorum* blotch (SNB) disease.

SnTox1 was the first toxic peptide produced by *S. nodorum* that interacts with a corresponding host susceptibility gene *Snn1* (Friesen *et al*., 2007; Liu *et al*., 2006). SnToxA is encoded by a gene with a high degree of similarity to the Ptr ToxA gene from *P. tritici-repentis*, the causal agent of tan spot of wheat with the matching susceptibility gene *Tsn1*. Tsn1-disrupted mutants were insensitive to both Ptr ToxA and SnToxA, suggesting that both toxins are functionally similar as they are recognized by the same locus in the host. Therefore, the Tsn1–ToxA interaction in the wheat–*S. nodorum* pathosystem parallels that of the wheat–tan spot system, and the wheat *Tsn1* gene serves as the major determinant for susceptibility to both SNB and tan spot (Liu *et al*., 2006).

The SnTox2–*Snn2* interaction was the third gene pair identified in this system. SnTox2 is a small secreted peptide of about 7 kDa. Sensitivity to SnTox2 is conferred by the single dominant gene *Snn2*. In contrast with the classical gene-for-gene model, the Tsn1–SnToxA and Snn2–SnTox2 interactions are additive in their contribution to susceptibility (Friesen *et al*., 2007).

SnTox3 is the fourth necrosis-inducing peptide that gives *Snn3*-dependent necrosis. However, unlike the SnToxA–Tsn1 and SnTox2–Snn2 interactions, which both have largely additive effects relative to each other, both the SnToxA–Tsn1 and SnTox2–Snn2 interactions are epistatic to the SnTox3–Snn3 interaction (Friesen *et al*., 2008b; Zhang *et al*., 2009).

ToxA was the first peptide toxin produced by the most common races of *P. tritici-repentis*. Ptr ToxA is a 13.2-kDa protein that causes necrosis in particular genotypes of wheat (Ciuffetti *et al*., 1997). ToxA interacts with a high-affinity binding site present on wheat mesophyll cells through the Arg-Gly-Asp (RGD)-containing, solvent-exposed loop, resulting in toxin internalization causing cell death (Manning *et al*., 2008). ToxA interacts with a chloroplast ToxA binding protein 1 (ToxABP1). ToxABP1 contains a lysine-rich region within a coiled-coil domain that is similar to the phosphatidylinositol binding sites present in animal proteins involved in endocytosis. ToxABP1 protein is present in both chloroplast membranes and chloroplast stroma. Surprisingly, *ToxABP1* is expressed at similar levels and encodes an identical protein in both ToxA-sensitive and ToxA-insensitive cultivars, indicating that ToxA should have other targets apart from ToxABP1 (Manning *et al*., 2007).

Just like the effectors of biotrophic fungal pathogens, toxic peptides from necrotrophic pathogens represent effectors that interact with host targets. However, the effectors of necrotrophic pathogens cause necrosis, facilitating disease, and might therefore be considered as basic effectors for which no true gene-for-gene-based resistance has been developed to date as a result of a low level of co-evolution. It could be that host targets of effectors of necrotrophic and biotrophic pathogens are intrinsically similar, but that those of biotrophic pathogens have evolved further as a result of co-evolution and no longer induce necrosis, but manipulate host targets in a more gentle manner.

EFFECTORS OF ECTOMYCORRHIZAL FUNGI

Little is known about effectors from ectomycorrhizal fungi that show both a saprophytic and biotrophic lifestyle. Recently, the genome of *Laccaria bicolor* was sequenced, which revealed that this basidiomycete shows highest sequence similarity with the sequenced basidiomycete plant pathogens *U. maydis* and *M. lini* (Martin and Selosse, 2008; Martin *et al*., 2008). The 65-Mb genome is expected to code for approximately 20 000 proteins, approximately 3000 of which are predicted to be secreted, including approximately 300 effector-type SSPs. A number of these SSPs show homology to HESPs of rust fungi. Some of the genes encoding SSPs are strongly up-regulated during infection, whereas others are down-regulated, suggesting a complex regulation of SSPs during colonization of the host. Many SSPs are also cysteine-rich and probably belong to the cystine-knotted peptides, such as Avr9 of *C. fulvum* (van den Hooven *et al*., 2001). One of those mycorrhiza-induced cysteine-rich SSPs of 7 kDa (MISSP7) was highly up-regulated in the mycorrhizal tips of mycelium present in the Hartig net. However, additional studies are required to learn more about its function and that of other MISSPs. Further functional analysis of the *L. bicolor* genome will provide more insight into the differences between symbiotic, saprophytic and pathogenic fungi.

DISCOVERY OF NOVEL EFFECTORS

Recently, whole-genome sequencing of fungal pathogens has provided an enormous amount of data that can be analysed for the presence of putative (secreted) effectors. Enrichment for effector candidates can be achieved by the integration of genome, transcriptome, proteome and metabolome data, when available. Comparative secretome analysis and BLAST sequence similarity searches can also be used to identify putative effectors in sequenced genomes, but will only prove to be successful when sufficient homology exists among effector genes, such as seen for Avr4, Ecp6 and the functional homologue of ToxA peptide from *S. nodorum* in *P. tritici-repentis* (Friesen *et al*., 2006, 2008a). In addition, the genome of *L. bicolor* (Martin *et al*., 2008) has identified some MISSPs homologous to effectors in rust and other fungi. However, despite the great potential of genome-wide searches for the identification of candidate effector genes, their function still needs to be confirmed experimentally by over-expression, gene disruption or silencing in fungal isolates and subsequent (a)virulence assays on host plants.

TRANSLOCATION OF FUNGAL EFFECTORS

Fungal effector proteins can be roughly grouped into extracellular effectors that are secreted into the apoplast or xylem of their host plants, and cytoplasmic effectors that are translocated into host cells. Despite the low degree of sequence conservation amongst fungal effectors, most code for small secreted proteins, some of which are translocated into host cells by an as yet unknown mechanism. Extracellular effectors are often N- and sometimes C-terminally processed, as has been shown for the Avr and Ecp effectors of *C. fulvum* (Stergiopoulos and de Wit, 2009) and the Six effectors of *F. oxysporum* f. sp. *lycopersici* (Houterman *et al*., 2007). Another common feature of many fungal effectors is the presence of multiple cysteine residues that might be involved in disulphide bridge formation, providing protein stability. Some effectors active inside the host cell, such

as those of rusts, possibly require appropriate folding and disulphide bridge formation outside the host before being taken up (Dodds *et al*., 2004; Kemen *et al*., 2005). *Magnaporthe oryzae* induces a biotrophic interfacial complex (BIC) in the host which contains secreted effectors, such as Avr-Pita, Pwl1 and Pwl2. It has been shown by live-cell fluorescence imaging that these effectors that accumulate in BICs are translocated into invaded host cells (Valent *et al*., 2009).

Effectors of rusts, powdery mildews, *M. oryzae* and *F. oxysporum* f. sp. *lycopersici* are putatively translocated into the host cell, where they interact with cytoplasmic or nuclear R proteins (Catanzariti *et al*., 2007; Dodds *et al*., 2004, 2006; Ellis *et al*., 2007; Houterman *et al*., 2009; Jia *et al*., 2000; Shen *et al*., 2007). However, so far, in all fungal effectors, no clear consensus signature, such as the RXLR motif present in oomycete effectors, has been identified. The cytoplasmic effectors Avra10 and Avrk1 of *B. graminis* f. sp. *hordei* even lack a signal sequence for secretion (Ridout *et al*., 2006). The host-selective protein toxin ToxA of *P. tritici-repentis* (Ciuffetti *et al*., 1997) contains a solvent-exposed RGD motif that interacts with the host plasma membrane and is probably required for internalization (Manning *et al*., 2008). A similar RGD motif mediating interaction with the plasma membrane is also present in the IpiO (AvrBlb1) effector proteins of the oomycete *Phytophthora infestans* (Senchou *et al*., 2004; Vleeshouwers *et al*., 2008).

DIRECT AND INDIRECT INTERACTIONS BETWEEN EFFECTORS AND R PROTEINS

Direct interaction between the cognate R protein and effector is also referred to as the 'receptor–ligand' model. Direct interactions between a fungal effector and plant R protein have been described for Avr-Pita (Avr-Pita$_{176}$) from *M. oryzae* and the cognate Pi-ta resistance protein from rice (Jia *et al*., 2000), and all effectors of *M. lini* and their cognate R proteins in flax (Dodds *et al*., 2006; Ellis *et al*., 2007; Wang *et al*., 2007). One implication of the 'receptor–ligand' model is that plants must carry large numbers of R proteins to enable them to recognize all individual effectors. This problem seems to be partly overcome by plants that have developed R proteins that monitor modifications in plant targets of fungal effectors. In this so-called 'guard' model, R proteins do not interact directly with an effector, but guard its host target and respond to alterations in this target caused by the effector. The 'guard' model also enables the detection of multiple unrelated effectors that interact with the same host target guarded by a single R protein. A classical example of the

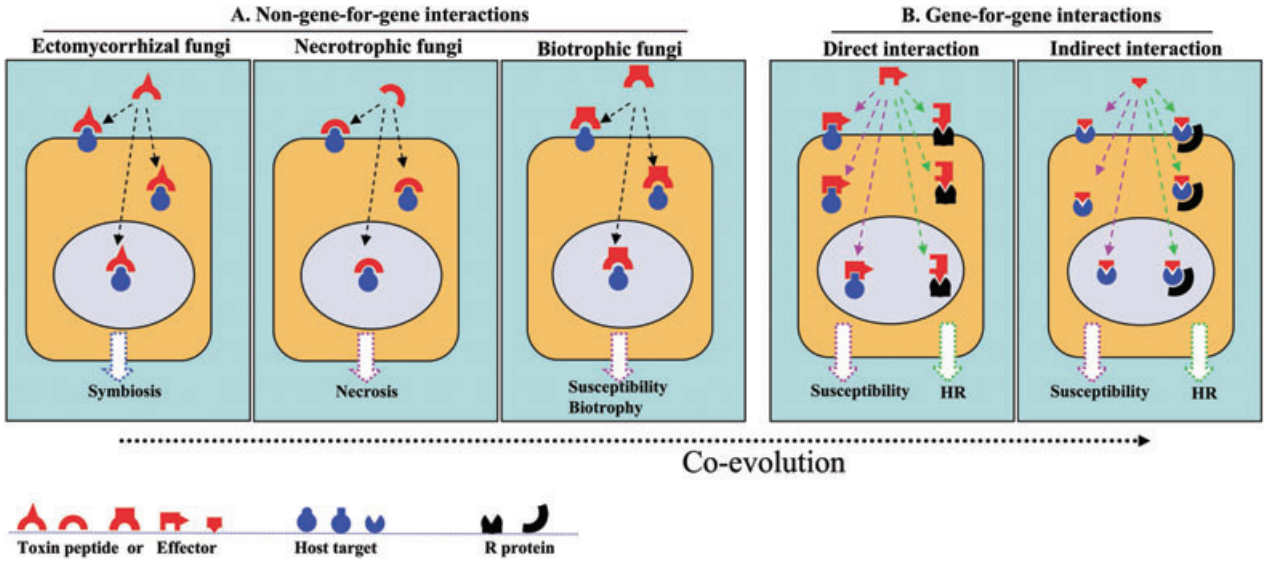

Fig. 1 Diagram showing effectors from non-gene-for-gene ectomycorrhizal, necrotrophic and biotrophic fungi, and from gene-for-gene biotrophic fungi, interacting with host targets and resistance proteins. (A) Non-gene-for-gene interactions. Ectomycorrhizal fungi: putative effectors of (ecto)mycorrhizal fungi interact with host targets to facilitate symbiosis and suppress or avoid host defence responses. Necrotrophic fungi: effectors of necrotrophic fungi interact with host targets, eventually causing necrosis of host plant cells to facilitate disease. Biotrophic fungi: effectors of biotrophic fungi interact with host targets and facilitate disease without killing host plant cells. (B) Gene-for-gene interactions. It is assumed that, as a result of co-evolution with their host plants, biotrophic fungi have evolved further. Direct interaction: it is assumed that effectors have two domains, one of which interacts with the virulence target, leading to host susceptibility, whereas the other is recognized by the R protein present in resistant plants, leading to the hypersensitive response (HR) and resistance. Indirect interaction: the effectors interact with the virulence target leading to susceptibility. Resistant plants have developed resistant proteins that do not interact directly with effectors, but guard the virulence target, and HR is triggered after sensing manipulation of the virulence target by the effector. Interaction between effectors and host targets (virulence targets and R proteins) can take place on the plasma membrane, in the cytoplasm or in the nucleus.

'guard' model is represented by the indirect interaction between Avr2 and Cf-2, mediated by Rcr3, in the *C. fulvum*–tomato pathosystem (Rooney *et al*., 2005). In many other (nonfungal) pathosystems, indirect interaction between R protein and cognate Avr has been reported, suggesting that the majority of interactions would fit into the 'guard' model (Jones and Dangl, 2006). Direct interaction between effector and R protein can be overcome more readily by mutations in effectors that abolish recognition, whereas jettison of effectors seems to mediate evasion of R-mediated resistance in the case of indirect interactions (De Wit, 2007; Jones and Dangl, 2006).

CONCLUSIONS AND FUTURE PROSPECTS

In Fig. 1, a short overview of the putative roles of effectors from necrotrophic, ectomycorrhizal and biotrophic fungi, and different types of interaction with their hosts, is depicted. Much additional research is required to confirm or reject the proposed models as they are in part speculative.

With the rapid advances in novel sophisticated and efficient sequencing platforms, many fungal genomes will be sequenced in the near future and, with parallel advances in bioinformatics tools, this will speed up the discovery of novel fungal effectors by comparative genomics. However, so far, homology among fungal effectors is limited. For most fungal effectors, a function in virulence can be investigated experimentally by gene disruption, gene silencing or overexpression, and it is expected that a number play a role in the suppression of PTI induced by fungal PAMPs. The discovery of more fungal PAMPs is anticipated and will enrich our understanding of the evolution of the plant defence system and the interplay between PTI and ETI. The finding that some fungal effector genes reside on pathogenicity islands is intriguing, and it will be interesting to determine how these islands have arisen during evolution. The elucidation of the mechanisms of translocation and the identification of interactors of fungal effectors in host cells remain major challenges for the future. Future research on effectors of mycorrhizal fungi, and of necrotrophic and biotrophic fungal pathogens, will reveal whether they represent similar biological functions, but only differ in their degree of co-evolution.

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