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# Roles and delivery mechanisms of fungal effectors during infection development: common threads and new directions

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Fungal effectors have often been referred as a 'sea of diversity', but recently, experiments have shed some light onto effector biology, including discovery that unrelated fungi utilize some common methods for creating a more compatible host environment. A wheat pathogen and a rice pathogen, for example, have evolved mechanisms to suppress chitin-mediated basal defenses in their respective plant hosts. Smut fungi, on the other hand, might have evolved a unique mechanism to manipulate their host environment by altering cell metabolism. Genome mining and bioinformatics pipelines have streamlined the suite of effectors in important pathogen genomes, so researchers can make more targeted strikes on potentially important effectors. This combination of informatics and empirical studies will allow greater insight into effector function.

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## Introduction

While fungal effectors may be identifiable by the known hallmarks like size (small), content (often cysteine-rich) and location (secreted), another important criterion is that they have neither significant homology to known sequences in other organisms nor obvious protein domains. This can make it difficult to group effectors together by function, and with several exceptions, to take advantage of the vast fungal resources available for reverse genetic approaches for effector identification. On the other hand, it perfectly reflects the mechanisms underlying plant and pathogen interactions; as more fungal genomes are sequenced, we find more fungal effectors residing in genomic locations that are subject to heavy selection pressure, similar to the 'two-speed' genome of the Potato Famine Oomycete pathogen *Phytophthora infestans* [1]. This ensures that the fungus can evolve to overcome selection pressures placed upon it by, for example, resistant plant cultivars, and generate virulence strategies in accord with its particular lifestyle and host. In recent years, there have been several exceptional review articles on fungal and oomycete effectors ([2–5], van West *et al.*, in this issue).

Here, we will examine the latest findings on effectors, such as their refined numbers within fungal genomes and their delivery mechanisms. We will also attempt to group fungal effectors together by mode of action; while effectors diverge in sequence, it would appear that some exert similar effects on their hosts. While we take a special interest in the hemi-biotrophic and biotrophic plant pathogens in this review, we will also draw from research into delivery mechanisms and modes of action of mammalian pathogen effectors, and effectors from necrotrophic fungi (for outstanding reviews, see [6,7], respectively). Impressively, since 2011 at least 18 research articles have been published on fungal effectors in plants; we will draw from these and a cohort of other related studies in the following review, and apologize in advance for those we were unable to include due to space constraints.

## Defining and refining the effector suite

In 2006, an oomycete genome paper was published comparing *Phytophthora sojae* and *P. ramorum*, and introduced us to the concept that fungal-like organisms, and probably fungi as well, could have suites of effectors numbering in the hundreds [8]. Since then, a wealth of fungal genomic data has provided information about sets of potentially secreted proteins and as we learn more about effectors and their varied roles, different bioinformatic tools have been adopted to increase the accuracy of defining effector suites. Between 2011 and 2012, at least ten plant pathogenic fungi have been bioinformatically scrutinized for their effector repertoire and a common pipeline has emerged for identifying likely effector candidates, which generally begins with SignalP [9] and TargetP [10] for signal peptide prediction (Table 1). The search continues with programs like TMHMM [11] and Fungal Big-PI [12], which identify transmembrane and GPI-anchoring domains, respectively, and proteins with these motifs are generally excluded from further analysis. Many pipelines then BLAST their shrinking list against the NCBI non-redundant protein database, and exclude proteins that return matches to known genes. Table 1 shows a list of pathogen genomes that have been put through this type of pipeline; however, numbers of effectors vary widely

**Table 1**

**Refined suites of effectors in plant pathogenic fungi**

| Name of fungus | No. predicted effectors | No. predicted genes | Search criteria[b] | Host | Lifestyle | Reference |
|---|---|---|---|---|---|---|
| *Colletotrichum higgensianum* | 102 | 16,172 | Big-PI; WolfPSORT | Crucifers | Hemi-biotroph | [13] |
| *Colletotrichum truncatum* | 11 | Unknown | SignalP; TMHMM; NCBInr; Big-PI | Legumes | Hemi-biotroph | [46] |
| *Fusarium graminearum* | 574 | 11,640 | SignalP; TargetP; TMHMM; Big-PI | Wheat | Necrotroph | [47] |
| *Golovinomyces oronotii* | 70 | Unknown | SignalP; TMHMM; NCBInr | Arabidopsis | Biotroph | [48] |
| *Hemileia vasatrix* | 382 | 6763[a] | SignalP; TargetP; TMHMM | Coffee | Biotroph | [49] |
| *Leptosphaeria maculans* | 122 | 12,469 | SignalP; TargetP; TMHMM | Crucifers | Necrotroph | [50] |
| *Sporosorium reilianum* | 442 | 6648 | NS | Maize | Biotroph | [14] |
| *Ustilago maydis* | 494 | 6652 | NS | Maize | Biotroph | [14••]; Broad Institute |
| *Verticillium albo-atrum* | 119 | 10,221 | SignalP; TMHMM; Phobius | BHR[c] | Necrotroph | [51] |
| *Verticillium dahliae* | 127 | 10,535 | As Above | BHR | Necrotroph | [51] |

[a] Contigs determined via 454 sequencing of mixed plant–pathogen genomes.
[b] SignalP and TargetP are used to predict secreted peptides; TMHMM is used to predict transmembrane domains, and these are eventually excluded from these analyses; NCBInr is the nonredundant protein database at NCBI used for finding homology and these are eventually excluded from the analyses; Big-PI is used to identify GPI-anchors and these are eventually excluded from the analysis; NS = not specified.
[c] Broad host-range.

from 70 to 574. Further, there is no correlation between effectors and the number of predicted genes in a genome; a larger set of genes does not necessarily ensure a larger number of effectors, as in the case of the brassica pathogen *Colletotrichum higgensianum*, which boasts 16,172 genes, with only 0.6% of them predicted to be effectors [13]. Conversely, the related smut fungi *Ustilago maydis* and *Sporosorium reilianum* have a much smaller number of predicted genes at 6648 and 6652, but a larger number of predicted effectors at 6.6% and 7.4%, respectively [14••]. Lifestyle niche does not appear to show any obvious connection to numbers of effectors either; while we may see the beginnings of a common thread when looking at obligate biotrophs like the rusts and smuts with larger numbers of effectors (Table 1), the toxin-producing necrotroph *Fusarium graminearum* also has a relatively large suite of predicted effectors at 4.9% of its genome. Analysis of effector function will in time reveal whether obligate biotrophs require greater numbers of effectors to modify their hosts' cells, keeping them alive throughout infection development.

## Special delivery!

### Exosomes and vesicles

Delivery of effectors from pathogens of mammals and oomycete pathogens into the cells of their hosts provide a wealth of information on which to build hypotheses for plant pathogens. Mode of entry into mammalian cells appears to mainly take place by either exosomes or secreted vesicles. Exosomes form via invagination of membranes and subsequent formation of multivesicular bodies (MVB), while vesicles form at the plasma membrane or at the membranes of organelles (reviewed in [15]). The mammalian fungal pathogens *Cryptococcus neoformans* and *Histoplasma capsulatum* deliver effectors by means of exosomes and secreted vesicles, respectively [16,17]. Evidence is now emerging that the powdery mildew obligate biotrophic fungus, *Golovinomyces orontii*, may also deliver its effectors in a similar manner. Powdery mildew fungi produce globose haustoria as feeding structures, and elaborate a multilayered extra-haustorial membrane (EHM) around the fungal cell such that plant and fungal cytoplasm never contact each other (reviewed in [18]). Recent isolation and subsequent electron microscopy of haustorial complexes has revealed a preponderance of MVB in the haustorium, thought to bud from the fungal plasma membrane [19••]. Importantly, the authors found the first evidence of vesicle clusters in the extra-haustorial matrix (EHMx) that fall within the size range of exosomes from human fungal pathogens (between 50 and 300 nm). Together, these data provide tantalizing evidence that an exosome-mediated and/or vesicle-delivery system may be shuttling effectors across the EHMx, and into the plant cell. Further experiments will be required to determine whether these vesicles are targeted for the plant cell.

### Biotrophic interfacial complexes

In 2007, Valent and colleagues began reporting on how the rice blast fungus, *Magnaporthe oryzae*, invades successive living plant cells during the biotrophic phase of its hemi-biotrophic infection cycle. In the first invaded cell, the fungus forms a thin filamentous hypha and subsequently differentiates into a bulbous invasive hypha (IH), filling the cell. The IH is enclosed in a host-derived extra-invasive hyphal membrane, preventing direct contact between fungal and plant cytoplasm [20]. The IH undergoes extreme constriction to cross into neighboring cells, which then further differentiates into bulbous IH similar to the initially invaded cells [20]. Using live-cell imaging and fluorescent labeling of known effectors, Valent and colleagues demonstrated that effectors preferentially accumulate in highly localized structures formed on the IH, called biotrophic interfacial complexes

(BICs) [21•]. Upon entering each rice cell, effectors are secreted at the tip of the initially filamentous hypha, into BICs. These tip BICs remain at the same location beside the first-differentiated bulbous IH cells as the fungus continues to colonize the host cell. Secondary BICs formed in subsequently invaded cells were comparatively smaller than initial BICs. Preferential BIC accumulation of effectors was conferred by motifs residing between the effector promoter and signal peptide-encoding sequences. BIC-localized secreted proteins, such as PWL2 and BAS1 (biotrophy-associated secreted protein 1) were translocated into the rice cytoplasm. By contrast, BAS4, which uniformly outlines the IH but is not found in the BICs was not translocated into the rice cytoplasm. The movement of fluorescent effectors that reached the invaded cell's cytoplasm moved into adjoining noninvaded rice cells in a manner dependent on protein size and rice cell type, consistent with transport through plasmodesmata [21•], and indicative of a putative 'priming' mechanism for hyphal invasion.

#### Pathogen-independent delivery

Recent evidence suggests the entry of fungal and oomycete effectors via receptor-mediated endocytosis, even in a pathogen-independent manner [22,23]. In 2010, Kale and coworkers found that oomycetes and fungal effectors with an RxLR motif or functional variants thereof enter into host cells via lipid raft-mediated endocytosis. This process involves binding of motifs to the host cell surface via phosphatidylinositol-3-phosphates (PI3P; [22], reviewed in [5]). In the flax rust pathogen *Melampsora lini*, evidence indicates that the avirulence proteins AvrM and AvrL567 are secreted from haustoria during infection, accumulate in the haustorial wall, and are delivered into the host cell [23]. N-terminal signal peptides were sufficient to direct the accumulation of fused fluorescent proteins into the host cytoplasm, in absence of the pathogen. When lipid binding activities of these effectors were tested, AvrM bound strongly to phosphatidylinositol, phosphatidylinositol monophosphates, and phosphatidyl serine, but AvrL567 did not bind phospholipids [24]. These results support a role for both phosphoinositols and an as yet unknown mechanism, for pathogen-independent internalization of effectors into host cells [24]. For additional information, see the oomycete effector review in this issue.

## Effector roles: from bioinformatics to the bench

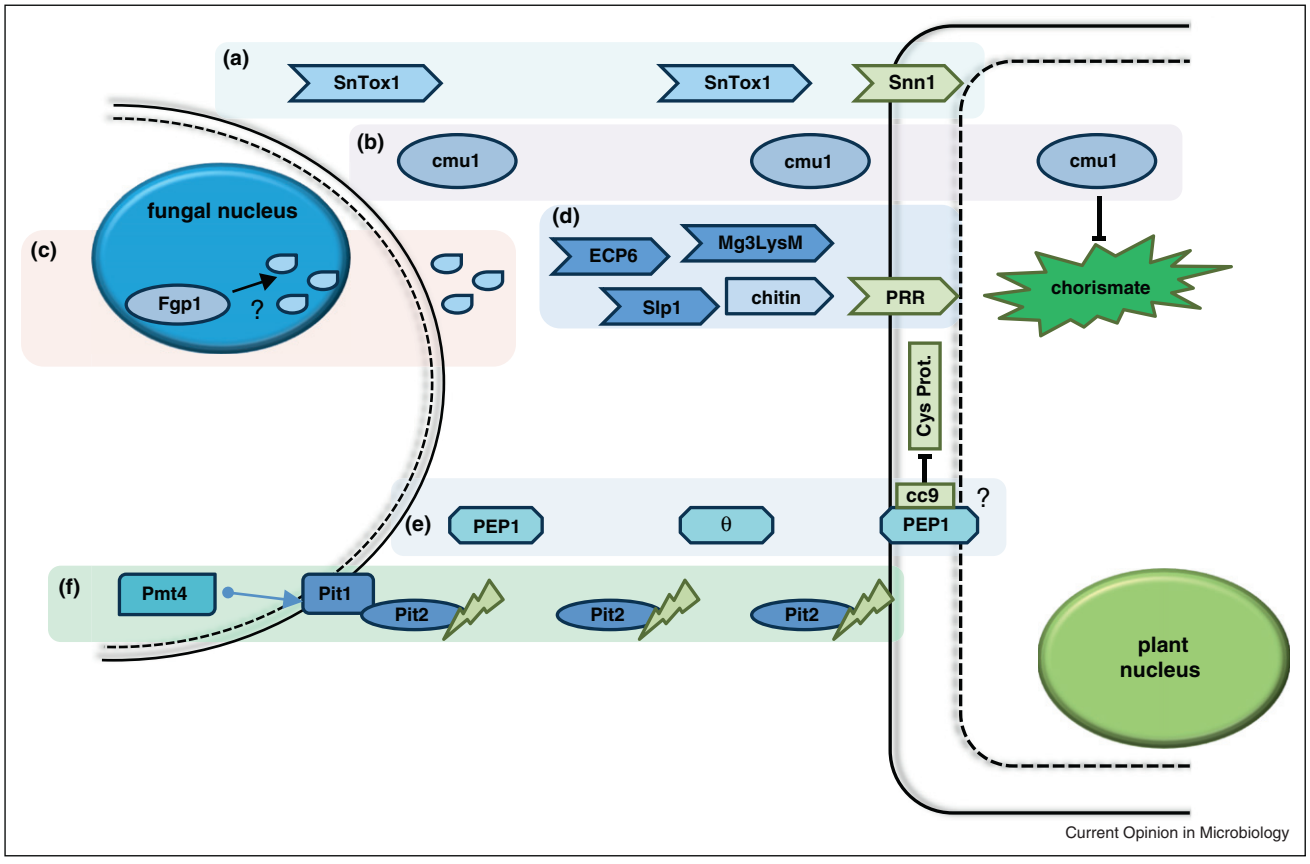
#### Suppressing basal immunity

Most plant pathogen effectors studied to date appear to play roles in suppressing basal defenses in plants. The zig-zag model, presented by Jeff Dangl and Jonathon Jones in 2006, describes the interplay between pathogen effectors and plant immune responses. It begins with the sensing of pathogen-associated molecular patterns (PAMPs) and activation of PAMP-triggered immunity (PTI), which gives a quick but low-strength defense response (basal defenses) to limit the spread of virulent pathogens [25]. In order to counter-act this response, pathogens will alter their PAMPs and/or secrete effectors that interfere with PTI, activating effector-triggered susceptibility (ETS). The following examples focus on the ETS section of the zig-zag model, and here is where we detect some common threads amidst the sea of effector diversity.

As part of its PTI mechanism, many plants have been shown to recognize chitin, a primary constituent of the fungal cell wall. Recognition takes place via pattern recognition receptors (PRR), for example the CEBiP proteins in rice and barley, both of which have been shown to bind chitin and elicit defense responses [26,27]. While a corresponding CEBiP in wheat has not yet been identified, recent work on fungal effectors in *Mycosphaerella graminicola*, a wheat pathogen, have revealed two genes that bind chitin, presumably competing with the plant's PRR, thereby blocking chitin-triggered basal defenses (Figure 1) [28]. Here, we find an example of commonalities between effectors in unrelated fungi; the chitin-binding effector from the tomato pathogen *Cladosporium fulvum*, ECP6 [29] was blasted against the *M. graminicola* genome, resulting in five candidates, two with signal peptides. The genes were named *Mg1LysM* and *Mg3LysM* for presence of Lysin domains, known to bind carbohydrates. *Mg3LysM* binds chitin and, when deleted, is significantly less virulent on wheat. Importantly, the mutant also triggers wheat defense responses, indicating that without this fungal gene present, chitin is sensed by the plant, activating PTI [28]. Similarly, Talbot and colleagues identified the *Slp1* gene in the rice blast fungus, *M. oryzae*, which contains two LysM domains and binds chitin [30]. Like *Mg3LysM*, it is also required for virulence and competes with CEBiP for chitin binding, suppressing basal defenses. Importantly, this study also demonstrated the importance of CEBiP in defense, as its silencing resulted in disease, even in the absence of *Slp1*. Although *Slp1* was identified in an earlier screen for secreted proteins associated with the biotrophic phase [31], it represents another example of where reverse genetics has been utilized to find similar effectors in an unrelated fungus.

Research on the maize smut fungus *U. maydis* has provided a wealth of information on effectors and their host targets in recent years. A current example includes the PEP1 protein [32••]. This fascinating story involves several host proteins, including the maize POX12, which encodes a peroxidase. The two proteins interact *in vivo*, and PEP1 can inhibit peroxidases *in vitro*. Importantly, *Pep1* is required for full fungal virulence, and when the mutants are inoculated onto maize, a strong reactive oxygen species (ROS) burst is observed under attempted penetration sites, with a concomitant increase in *POX12*

**Figure 1**




A model of putative molecular crosstalk between plants and fungal pathogens. For the sake of simplicity and inclusion, effectors from both biotrophic and necrotrophic fungi are incorporated into the same model, even though all strategies are obviously not used by all fungi. **(a)** The necrotrophic effector SnTox1 interacts with the resistance gene Snn1 (likely an NBS-LRR gene, but unknown whether it is membrane-bound) to promote cell death and infection. **(b)** Chorismate mutase, cmu1, enters the host cell and limits the amount of chorismate in the host cell, potentially effecting salicylic acid signaling. **(c)** Master regulators like Fgp1 are potentially in the nucleus, controlling production of secreted effectors. **(d)** Fungal effectors compete with the plant's PRR for chitin binding, thereby repressing basal defenses. **(e)** Pep1, and possibly other unknown fungal effectors (theta symbol) induce the plant's cc9, blocking cysteine protease-related defenses. **(f)** Pmt4 o-mannosylates Pit1 (blue arrow), which potentially interacts with the effector Pit2. Pit2 is potentially intercepting danger signals (lightening bolts) from the plant. Shapes in varying shades of blue indicate fungal-derived proteins, while those in green indicate plant-derived ones. Question marks indicate hypotheses that require testing.

expression. ROS is a well-known part of plant defenses, strengthening cell walls and having directly toxic effects on pathogens ([33] and Tudzynski *et al.*, in this issue). PEP1 appears to inhibit this important aspect of basal plant defenses. We will revisit *Pep1* in the concluding section of this review.

The second half of the zig-zag model includes effector-triggered immunity (ETI), which involves either direct or indirect recognition between a pathogen effector (usually an avirulence gene) and a plant gene (usually a resistance gene). This interaction results in a strong defense response, culminating in hypersensitive cell death. Necrotrophic pathogens turn ETI to their advantage; as per their lifestyle, they require dead cells as food sources and an ETI-like reaction that results in dead host cells provides them with the proper infection court. The wheat pathogen, *Stagonospora nodorum*, requires the small, secreted necrotrophic effector *SnTox1* for full virulence [34]. This gene interacts with the wheat resistance gene product Snn1 and leads to all the hallmarks of a hypersensitive response, including cell death, DNA laddering and defense gene expression (Figure 1). In absence of this protein interaction, disease does not occur.

## Altering host metabolism

Apart from suppressing basal defenses, a recent finding also shows that fungal effectors could be altering host cell metabolism for its own benefit. The *cmu1* gene, for chorismate mutase, from *U. maydis* produces such an effector [35••]. This gene is expressed during the biotrophic fungal stage, and its protein product is translocated into the host cell cytoplasm, where it subsequently spreads to neighboring cells. Fascinatingly, this movement into neighboring

cells might be 'priming' them for ensuing invasion, altering their metabolism for a more hospitable environment. How does it achieve this goal? The researchers' evidence suggests that it reduces the chorismate available for biosynthesis of salicylic acid, the potent signaling molecule involved in plant defense (reviewed in [36,37,35••]; Figure 1). Less SA could potentially mean less defense signaling potential, generating a more pathogen-friendly environment. Currently, this is the only confirmed example of a secreted chorismate mutase that alters plant metabolism; a quick database search uncovers no obvious matches to *cmu1* in the ascomycetes *M. oryzae*, *Neurospora crassa*, or *Fusarium* species. Interestingly, copies are found in the related basidiomycete biotrophic pathogens *S. reilianum* and *Ustilago hordei*, but not in the basidiomycete wheat rust *Puccinia* species. Is it possible that this particular strategy of altering host metabolism via limiting salicylic acid is an adaptation unique to smut fungi?

### Degrading host substrates

Fungalysins are metallopeptidases whose function could include the breakdown of plant compounds for fungal nutrients, or the breakdown of host tissues. In 2012, Vargas *et al.* showed the expression of a putatively secreted fungalysin from the maize hemi-biotroph *Colletotrichum graminicola*, to correlate with the pathogen's switch from a biotrophic to necrotrophic lifestyle [38]. While the role of these proteins as fungal effectors is not completely clear, it is tempting to probe further into their function, especially since the well-characterized avirulence effector *AVR-PITA* from *M. oryzae* also encodes a metalloprotease [39].

## Future challenges and new directions in effector biology

### Unknown or 'indirect' effectors?

How can we classify fungal proteins that are either secreted but with as yet unknown functions, or working *inside* the fungal cell, but impacting secreted effectors? Both of these classes need to be paid proper attention, as their roles could turn out to be pivotal in pathogenesis. An example of each comes again from *U. maydis*. *Pmt4* encodes an o-mannosyl transferase, modifying proteins as they leave the endoplasmic reticulum; its targets include a mucin involved in appressorium formation encoded by the *Msb2* gene, and the *Pit1* gene, whose product is involved in later stages of virulence, allowing the fungus to spread throughout the leaf [40]. *Pit1* is genetically linked to a secreted putative effector encoded by *Pit2*; the Pit2 protein is membrane bound at fungal tips, and the authors hypothesize that Pit2 shuttles defense signals to Pit1, helping disguise the fungus from being detected [41]. While Pmt4 is not secreted, could we perhaps consider it an *indirect effector*, as it is required for proper functioning of proteins that are? This hypothesis could be tested by identifying additional Pmt4 secreted targets and their characterization during infection.

A recent discovery by Jonkers *et al.* [42] in the necrotrophic fungus *F. graminearum* could also provide an example of an indirect effector. The *Fgp1* gene controls both tricothecene toxin production, virulence and sexual and asexual spore development in this fungus and interestingly, this master regulator is homologous to the nuclear-localized *Sge1* gene from *F. oxysporum*, which regulates the expression of the small, secreted *SIX* genes. The *SIX* genes function in either virulence or avirulence in tomato plants and are located on the mobile, lineage-specific chromosomes horizontally transferred to give rise to newly pathogenic strains [43]. While homologs of the *SIX* genes have not yet been found in *F. graminearum*, it is tempting to speculate that *Fgp1* controls a functionally, if not structurally, similar set of genes. Could genes like *Fgp1* and *Sge1* also be considered indirect effectors?

### Insight from the flip side

Pep1 was introduced above as the fungal effector that suppresses basal defenses in maize, but there is more to this story. When *pep1* mutants are inoculated onto maize, the plant's cystatin-encoding gene *cc9* is no longer expressed [44]. Moreover, host cysteine proteases, once suppressed by cc9, now become active and contribute to defenses. Cysteine proteases are known targets of oomycete effectors such as EPIC1 and 2B [45]; could other fungal effectors usurp host cystatin genes in order to shut down Cys proteases? And moreover, can we utilize plant responses to work our way backwards to identify fungal effectors? Much insight can be gained by carefully dissecting plant defenses, or lack thereof, during infection with various fungal mutants.

While bioinformatics pipelines have streamlined the *in silico* search for effectors, there is promise in effector identification from inferences made during the host response, as well as fungal genes that are modifying or controlling small secreted proteins into the plant cell.

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