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How filamentous pathogens co-opt plants: the ins and outs of fungal effectors

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Research on effectors secreted by pathogens during host attack has dominated the field of molecular plant–microbe interactions over recent years. Functional analysis of type III secreted effectors injected by pathogenic bacteria into host cells has significantly advanced the field and demonstrated that many function to suppress host defense. Fungal and oomycete effectors are delivered outside the host plasma membrane, and although research has lagged behind on bacterial effectors, we are gradually learning more and more about the functions of these effectors. While some function outside the host cell to disarm defense, others exploit host cellular uptake mechanisms to suppress defense or liberate nutrients intracellularly. Comparative genomics suggests that the organization of effector genes drives effector evolution in many pathogen genomes.

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Introduction

Inheritance of plant immunity to pathogens is controlled by corresponding gene pairs, as plants carry resistance (*R*) genes that interact with pathogen avirulence (*Avr*) genes in a gene-for-gene manner. Since direct interaction between R and Avr proteins could often not be demonstrated experimentally, it was recognized that R proteins may also monitor the state of host components targeted by pathogen Avr molecules to establish disease. Presently, the term 'effector' is commonly used for these molecules [1•]. Similar morphological growth characteristics, virulence mechanisms, and infection strategies are generally shared in the taxonomically distinct fungi and oomycetes, despite differences in physiology, biochemistry, and genetics. Both types of pathogens target effectors to the apoplast or cytoplasm where they function to modulate host physiology, often through suppression of host defenses, or to protect the pathogen from host defense responses employed to halt pathogen growth. In this review, we focus on recent progress in research on the function and evolution of effectors from filamentous plant pathogens, guided by the consecutive stages occurring during disease establishment (Figure 1).

Effector production

Fungal effector genes are typically not, or lowly, expressed in axenic cultures, but are induced upon host colonization. Since some effector genes are induced by nitrogen starvation *in vitro*, nitrogen limitation was proposed as an *in planta* trigger of their induction. However, nitrogen availability may not be limited in plants, and many *in planta*-induced effector genes do not respond to nitrogen deprivation [2]. Thus, the *in planta* signals that trigger induction of effector genes presently remain largely unknown.

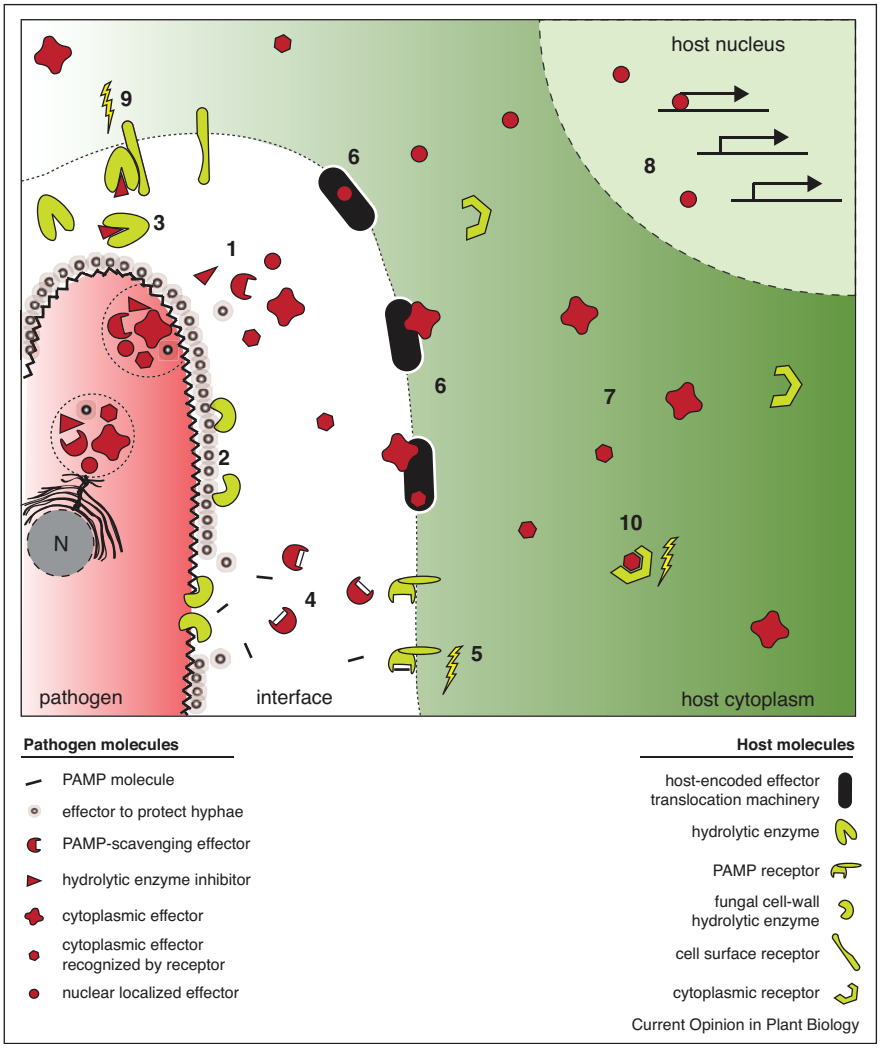
Transcriptional regulators important for early infection stages were recently identified. In the root invading fungus *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), the transcriptional regulator Sge1 is required for *in planta* expression of various effector genes [3•]. Interestingly, *SGE1* orthologs occur widely in fungi and include master regulators of morphological switching in dimorphic fungi. Recently, the *Magnaporthe oryzae* zinc finger transcription factor MoCRZ1 was found to regulate various virulence factors [4]. *MoCRZ1* is important for virulence on rice, and homologs were identified as pathogenicity regulators in various fungi [5,6]. Intriguingly, MoCRZ1 also regulates genes involved in vesicle-mediated secretion, potentially implicating MoCRZ1 in effector secretion [4].

Recently it was elegantly demonstrated that pathogens may tailor their effectors to individual host tissues. A gene expression study in *Ustilago maydis*-infected maize tissues revealed differential timing and organ-specific expression of particular effector proteins. Subsequent inactivation of effector clusters revealed differential impact on pathogenicity in various plant tissues. The data suggest that *U. maydis* employs universal effectors for establishment of host compatibility, followed by deployment of effectors with organ-specific properties to redirect physiology [7].

Effector delivery

Filamentous pathogen effector proteins are typically produced in the endoplasmic reticulum and secreted through

Figure 1



The role of effectors in the interactions between fungi and their host plants. Fungi secrete effectors in the interface between pathogen and host after host penetration (1). Some effectors contribute to fungal virulence by shielding hyphae against hydrolytic host defense enzymes in the host–pathogen interface (2), by inactivating these enzymes (3), or by scavenging potential PAMP molecules (4) that may alarm host defense (5). Many effectors do not remain in the host–pathogen interface but are translocated to the host cytoplasm without the use of pathogen-encoded translocation machinery (6). Although the molecular mechanism explaining how translocated effectors contribute to fungal virulence largely remains obscure, some of them are expected to affect cytoplasmic processes related to host defense (7). Recent evidence suggests some effectors are translocated to the nucleus where they may regulate transcription of target genes (8). Host recognition of filamentous pathogen effectors occurs through cell surface receptors in the host–pathogen interface (9), or in the host cytoplasm through NB-LRR-type receptors (10).

Golgi-derived vesicles. While fungi limited to the extracellular space presumably secrete effectors mainly at hyphal tips, rusts, downy, and powdery mildews deliver their effectors via haustoria [8]. These structures were originally only considered as feeding structures that invaginate the host plasma membrane and are surrounded by an extracellular matrix. A remarkable structure for effector delivery is described as the biotrophic interfacial complex of *M. oryzae*. Upon penetration of rice cells by this fungus, the penetration peg differentiates into a primary hypha that invaginates the host plasma membrane [9]. At the tip of the entering hypha, effectors are secreted where the biotrophic interfacial complex develops. Subsequently, the hypha differentiates into a bulbous pseudohypha and continues to grow into neighboring plant cells while the biotrophic interfacial complex remains at the same position and delivers newly synthesized effectors [10••].

Effectors with apoplastic functions

Cell-wall-degrading enzymes (CWDEs) are relatively well-characterized apoplastic effectors. Comparative genomics demonstrates that CWDE catalogs differ significantly between fungal pathogens [11•,12]. Although in several fungi the sucrose nonfermenting 1 protein (SNF1)

regulates CWDE expression and *SNF1* mutants display impaired virulence, functional redundancy complicates investigations into the contribution of individual CWDEs in virulence [13]. As CWDEs are also produced by saprophytic fungi, they are likely recruited as pathogenicity factors in pathogenic species that evolved from saprophytes, but do not determine host range or host specificity.

A second group of apoplastic effectors are the necrosis and ethylene-inducing protein (NEP1)-like proteins (NLPs) present in many pathogenic bacteria, fungi, and oomycetes that generally induce cell death in dicotyledonous plants through plasma membrane permeabilization [14•]. Curiously, pathogens of monocotyledonous plants also carry *NLP* genes, but their role in pathogenicity remains obscure since they do not elicit necrosis. For example, heterologous expression of the single *NLP* gene from *Mycosphaerella graminicola* (*MgNLP*) did not induce cell death or elicit immune responses in wheat leaves, and gene knockouts did not affect virulence on wheat. However, MgNLP induced cell death in *Arabidopsis* leaves [15].

Perhaps the most intriguing apoplastic effectors are generally referred to as small cysteine-rich secreted proteins with unknown function. These effectors are generally species-specific or even isolate-specific. For few, their role in virulence has recently been elucidated. Several of these effectors from *Cladosporium fulvum*, but also from the oomycete *Phytophthora infestans*, have been characterized as inhibitors of extracellular host proteases important for basal defense [16–18]. Others appear to play key roles in protecting the fungus from chitin-triggered host defenses [19,20••]. Plants produce apoplastic exochitinases that are not detrimental to fungal growth, but release chitin oligosaccharides from fungal cell walls that act as recognition patterns for host defense receptors. The rice lysin motif (LysM)-containing chitin oligosaccharide elicitor-binding protein (CEBiP) was characterized that, together with the LysM-containing chitin elicitor receptor kinase-1 (OsCERK1), is required for chitin-triggered immune responses [21,22]. These responses include vacuolar accumulation of basic endochitinases that act as powerful antifungal agents once they are released. Orthologous chitin receptors are found in other plant species, including *Arabidopsis* [23]. Upon stomatal entry, *C. fulvum* secretes a repertoire of effector proteins that include the chitin-binding effectors Avr4 and Ecp6. Avr4 contributes to virulence by binding to fungal cell walls through an invertebrate chitin-binding domain in order to protect hyphae from host chitinases [19]. In contrast, Ecp6 sequesters chitin oligosaccharides through its LysM domains in order to prevent the activation of plant immune receptors [20••]. Ecp6 homologous LysM effectors widely occur in fungi [24,25], suggesting that scavenging of chitin oligosaccharides is a conserved strategy of fungal pathogens to avoid detection [20••]. Interestingly, although the secretion of chitinases by the plant is a widespread strategy in antimicrobial defense, Avr4 homologs appear restricted to only few *C. fulvum*-related fungi [26]. Possibly, in other pathogens LysM effectors may also be able to protect fungal hyphae against plant chitinases [25].

Effector uptake into host cells

Although effectors are delivered apoplastically, many appear to be subsequently translocated into the host cytoplasm. Initial evidence for cytoplasmic translocation derives from cytoplasmic R proteins that recognize fungal effectors. Flax rust (*Melampsora lini*) Avr effectors induced cell death in plants containing cytoplasmic R proteins, and direct interaction between the effectors and corresponding R proteins was demonstrated [27]. Recently, host cell internalization of haustorial effectors in the absence of *M. lini* was demonstrated, showing that pathogen-encoded components are not required for translocation [28]. Cytoplasmic recognition of effectors occurs in host cells for other pathogens also [1•,8]. Interestingly, some *M. oryzae* biotrophic interfacial complex-secreted effectors autonomously move from the cytoplasm of invaded cells into neighboring cells, possibly preparing these for fungal invasion [10••].

Ground-breaking work on a possible mechanism of effector uptake was recently reported [29••]. Many predicted oomycete effectors contain an N-terminal RxLR motif [30] that was proposed to mediate autonomous effector uptake [31,32]. It is proposed that RxLR motifs enable oomycete effectors to bind to host cell surface phosphatidylinositol-3-phosphate (PI3P) and subsequently enter host cells though vesicle-mediated endocytosis [29••]. Similarly, the N-termini of various fungal effectors were reported to carry degenerate RxLR motifs that bind to PI3P and mediate effector translocation, although this may not be a universal means of effector uptake [29••,33]. Furthermore, whether effector uptake mediated by PI3P binding is functionally involved in the physiology of plant infection by fungi and oomycetes presently remains unknown. Conceivably, lipid-targeting may be one of several means for effectors to enter host cells since different uptake mechanisms are likely to exist to prevent hosts from intercepting effector trafficking.

Evidence for another conserved oomycete host translocation motif was provided for crinkler effectors, many of which appear to be targeted to the host nucleus [34•]. Furthermore, powdery mildew and rust fungi encode small secreted proteins that share an N-terminal Y/F/WxC motif that is not found in effectors from non-haustorial fungi or oomycetes, and it is tempting to speculate that this motif mediates translocation of fungal haustorial effectors [35].

Effectors with cytoplasmic functions

In contrast to many bacterial type III effectors that suppress host defense responses [1•], the function of few cytoplasmic fungal effectors has been elucidated. Houterman *et al.* showed that the *Fol* effector Avr1 (Six4) suppresses resistance mediated by the tomato cytoplasmic R protein I-2 [36]. Recently, 'SWEET' sugar efflux transporters were identified in plants [37••]. Several pathogens, including fungi with diverse feeding styles, induce expression of distinct *SWEET* genes, and *SWEET* induction by pathogenic bacteria was type III secretion dependent. Moreover, direct binding of a type III effector to a *SWEET* promoter was demonstrated, suggesting that sugar efflux is hijacked by cytoplasmic pathogen effectors in order to release nutrients [37••].

Previous studies identified fungal hexose transporters in obligate biotrophs that were assumed to act in concert with fungal cell-wall-derived invertases to take up glucose or fructose after sucrose hydrolysis [38]. However, a recent study identified a plasma membrane-localized sucrose transporter in the smut fungus *U. maydis* that is specifically produced during plant infection, required for virulence, and able to outcompete plant transporters. In this way, *U. maydis* can utilize sucrose without prior extracellular hydrolysis by invertases [39]. Direct utilization of sucrose circumvents invertase-induced changes in apoplastic glucose concentrations known to induce defense [38].

Effector evolution

Effector genes are frequently under selection pressure, illustrating the coevolutionary arms race between host and pathogen [40–45]. They are often located at genomic sites that promote evolution through mutation or recombination. Comparative genomics among *Aspergillus* spp. revealed the accumulation of species-specific genes in chromosomal islands enriched for transposons [46]. Tomato pathogenic *Fol* strains contain a transposon-enriched pathogenicity chromosome that can be exchanged between isolates [12]. The extreme impact of transposons is illustrated in the size-expanded genomes of obligate powdery mildew pathogens that are largely composed of transposons. These pathogens lost many genes that are dispensable for obligate biotrophy, likely explaining why they can no longer grow in the absence of their host [11•]. Intriguingly, of the ~250 effector genes identified in barley powdery mildew, only a handful are shared with pea and *Arabidopsis* powdery mildews, illustrating extreme host adaptation [11•].

Repeat-induced point mutation (RIP) is a fungal defense mechanism to protect genomes against transposable elements by accumulating mutations in repetitive DNA. In a large-scale study of *Leptosphaeria maculans* field isolates, a transposon-enriched cluster of effector genes was found to be degenerated by RIP, presumably as consequence of imposed selection pressure through the introduction of resistant canola varieties with matching R genes [41]. Intriguingly, one-third of the *L. maculans* genome is composed of AT-rich blocks that contain effector genes and transposons that are both affected by RIP [42•]. Taken together, transposon and RIP activity orchestrate rapid effector diversification, and aid in the rapid generation of effector variants that escape host recognition [42•]. Transposon activity appears to play an important role in effector evolution in oomycete genomes as well [43].

Effector discovery

Pathogen effector catalogs are highly lineage-specific and determination of effector catalogs is a challenge. Typical effector calling based on the presence of signal peptides and absence of transmembrane domains has resulted in the prediction of catalogs that often contain up to hundreds of potential effectors for individual pathogens. To enhance prediction accuracy, secreted protein prediction pipelines have been developed that combine different algorithms [47,48]. However, more sophisticated methods are required to identify the most relevant effectors for disease establishment within large effector catalogs. A rather obvious criterion is whether candidate effector expression *in planta* can be detected [11•,35,44]. Furthermore, several studies have now shown that signatures of positive selection can be used to pinpoint candidate effector genes in sequenced genomes [40–45]. Comparative genomics on the related maize pathogens *U. maydis* and *Sporisorium reilianum* identified regions of low sequence conservation that primarily encode clusters of secreted effectors in otherwise well-conserved syntenic genomes. Interestingly, this effector differentiation suggests that the two maize pathogens target different host molecules. Furthermore, mutational analysis of several effector clusters confirmed their role in virulence [49].

Effector recognition

As discussed above, successful pathogens exploit effectors to subvert their hosts, resulting in effector-triggered susceptibility (ETS). Plants have responded by evolving R proteins that recognize effectors and activate effector-triggered immunity (ETI). Necrotrophic fungal pathogens were considered rather nonspecific in their host attack. However, many necrotrophic pathogens evolved mechanisms to attack plants in sophisticated ways, even exploiting host resistance mechanisms [50–52]. Since various effectors (toxins) interact with disease resistance protein analogs, it is now suggested that necrotrophic pathogens deliberately activate host ETI responses directed against biotrophic pathogens to establish ETS [52].

Nowadays, cultivar-specific resistance activated by species-specific, race-specific or strain-specific effectors is generally discriminated from immune responses

triggered by pathogen-associated molecular patterns (PAMPs) that are conserved throughout classes of microbes. However, some pathogens deploy evolutionarily ancient and well-conserved effectors that are instrumental for pathogenicity, forcing plants to evolve recognition of these molecules to become resistant to these pathogens. Essentially, such effectors now act as PAMPs that blur the PAMP-effector dichotomy and illustrate a continuum between immune responses triggered by PAMPs and by effectors. Ultimately, plant resistance is determined by immune receptors that recognize appropriate ligands, the nature and intrinsic function of which is not relevant as long as they accurately betray the microbial invader to the plant [53•].

Conclusions

Although all fungal effectors are delivered to the apoplast, they can be divided into two groups: those that remain in the apoplast and those that translocate into host cells. Recently, a mechanism for effector uptake has been proposed, but the universality of this mechanism is not certain and other means of effector uptake are likely to exist. Without doubt, the major challenge for the future will be to assign biological functions to the increasing number of effector molecules identified in fungal genomes. Typical effector calling based on motifs for extracellular secretion has resulted in the prediction of catalogs containing hundreds of effectors for individual pathogen strains. More sophisticated methods of effector discovery are required to identify those that make major contributions to virulence. Comparative genomics upon resequencing of multiple isolates of a single species or related species with overlapping or differential host ranges can identify signs of evolutionary pressure on specific genes that may be of interest to focus research efforts. Ultimately, understanding the function of individual pathogen effectors is expected to provide new avenues for disease control.

Box 1.

Box 1 Outstanding questions:

- Why do pathogens employ highly lineage-specific effector catalogs while many of their host targets appear to be conserved across host species?
- Which are the targets of filamentous pathogen effectors?
- Why do genomes of filamentous pathogens often encode hundreds of effector proteins?
- Which are the *in planta* triggers of effector gene expression and how are these triggers perceived?
- What is the role of NLP effectors in biotrophic pathogens and the role of LysM effectors in non-pathogenic fungi?
- Do mycorrhizal fungi utilize effectors that target host defense to establish symbioses?

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