Fungal Effectors and Plant Susceptibility

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Abstract

Plants can be colonized by fungi that have adopted highly diverse lifestyles, ranging from symbiotic to necrotrophic. Colonization is governed in all systems by hundreds of secreted fungal effector molecules. These effectors suppress plant defense responses and modulate plant physiology to accommodate fungal invaders and provide them with nutrients. Fungal effectors either function in the interaction zone between the fungal hyphae and host or are transferred to plant cells. This review describes the effector repertoires of 84 plant-colonizing fungi. We focus on the mechanisms that allow these fungal effectors to promote virulence or compatibility, discuss common plant nodes that are targeted by effectors, and provide recent insights into effector evolution. In addition, we address the issue of effector uptake in plant cells and highlight open questions and future challenges.

Contents

INTRODUCTION

Fungal plant pathogens are of huge economic importance because they threaten the production of crops already growing in the field and can cause postharvest diseases. Estimates suggest that approximately 10% of agricultural production is lost annually owing to fungal infection (103). With the growing consequences of climate change, these losses are expected to increase (46). To combat fungal infections, farmers rely on resistant crop varieties or multiple fungicide treatments, which can have negative effects on the environment. In addition, current agricultural practices that rely largely on planting one crop genotype on huge areas of land promote the selection of fungal strains that overcome genetic resistance quickly, necessitating the constant development and introduction of new resistance traits into crops by breeding approaches (153).

The ability to determine the sequence of fungal genomes has enabled unprecedented insights into genome composition, structure, and plasticity as well as genome evolution and adaptation. Since the first genome of a fungal plant pathogen, *Magnaporthe oryzae*, was published by Dean et al. (21) in 2005, the number of sequenced genomes from fungal plant pathogens has exploded. In addition, the genomes of several fungal endophytes that colonize plants without disease symptoms have become available, along with those of ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungal species, which establish mutually beneficial interactions with their plant hosts that represent the most ecologically and agriculturally important symbiosis in terrestrial ecosystems. The explosion of available genome sequences is not restricted to fungal genomes; it also includes an exponentially growing number of sequenced genomes from bacterial, oomycete, and nematode plant pathogens as well as parasitic plants, allowing cross-kingdom comparisons to detect conserved patterns and features and unprecedented insights into pathogen evolution (122, 133, 152).

Fungi have diverse lifestyles in which they deploy distinct strategies to interact with their host plants (**Figure 1**), including necrotrophic, biotrophic, and hemibiotrophic strategies; they also differ vastly in the range of plants they can infect. All fungi that colonize plants are recognized by the plant immune system and elicit host defenses. These initial defense responses are triggered by

Endophytes: fungi that establish a biotrophic interaction with host plant cells without causing visible symptoms

Necrotrophs: fungi that kill host plant cells and feed on their dead tissue

Biotrophs: fungi that colonize living plant tissue that represents either their sole (obligate) or an alternative (facultative) source for nutrients

Hemibiotrophs: fungi that establish an initial biotrophic phase with host plant cells and subsequently kill them to feed on dead tissue

invariant molecular patterns exposed by the microbe, referred to as pathogen-associated molecular patterns (PAMPs) and microbe-associated molecular patterns (MAMPs). In fungi, the cell wall component chitin functions as such a PAMP: After fungal contact, chitin oligomers are released from the fungal cell wall through plant chitinases. PAMPs are recognized through membrane-localized pattern recognition receptors (PRRs), which trigger a first line of defense reactions called PAMP-triggered immunity (PTI) (57). PRR signaling can also be triggered by host-derived damage-associated molecular patterns (DAMPs) (2, 6, 110). The activation of PRR signaling results in rapid responses that include the accumulation of reactive oxygen intermediates; activation of ion channels; activation of specific, defense-related mitogen-activated protein kinase cascades; and extensive transcriptional reprogramming of the host, collectively leading to an accumulation of antimicrobial compounds. Among these compounds are proteinases, chitinases, and glucanases that damage pathogen structures; enzyme inhibitors directed toward molecules produced by the pathogen; and nonproteinaceous antimicrobial molecules (25, 57, 84).

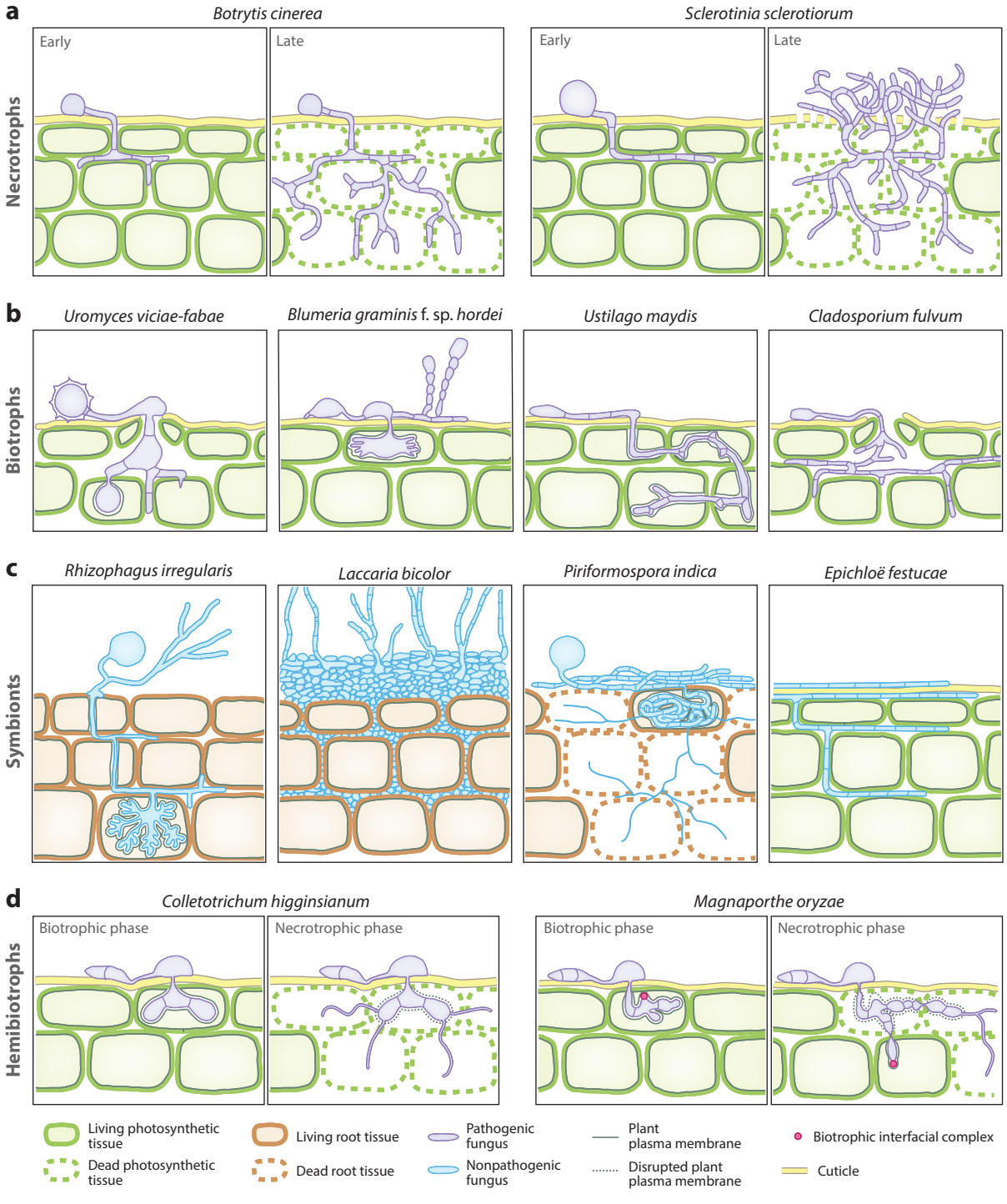
Fungal effector: any secreted molecule that modulates the interaction between the fungus and its host

After PRR activation, changes in hormone biosynthesis occur and plant cell walls are reinforced by callose deposition (84). In *Arabidopsis thaliana*, oligomers of fungal chitin are perceived by the LysM-RLK (receptor-like kinase) CERK1/RLK1/LYK1 receptor through three extracellular LysM domains that bind chitin oligomers directly (79, 94). Chitin-induced homodimerization of *A. thaliana* CERK1 is essential for the activation of downstream signaling (79). In rice, a LysM-containing protein called chitin elicitor–binding protein (CEBiP) is required for chitin perception and signaling in addition to CERK1 (143). For more detailed discussions on the mechanism of PAMP perception, we refer readers to other recent reviews (25, 84). PTI is considered to be effective against nonadapted fungi (nonhost resistance) and can determine at the level of penetration whether a plant can serve as its host (25, 161).

To establish a compatible interaction leading to proliferation, fungi must avoid eliciting PTI or either cope with or suppress it. To do so, the fungus must inactivate toxic metabolites or secrete so-called fungal effectors, which may be either toxic secondary metabolites or proteins that kill the host plant (in necrotrophic and hemibiotrophic fungi during their necrotrophic stage). Alternatively, effectors can be secreted proteins that shield the fungus, suppress the host immune response, or manipulate host cell physiology (17, 40, 105, 150, 180).

Protein effectors are most often secreted via the conventional endoplasmic reticulum–Golgi apparatus route. To enter this route, they must contain an N-terminal secretion signal. Effector candidates can thus be identified bioinformatically by the presence of this signal. In general, effectors are expressed only after contact with the plant. Their expression profile is tightly tuned to the different infection stages and may be affected by the cell type and/or organ being infected (105). Ongoing coevolutionary processes between plants and fungi that colonize them have shaped the genomes of both partners. In addition, we are beginning to see trends that effector genes are arranged in flexible genomic regions and to understand how this aids and promotes rapid effector gene evolution and affects the gain and loss of effector genes (122, 125). Furthermore, we are beginning to see how recent host jumps have affected the effector repertoire and promoted enhanced diversification (29, 141).

Although secreted effectors are key players in suppressing PTI, they can also be recognized by the plant surveillance system, triggering a second layer of defense termed effector-triggered immunity (ETI). Effectors that trigger ETI are usually perceived by plant resistance proteins (R proteins), which are conserved intracellular receptors of the nucleotide-binding leucine-rich receptor (NB-LRR) class (25). Effector perception by NB-LRRs is highly specific and can be either direct (with the receptor binding the effector) or indirect (involving accessory proteins). Accessory proteins can be pathogen virulence targets or structural mimics of such targets (25). PTI and ETI responses are similar but may differ in their strength. ETI defenses often trigger a


a
Botrytis cinerea
Sclerotinia sclerotiorum
Early
Late
Early
Late
Necrotrophs
b
Uromyces viciae-fabae
Blumeria graminis f. sp. hordei
Ustilago maydis
Cladosporium fulvum
Biotrophs
c
Rhizophagus irregularis
Laccaria bicolor
Piriformospora indica
Epichloë festucae
Symbionts
d
Colletotrichum higginsianum
Magnaporthe oryzae
Biotrophic phase
Necrotrophic phase
Biotrophic phase
Necrotrophic phase
Hemibiotrophs
Living photosynthetic tissue
Dead photosynthetic tissue
Living root tissue
Dead root tissue
Pathogenic fungus
Nonpathogenic fungus
Plant plasma membrane
Disrupted plant plasma membrane
Biotrophic interfacial complex
Cuticle

localized cell death response, or hypersensitive response. In contrast to the conserved molecules involved in triggering PTI, effectors and receptors triggering ETI are highly variable and often dispensable, reflecting the antagonistic coevolution of these components (25). Given the strong hypersensitive-response phenotype elicited in many of these gene-for-gene interactions, in which an effector [in this case termed an avirulence (Avr) protein] in many cases interacts directly with the product of an *R* gene, it is not surprising that effectors encoding fungal Avr proteins were the first to be molecularly characterized (166). More than 26 fungal *Avr* genes have now been cloned (131), and in many cases the matching plant R protein has also been identified (3, 47, 150). Most of the R proteins are cytoplasmic plant proteins, suggesting that the interacting effectors are translocated from the fungus to the plant (150). The Avr function of effectors is relatively easy to assess because of its typical outcome (cell death or strong plant defense response); determining whether Avr proteins also have a virulence function in susceptible cultivars and identifying the underlying molecular mechanisms are much more challenging.

Avirulence (*Avr*) gene: a gene whose product triggers a multifaceted defense response upon recognition by a cognate plant R protein

The broad and growing field of microbial effectors has been extensively reviewed recently (8, 17, 40, 105, 115, 122, 133, 150, 152). In this review, we provide an overview of effectors in fungi with different lifestyles (i.e., including both beneficial and pathogenic fungi) and describe the processes that contribute to their rapid evolution. We then focus on fungal effectors that contribute to virulence and discuss recent progress in elucidating their functions and how they affect compatibility. Finally, we address the issue of fungal effector delivery to plants and briefly review posttranscriptional modifications of fungal effectors.

THE MANY WAYS TO COLONIZE A PLANT: A DIVERSITY OF INFECTION STRUCTURES

The initial phases of infection, involving fungal adhesion to the cuticle, growth of germ tubes on the plant surface, and differentiation of infection structures (appressoria or hyphopodia), are similar in all plant-colonizing fungi. However, fungi differ in the surface cues that they perceive: The appressorium (in pathogenic fungi) and the hyphopodium (in beneficial fungi) can form in response to plant topographical cues, such as stomatal pores; plant chemical cues, such as epicuticular

Figure 1

Plant colonization by fungi with different lifestyles. (*a*) Necrotrophic fungi such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* generally grow subcuticularly and kill epidermal cells by secreting toxic metabolites and proteins. Their hyphae eventually replace large parts of the plant epidermis. Both early and late developmental stages are shown. (*b*) Obligate biotrophic pathogens such as rust fungi (*Uromyces viciae-fabae*) and powdery mildew (*Blumeria graminis* f. sp. *hordei*) undergo a complex series of developmental steps and eventually form a haustorial mother cell from which the haustorium, a balloon-shaped feeding structure, develops. After initial intracellular growth, the biotrophic maize pathogen *Ustilago maydis* switches to predominantly intercellular growth at late stages, when massive fungal proliferation occurs and large plant tumors are induced. The biotrophic tomato pathogen *Cladosporium fulvum* colonizes the extracellular compartment of tomato leaves and later produces large numbers of conidiophores that block stomata and cause chlorosis or cell death (necrosis). (*c*) The obligate arbuscular mycorrhizal root symbiont *Rhizophagus irregularis* colonizes individual cortical cells with highly branched feeding structures called arbuscules. The ectomycorrhizal fungus *Laccaria bicolor* grows exclusively intercellularly; colonizes roots by forming a mantle or sheath of hyphae, which covers the root epidermis; and grows between cortical cells, generating the so-called Hartig net. Endophytes can colonize either plant roots (*Piriformospora indica*) or the aerial plant organs (*Epichloë festuca*) and can grow either intracellularly (*P. indica*) or intercellularly (*E. festuca*). (*d*) Hemibiotrophic fungi such as *Colletotrichum* spp. and *Magnaporthe oryzae* initially develop bulged biotrophic invasive hyphae that later change into thin necrotrophic hyphae. Both biotrophic and necrotrophic phases are shown. Hyphae are shown in blue (nonpathogenic fungi) or violet (pathogenic fungi), photosynthetic tissue in green, and root tissue in brown; solid green or brown lines indicate living tissue, and dashed green or brown lines indicate dead tissue. Note that all intracellular structures are encased by the plant plasma membrane, indicated by a solid gray line; a dashed gray line surrounding the hyphae indicates a switch to necrotrophy. The membranous biotrophic interfacial complex structure in *M. oryzae*–infected cells is shown in pink.

Symbionts: fungi that establish a biotrophic interaction with host plants that has beneficial effects for both the host and the microbe

waxes; or physical cues, such as hydrophobicity and/or thigmotropism in different systems (101, 127, 160). Pathogenic fungi with different lifestyles develop different types of appressoria. The hemibiotrophs *M. oryzae* and *Colletotrichum* spp. form dome-shaped, melanized appressoria that accumulate turgor pressure to allow mechanical entry of the infection hyphae into the host (160). Most necrotrophs form inconspicuous appressoria and penetrate the plant cuticle by secreting large amounts of plant cell wall–degrading enzymes (PCWDEs). Many pathogenic biotrophs use a combination of turgor pressure and PCWDEs to breach the cell wall without affecting host cell viability (101), whereas beneficial biotrophs rely heavily on host-derived cell wall–loosening enzymes (127).

Successful penetration is followed by colonization of the host plant and accompanied by the development of fungal growth structures that can differ even in fungi that adopt the same lifestyle. Necrotrophic fungi like *Botrytis cinerea* and *Sclerotinia sclerotiorum* generally grow subcuticularly and kill epidermal cells by secreting toxic metabolites and proteins, and eventually their hyphae replace large parts of the plant epidermis (**Figures 1*a*** and **2*a*,*b***). Beneficial and pathogenic biotrophs as well as hemibiotrophs can grow inside the host as either intracellular or intercellular hyphae, the latter of which may insert dedicated feeding structures (haustoria or arbuscules) into the host cell (101) (**Figures 1*b*,*c*** and **2*c–f***). Hemibiotrophic fungi such as *M. oryzae* and *Colletotrichum* spp. initially develop bulged biotrophic invasive hyphae that subsequently change into thin necrotrophic hyphae (101, 107) (**Figures 1*d*** and **2*g*,*h***).

A conserved feature of biotrophic fungi that grow intracellularly or insert haustoria or arbuscules into their host cells is that these structures are tightly encased by the plant plasma membrane (**Figure 1**). This membrane, also termed the extrahaustorial membrane in haustorium-forming biotrophs and the periarbuscular membrane in AM symbionts, is continuous with the plant plasma membrane but appears to lack several common plant plasma membrane proteins, instead possessing a unique set of transmembrane proteins (70, 93, 101, 127). Although the molecular mechanisms by which haustoria and arbuscules are formed remain to be elucidated (70), it is clear that these structures not only function in nutrient uptake or exchange but also represent the primary site for effector secretion (101). Similar to haustoria, the intracellular hyphae of *Ustilago maydis* and the biotrophic hyphae of *M. oryzae* and *Colletotrichum* spp. are completely encased by the plant plasma membrane, forming a tight biotrophic interface (**Figure 1**). In *M. oryzae*, a membrane-rich structure forms at the primary hyphal tips of invasive hyphae, the so-called biotrophic interfacial complex (BIC). This structure, which was recently shown to be of plant origin and to lie outside the fungal plasma membrane, may serve to translocate a subset of effectors into the host cell (39, 66).

THE EFFECTOR REPERTOIRE IN PLANT-COLONIZING FUNGI

The fungal repertoire of secreted proteins involved in fungus-plant interaction likely influences the fungal lifestyle and the level of host specialization. Although identifying proteins containing conserved functional domains is generally not a problem, the classification of secreted effector proteins from genome sequences often involves different criteria and bioinformatic approaches that prevent direct comparisons. Frequently, effector proteins are defined as small secreted proteins containing $\leq$300 amino acids (31, 37, 89, 90, 179). Many of these proteins are cysteine rich, and their tertiary structures are stabilized by disulfide bridges (148). Therefore, they are well suited to survive the harsh physiological conditions in the plant apoplast. However, much larger proteins can also act as effector proteins (23), and therefore we consider a cutoff of 300 amino acids to be rather arbitrary. A criterion frequently used to define effectors is the absence of detectable orthologous proteins outside the genus (102, 146, 172); however, some effector proteins are conserved or possess conserved functional domains (18, 23, 37, 92, 109, 149). Because of these ambiguities in

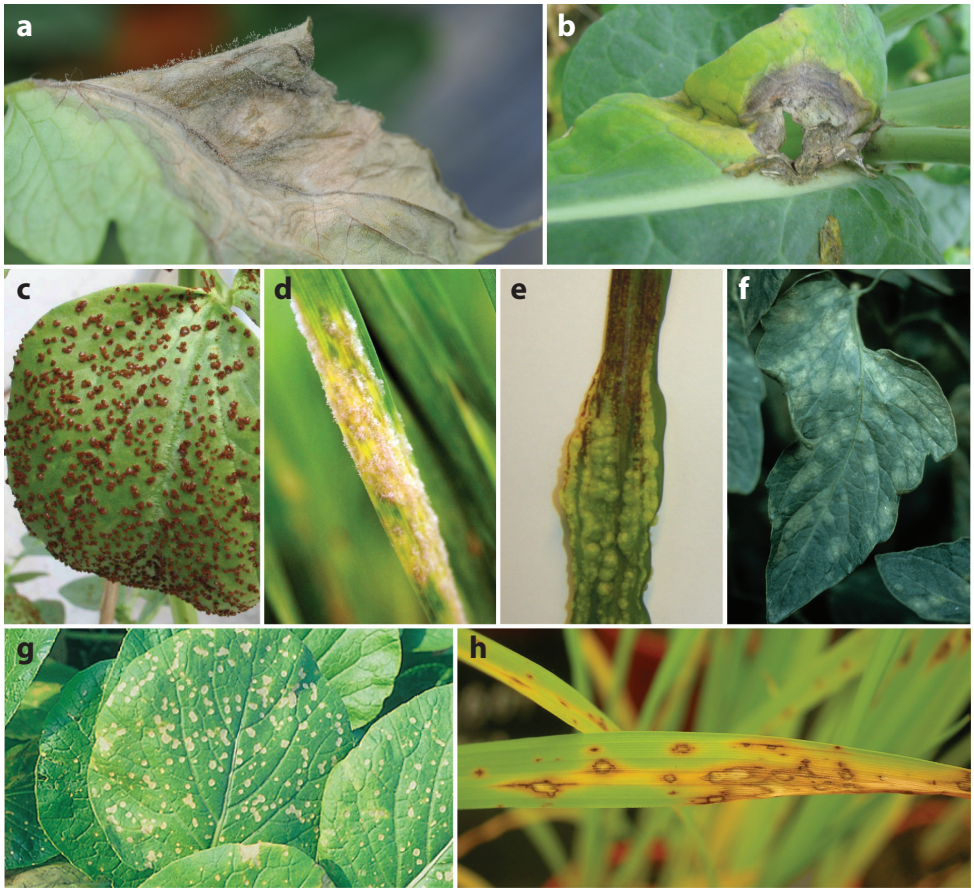


Figure 2

Disease symptoms caused by phytopathogenic fungi with different lifestyles: (*a*) *Botrytis cinerea* infecting tomato (image courtesy of D. Blancard), (*b*) *Sclerotinia sclerotiorum* infecting rapeseed (image reproduced with permission from *Paysan Breton*; **http://www.paysan-breton.fr**), (*c*) *Uromyces viciae-fabae* infecting bean (image courtesy of K.D. Zinnert), (*d*) *Blumeria graminis* infecting barley (image courtesy of P. Spanu), (*e*) *Ustilago maydis* infecting maize, (*f*) *Cladosporium fulvum* infecting tomato (image courtesy of D. Blancard), (*g*) *Colletotrichum higginsianum* infecting mustard spinach (image courtesy of H. Horie; **http://www.boujo.net**), and (*h*) *Magnaporthe oryzae* infecting rice (image courtesy of N.J. Talbot).

defining effector proteins, here we consider any secreted fungal protein to potentially act as an effector.

To obtain a comparable data set of putative secreted proteins, we defined the secretomes of 84 plant-colonizing fungi with various lifestyles for which the genome sequence is publicly available as well as those of five saprophytic fungi as a contrasting set (**Supplemental Table 1**; follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**). We grouped individual secretomes into PCWDEs, secreted proteins with functional annotation except PCWDEs, and secreted proteins without functional annotation (**Figure 3**). The majority of secreted protein effectors most likely fall into the class of proteins without predictable function. The group of PCWDEs may include effectors specifically needed for penetration or spore dispersal. Among the secreted proteins with functional domains,

Supplemental Material

we also expect effectors needed for degrading, modifying, inhibiting, altering the activity of, or modulating the stability of plant targets.

Saprotrophs: fungi that derive energy from the degradation of nonliving organic matter and do not colonize a living host

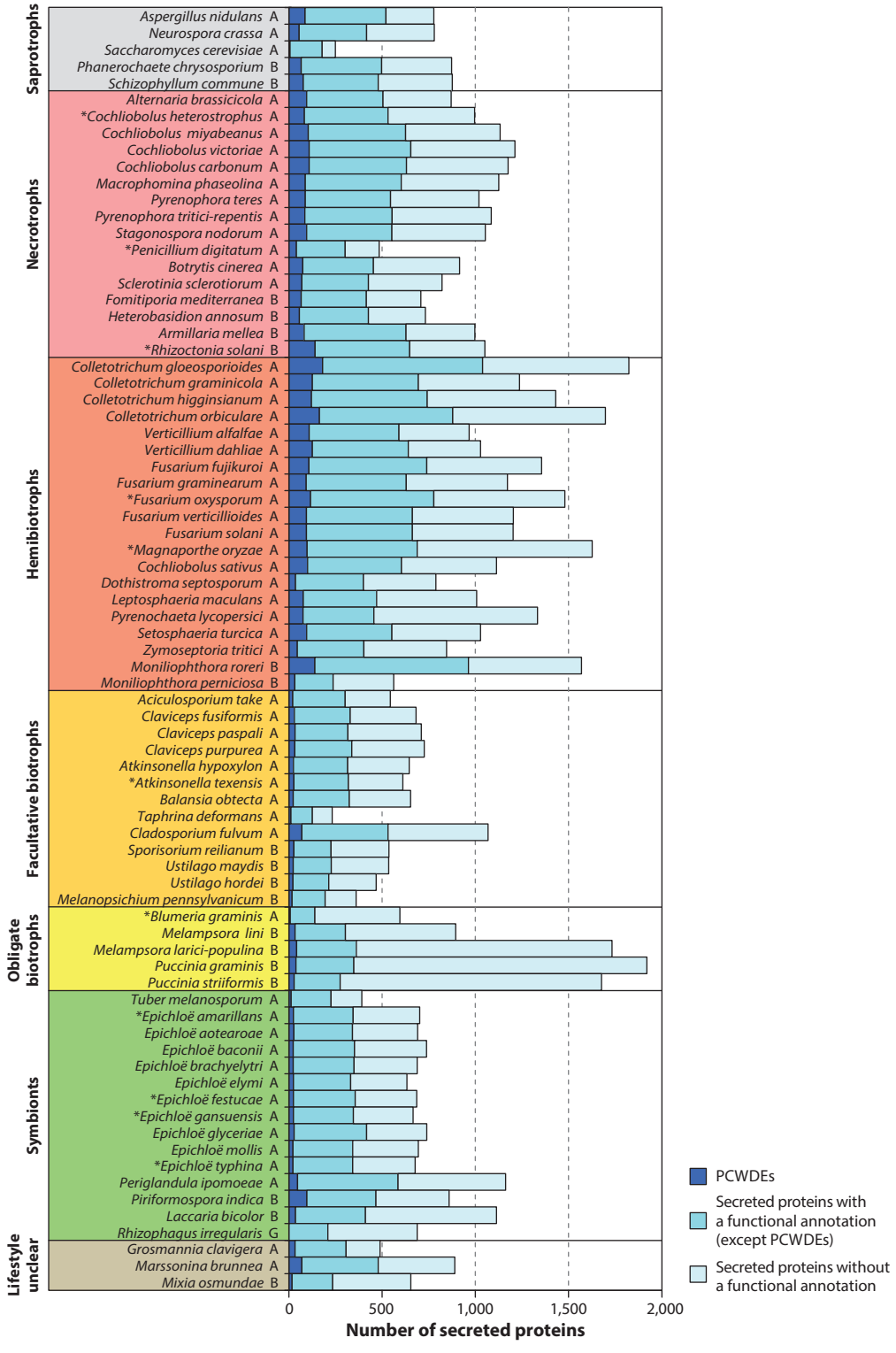
A frequently used criterion for effector gene identification is exclusive expression during host colonization (150). Recent data from several systems have shown that effectors can be stage-, organ-, and host-specifically expressed (73, 102, 144). However, comprehensive data where this has been addressed in various systems are largely lacking. Therefore, our analysis does not include the expression profile as a criterion for effector classification. Furthermore, our data set excludes unconventionally secreted proteins such as the barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) effectors Avr-k1 and Avr-a10 (128). Our analysis also does not include secondary metabolites that can shape the fungus-plant interaction in a similar way as protein effectors do. We refer readers to a recent review showing that genes involved in secondary metabolism are overrepresented in saprotrophic, necrotrophic, and hemibiotrophic fungi, whereas biotrophy is associated with a convergent loss of secondary metabolites (180).

To extract lifestyle specific features, we grouped the fungi according to their feeding strategies (**Figure 3**). We plotted the absolute number of secreted proteins without relating this to the total number of predicted genes, as we believe that the outcome of a fungus-plant interaction depends on the actual effector repertoire available. However, fungi with small proteomes (e.g., *Penicillium digitatum* and *Taphrina deformans*) likely also possess a smaller set of secreted proteins (**Figure 4*a***), and, indeed, for most species the proportion of predicted secreted proteins relative to the total proteome is between 5% and 10% (**Figure 4*a***).

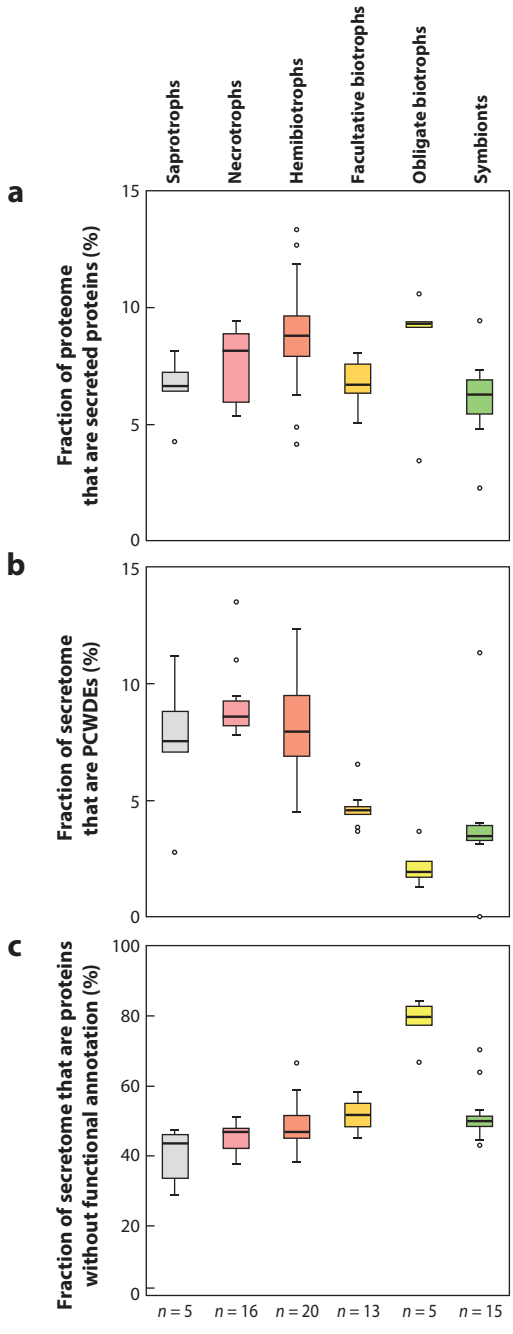
The proportion of secreted PCWDEs in necrotrophic and hemibiotrophic fungi is higher than that in biotrophs but comparable to that in saprotrophic fungi (see 72, 102, 177) (**Figure 4*b***). This is in agreement with the adaptation of biotrophic organisms to living plant tissue and the need to avoid plant cell damage, which could trigger plant cell death. Interestingly, obligate biotrophic species show the lowest proportion of PCWDEs (**Figure 4*b***), possibly reflecting their inability to proliferate outside the plant as saprotrophs do. An exception is the facultative

Figure 3

The secretome composition of fungi with different lifestyles. The secretomes of 84 plant-colonizing fungi and 5 saprophytic fungi were sorted into secreted plant cell wall–degrading enzymes (PCWDEs; *dark blue*), secreted proteins with a functional annotation except PCWDEs (*blue*), and secreted proteins without a functional annotation (*light blue*). To define the secretomes, we discarded pseudogenes from the set of predicted gene models and defined secreted proteins based on the presence of an N-terminal signal peptide as predicted by SignalP 4.0 (113) and on the absence of transmembrane domains as predicted by TMHMM 2.0c (TMHMM score <2) (71). We then used the Pfam database (**http://pfam.xfam.org**) to assign functional domains to the determined set of secreted proteins, as described recently (180). We used the CAZymes Analysis Toolkit (**http://mothra.ornl.gov/cgi-bin/cat/cat.cgi**) to filter out proteins with Pfam annotations corresponding to CAZymes that are PCWDEs. To define the set of PCWDEs, we extracted from the Carbohydrate-Active Enzymes (CAZy) database (80) all glycoside hydrolase families that contain cellulases (EC 3.2.1.4 and 3.2.1.91) and xylanases (EC 3.2.1.8 and 3.2.1.37) based on the EC numbers (67). Similarly, we identified all polysaccharide lyase and carbohydrate esterase families that contain pectinolytic enzymes, as previously defined (52). As a result, the following Pfam IDs were considered (the corresponding CAZy families are in parentheses): Glyco_hydro (GH)_1, 3, 4, 6, 7, 8, 9, 10, 11, 12, 16, 26, 28, 30, 39, 43, 44, 45, 48, 51, 52, 54, 61 (AA9), 62, 88, 105, and 116; Cellulase (GH5); Pec_lyase_C (PL1); Pectate_lyase_2 (PL2); Pectate_lyase (PL3); Pec_lyase (PL10); and Pectate_lyase22 (PL22). Note that the following CAZy families were not present in the analyzed secretomes: GH4, 8, 48, 52, and 116, and PL2, 10, and 22. Proteins that contain at least one of the Pfam domains that define a PCWDE were grouped as secreted PCWDEs. Proteins that exclusively possess Pfam domains of unknown function or contain no Pfam annotation were grouped as secreted proteins without a functional annotation. All other proteins were grouped as secreted proteins with functional annotation except PCWDEs. Based on information in the literature (**Supplemental Table 1**), we grouped all sequenced fungi according to their lifestyle during plant colonization. The five saprotrophic fungi serve as a contrasting set. If the genome sequence for more than one isolate of a species was publicly available, then we separately analyzed each isolate and displayed the average value of all isolates; species for which this applies are indicated by an asterisk. **Supplemental Table 1** lists all isolates used for this analysis. Abbreviations: A, ascomycete; B, basidiomycete; G, glomeromycete.


Saprotrophs
Aspergillus nidulans A
Neurospora crassa A
Saccharomyces cerevisiae A
Phanerochaete chrysosporium B
Schizophyllum commune B
Necrotrophs
Alternaria brassicicola A
*Cochliobolus heterostrophus A
Cochliobolus miyabeanus A
Cochliobolus victoriae A
Cochliobolus carbonum A
Macrophomina phaseolina A
Pyrenophora teres A
Pyrenophora tritici-repentis A
Stagonospora nodorum A
*Penicillium digitatum A
Botrytis cinerea A
Sclerotinia sclerotiorum A
Fomitiporia mediterranea B
Heterobasidion annosum B
Armillaria mellea B
*Rhizoctonia solani B
Hemibiotrophs
Colletotrichum gloeosporioides A
Colletotrichum graminicola A
Colletotrichum higginsianum A
Colletotrichum orbiculare A
Verticillium alfalfae A
Verticillium dahliae A
Fusarium fujikuroi A
Fusarium graminearum A
*Fusarium oxysporum A
Fusarium verticillioides A
Fusarium solani A
*Magnaporthe oryzae A
Cochliobolus sativus A
Dothistroma septosporum A
Leptosphaeria maculans A
Pyrenochaeta lycopersici A
Setosphaeria turcica A
Zymoseptoria tritici A
Moniliophthora roreri B
Moniliophthora perniciosa B
Facultative biotrophs
Aciculosporium take A
Claviceps fusiformis A
Claviceps paspali A
Claviceps purpurea A
Atkinsonella hypoxylon A
*Atkinsonella texensis A
Balansia obtecta A
Taphrina deformans A
Cladosporium fulvum A
Sporisorium reilianum B
Ustilago maydis B
Ustilago hordei B
Melanopsichium pennsylvanicum B
Obligate biotrophs
*Blumeria graminis A
Melampsora lini B
Melampsora larici-populina B
Puccinia graminis B
Puccinia striiformis B
Symbionts
Tuber melanosporum A
*Epichloë amarillans A
Epichloë aotearoae A
Epichloë baconii A
Epichloë brachyelytri A
Epichloë elymi A
*Epichloë festucae A
*Epichloë gansuensis A
Epichloë glyceriae A
Epichloë mollis A
*Epichloë typhina A
Periglandula ipomoeae A
Piriformospora indica B
Laccaria bicolor B
Rhizophagus irregularis G
Lifestyle unclear
Grosmannia clavigera A
Marssonina brunnea A
Mixia osmundae B
0
500
1,000
1,500
2,000
Number of secreted proteins
PCWDEs
Secreted proteins with a functional annotation (except PCWDEs)
Secreted proteins without a functional annotation

biotrophic fungus *Cladosporium fulvum*, which has a set of carbohydrate-degrading enzymes that is more similar to those of necrotrophs and hemibiotrophs (20) (**Figure 3**). *C. fulvum* is a close relative of the hemibiotroph *Dothistroma septosporum* and may have only recently adapted to a new host in which its lifestyle changed from hemibiotrophic to biotrophic, as inferred from the finding that many PCWDEs are not expressed during colonization of tomato (20). An exception among



the hemibiotrophs is the wheat pathogen *Zymoseptoria tritici*, which has a reduced set of secreted PCWDEs (45) (**Figure 3**); this fungus instead expresses a large number of secreted proteases, indicating nutrition intake via protein degradation during the cell death–associated phase (45).

A strong reduction of PCWDEs also occurred in the symbiont group (**Figure 4*b***), again presumably resulting from adaptation to a strictly biotrophic lifestyle. The AM fungus *Rhizophagus irregularis* lacks all secreted PCWDEs, and Rich et al. (127) have suggested that plant penetration and development of intracellular arbuscules may rely on plant genes encoding cell wall–modifying enzymes and expansins. Fungal proteins with expansin domains, which are considered to aid in plant penetration, were also expressed in the Hartig net structure that is established during root colonization by the ECM fungus *Laccaria bicolor*. The mutualistic root endophyte *Piriformospora indica* possesses a set of secreted PCWDEs comparable to those of saprotrophic fungi, and this may represent an ancestral saprophytic trait (74, 179). Notably, many of these enzymes are not expressed during the biotrophic phase but instead are induced in axenic culture or during the plant cell death–associated phase that is established on certain hosts (73, 179).

We observe that fungi with the highest total number of secreted proteins are overrepresented in the hemibiotroph group (**Figure 3**), in line with the fact that their secretomes combine distinct features of both necrotrophic and biotrophic fungi (82). Global transcriptomic analyses of the hemibiotroph *Colletotrichum higginsianum* revealed that genes encoding secreted proteins without a functional annotation are expressed predominantly during the initial biotrophic phase, whereas expression of secreted lytic enzymes (including PCWDEs) was higher in the subsequent necrotrophic phase (102). A similar trend was observed in the endophyte *P. indica*, which switches to a cell death–associated phase during the late stages of infection (73). Collectively, these data suggest that expression of secreted proteins without functional annotation is a general feature of biotrophy, whereas expression of PCWDEs is generally associated with necrotrophy.

In addition, with the exception of *Melampsora lini*, the obligate biotrophic rust fungi feature an exceptionally large set of secreted proteins (**Figures 3** and **4*a***), and the proportion of secreted proteins without functional annotation is particularly high within this group (**Figures 3** and **4*c***). This may result from the expansion of gene families coding for small secreted proteins, as described for *Puccinia graminis* f. sp. *tritici* and *Melampsora larici-populina* (31, 114). In contrast to the other

←

Figure 4

Lifestyle-specific trends within the secretome composition of plant-colonizing fungi. Secretomes were analyzed and categorized for each fungus separately as described in **Figure 3**, and for each category the values from fungi with the same lifestyle were combined. Results are shown in the form of box plots, where the top and bottom of the boxes indicate the 25% and 75% quartiles, respectively, and the thick middle line indicates the 50% quartile (median). The whiskers correspond to the lowest and highest data points within the 1.5 interquartile range of the lower and upper quartiles, respectively. Outliers are indicated by open circles. (*a*) Number of secreted proteins relative to the total proteome. Outliers are as follows: saprotrophs, *Saccharomyces cerevisiae* (4.2%); hemibiotrophs, *Magnaporthe oryzae* (13.3%), *Colletotrichum orbiculare* (12.7%), *Pyrenochaeta lycopersici* (4.9%), and *Moniliophthora perniciosa* (4.2%); obligate biotrophs, *Melampsora larici-populina* (10.6%) and *Melampsora lini* (3.5%); symbionts, *Periglandula ipomoeae* (9.4%) and *Rhizophagus irregularis* (2.3%). (*b*) Number of secreted plant cell wall–degrading enzymes (PCWDEs) relative to the total number of secreted proteins. The outliers are as follows: saprotrophs, *Saccharomyces cerevisiae* (2.8%); necrotrophs, *Rhizoctonia solani* (13.5%), *Alternaria brassicicola* (11.0%); facultative biotrophs, *Cladosporium fulvum* (6.5%), *Aciculosporium take* (3.9%), *Balansia obtecta* (3.7%); obligate biotrophs, *Melampsora lini* (3.7%); symbionts, *Piriformospora indica* (11.3%) and *Rhizophagus irregularis* (0%). (*c*) Number of secreted proteins without functional annotation relative to the total number of secreted proteins. The outliers are as follows: hemibiotrophs, *Pyrenochaeta lycopersici* (65.8%); obligate biotrophs, *Melampsora lini* (66.0%); symbionts, *Rhizophagus irregularis* (69.5%), *Laccaria bicolor* (63.1%), and *Tuber melanosporum* (42.3%).

rust fungi included in our study, *M. lini* does not switch hosts to complete its life cycle and interacts only with flax plants (77). The large number of secreted proteins without functional annotation observed in the three rust fungi that perform a host switch during their life cycle may thus reflect different effector requirements in the two hosts.

The symbiotic fungi show large variations in both numbers of secreted proteins and composition, which may not be immediately apparent from **Figure 3** owing to ascertainment bias (there are ten related *Epichloë* species included in this group) (**Figures 3** and **4*c***). The secretome of the AM fungus *R. irregularis* harbors a relatively large set of secreted proteins without known functional domains (**Figure 4*c***), possibly reflecting its broad host range. The two ECM fungi, the ascomycete *Tuber melanosporum* and the basidiomycete *L. bicolor*, differ greatly in their repertoire of secreted proteins. *L. bicolor* possesses a large set of secreted proteins particularly enriched in proteins without functional domains (89) (**Figure 4*c***), and of these, several small secreted proteins (MiSSPs) are highly induced in mycorrhizal tissue (89). By contrast, the genome of *T. melanosporum* encodes only a few secreted proteins, and induction of genes encoding small secreted proteins has not been detected in mycorrhizal roots (90). *Periglandula ipomoeae* stands out, with a relatively large set of secreted proteins among the symbionts (**Figures 3** and **4*b***); this could suggest an ancestral trait, an additional undiscovered saprophytic lifestyle, or a unique symbiotic strategy.

MiSSPs: mycorrhiza-induced small secreted proteins

Arms race: competitive host-pathogen coevolution based on the continuous development of new resistance and virulence alleles

Trench warfare: host-pathogen coevolution based on fluctuations in the resistance and virulence allele frequencies in the population

Positive selection: an increase of beneficial alleles within a population, reflected by an excess of nonsynonymous nucleotide substitutions in homologous genes

EFFECTOR GENE EVOLUTION

As plant-pathogen interactions evolve, plants are selected for an incompatible (resistant) interaction and parasites are selected for a compatible (susceptible) interaction. The underlying principle for this antagonistic coevolution is based on the gene-for-gene model (35). In this model, *R* gene products from the host plant detect Avr effectors from the pathogen, leading to an incompatible interaction; by contrast, a failure of detection, resulting from either allelic variation or the absence of at least one of the components, results in a compatible interaction (25). This relationship can result in a boom-and-bust cycle (10), in which pathogen *Avr* genes are selected for when host *R* genes are rare, *R* genes are selected for when *Avr* genes are common, *Avr* genes are counterselected when *R* genes are common, and *R* genes are counterselected when *Avr* genes are rare. Within the boom-and-bust cycle, two dynamic coevolutionary scenarios can be distinguished, which not only apply to the relationship between *Avr* genes and *R* genes but can be extended to any effector-plant target gene pair (**Figure 5**). In the arms race model, both the pathogen and the host develop in continuous cycles new effector and plant target alleles that become temporarily fixed in the population (15) (**Figure 5**). In the trench warfare model, effector and plant target alleles are maintained in populations, but their frequencies oscillate over time (147) (**Figure 5**). Although trench warfare may be common in natural systems, agricultural systems likely follow the arms race model because of the constant perturbations of humans on the plant cultivars (10). Indeed, several studies of fungal pathosystems in agricultural settings support the arms race scenario (24, 61), whereas few examples of the trench warfare scenario exist (157).

Effector evolution is a trade-off between escaping from detection and optimizing the virulence function. The long-term fitness of a pathogen may additionally rely on the continuous emergence of novel effectors to be able to substitute an effector that has lost the arms race or to capture new host targets. These enormous demands on the effector repertoire implicate a strong evolutionary pressure leading to accelerated evolution. Consistent with this idea, genome-wide analyses of plant pathogenic fungi have demonstrated a higher degree of positive selection in genes encoding secreted proteins compared with genes encoding nonsecreted proteins (48, 56, 141, 172). Presence/absence polymorphisms of effectors between or within species are equally frequent (141, 150, 172). Many effectors belong to multigene families and have thus diversified from a common

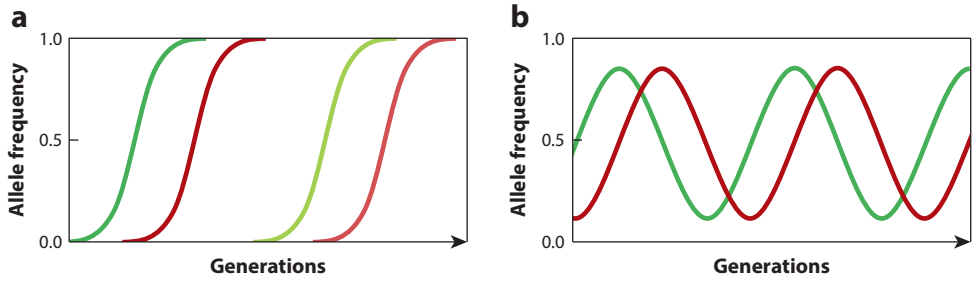


Figure 5

Coevolutionary principles driving effector and plant target evolution. Population-wide allele frequencies of a pathogen-derived effector molecule (*red line*) and a host-derived interactor (*green line*) can follow (*a*) the arms race model or (*b*) the trench warfare model. Allele fixation (selective sweeps) and recurrent development of new alleles (indicated by *light-colored lines*) in the arms race model contrast with the fluctuation of allele frequencies in the trench warfare model.

ancestor (99, 112, 151, 179). These families can be either lineage specific or widespread across the fungal kingdom, undergoing multiple expansions and losses (151). A link between effector gene family diversification and host adaptation was established for the oomycetes *Phytophthora infestans* and *Phytophthora mirabilis*, infecting Solanales and Magnoliales, respectively. Here, diversification of a member of the cystatin-like effector family changed its specificity toward its associated cysteine protease from the host plant, which likely facilitated the host jump (29). Thus, there is emerging evidence that pathogen effectors evolve as diversifying multigene families according to the birth-and-death evolution model (98, 151).

Although sexual recombination is one major contributor to genetic diversity in eukaryotic organisms, a characteristic feature of pathogenic microbes, including most plant pathogenic fungi, is their limited rate of sexual reproduction (49, 139). The strategy of clonal propagation allowing the rapid spread of favorable effector alleles appears attractive but at the same time reduces the exchange of new genetic material. An alternative way to gain new virulence traits is horizontal gene transfer, which is exemplified by the parasexual transfer of mobile pathogenicity chromosomes in *Fusarium* spp. (83). These accessory chromosomes are probably key to successfully transferring virulence traits between populations (1, 83). A paradigm for effector evolution relying on horizontal gene transfer is the fate of the avirulence gene *Avr-Pita* from *M. oryzae*, which is under strong selection pressure owing to regular agricultural deployment of resistant, *Pi-ta*-containing rice cultivars. An analysis of different *M. oryzae* field isolates revealed that *Avr-Pita* has translocated several times via mobile elements (13), and the authors suggested that this multiple translocation reflects deletions and recoveries mediated by its parasexual transfer among individuals. In the course of a boom-and-bust cycle, this scenario would explain how *Avr* genes are efficiently recovered after *R* genes are removed from the field.

In addition to intraspecies horizontal gene transfer, interspecies horizontal gene transfer also demonstrates the transfer of effector genes. A prominent example is the transfer of the host-specific toxin ToxA from the cereal pathogen *Stagonospora nodorum* to *Pyrenophora tritici-repentis*, which led to the emergence of a highly virulent pathogen population that causes tan leaf spot in wheat fields worldwide (36). In a cross-kingdom horizontal gene transfer, the fungus *Verticillium dahliae* acquired a plant gene encoding a natriuretic regulatory peptide (19), which now acts as a secreted virulence-promoting effector in susceptible tomato (19). These examples demonstrate that horizontal gene transfer contributes substantially to the evolution of virulence-promoting effectors.

Birth-and-death evolution: multigene family evolution in which the duplicated genes within a species evolve independently from one another by natural selection

Horizontal gene transfer: the transmission of genetic material between two organisms belonging to the same or different species

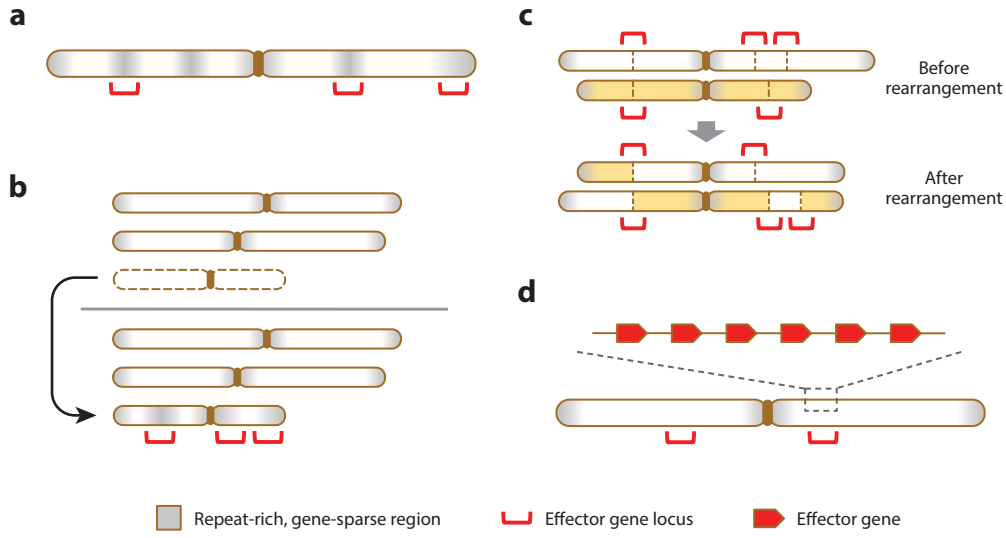


Figure 6

Effector genes residing in distinct genome compartments. (*a*) Effector genes are located in repeat-rich, gene-sparse regions in *Leptosphaeria*, *Magnaporthe*, and *Phytophthora* spp. (*b*) Effector genes are located on mobile, conditionally dispensable chromosomes consisting mainly of repeat-rich DNA in *Fusarium* spp. (*c*) Effector genes are located at chromosomal breakpoints of highly rearranged chromosomes in *Verticillium* spp. (two nonhomologous chromosomes are depicted in *white* and *yellow* before and after rearrangement). (*d*) Effector genes are located in gene clusters in smut fungi.

Plant pathogens are faced with an evolutionary conflict, in which effector genes require fast and flexible evolution but the majority of the genome requires evolution at moderate rates. This balancing act is carried out by the compartmentalization of the genome. Many pathogenic fungi (e.g., *M. oryzae*, *Leptosphaeria maculans*, and *Fusarium* and *Blumeria* spp.) as well as plant pathogenic oomycetes have gene-sparse genomic regions that are highly enriched in repetitive elements and putative effector genes (122) (**Figure 6*a***). The compartmentalization culminates in accessory chromosomes that are devoid of essential genes and harbor solely pathogenicity-relevant genes (83) (**Figure 6*b***). The activity of transposable elements in these genome compartments leads to gene duplications and the subsequent dispersal of the duplicated genes (122). The dispersal promotes diversification because it hinders gene conversion and unequal crossovers that would otherwise homogenize the duplicated sequences (98). The transposons also promote horizontal gene transfer, gene losses, and the production of chimeras, making this genomic context an ideal playground for the evolution of virulence traits.

The genome of the canola pathogen *L. maculans* has an unusual isochore structure consisting of alternating GC- and AT-rich blocks. Most putative effector genes localize to the AT blocks, which consist mainly of transposon-rich repetitive DNA and are affected by repeat-induced point mutation (132). This localization may be beneficial for rapid effector diversification, as repeat-induced point mutation results in an enhanced mutation rate and can affect even single-copy genes when they are in close proximity to repetitive sequences (104).

In *V. dahliae*, various isolates display extensive chromosomal reshuffling, and the highly dynamic regions flanking the chromosomal breakpoints are enriched for virulence-related genes and effectors (16) (**Figure 6*c***). The chromosomal rearrangements most likely create a sufficient source of genetic variation to compensate for the lack of meiotic recombination in this strictly asexual pathogen (16).

Smut fungi and the distantly related fern pathogen *Mixia osmundae* have small genomes with a low content of repetitive DNA. In these genomes, many genes encoding secreted proteins reside in clusters of three or more genes (60, 76, 137, 141, 159) (**Figure 6*d***). Effector gene clusters most likely originate from gene duplications without subsequent dispersal. Genome comparisons of related smut fungi revealed that these clusters show low sequence conservation in an overall conserved genomic context, indicating accelerated evolution within the clusters (137).

These examples illustrate that fungal pathogens use different strategies of genomic compartmentalization to outsource effector gene evolution. Ultimately, such strategies generate effector repertoires that determine both the lifestyles and host ranges of pathogens.

EFFECTORS AND FUNGAL VIRULENCE

In this section, we discuss the tools used by diverse fungi to colonize plants. These are mostly protein effectors but also include protein toxins and other metabolites that interfere with or induce certain plant processes (**Figure 7**). We do not cover secreted PCWDEs involved in penetration or fungal nutrition during colonization, as these have recently been reviewed (72). Effectors either promote the virulence of fungal pathogens or allow symbionts to colonize a plant. Such effectors can be attached to the fungal cell wall, can reside in the apoplast, or can be transferred to plant cells, where they can function in various compartments. Deletion of effector genes leads to reduced fungal biomass in the infected tissue, resulting in less severe macroscopic disease symptoms and/or altering the plant response.

Compared with bacterial and oomycete effectors, relatively few fungal effectors have been functionally characterized. The most severe bottleneck in fungal effector research is the difficulty of manipulating the fungi, as several of the most devastating pathogens, such as rust fungi as well as the most relevant plant-growth-promoting AM fungi, are obligate biotrophs (**Figure 3**). An equally significant problem is that effector mutants often display no associated phenotype with respect to plant colonization, which could reflect functional redundancy, inadequate assay systems, or an inability to detect subtle phenotypes. For example, a large-scale gene disruption study of 78 effector genes upregulated early during rice colonization by *M. oryzae* identified only one gene contributing to virulence (*MC69*) (134), and deleting an entire six-member gene family in *U. maydis* did not affect virulence (32). However, continuously improving technologies such as bimolecular fluorescence complementation, host- and virus-induced gene silencing, and transient expression techniques have facilitated significant progress in the functional characterization of secreted fungal effectors.

Fungal effectors may be needed even before penetration to suppress or downregulate PTI. It is therefore not surprising that expression of several effectors can already be induced by plant surface contact (75, 102). At this stage, fungi also secrete proteins (such as hydrophobins and repellents) that alter hyphal surface structure, but because they have no clear function in modulating plant processes (65), we do not discuss them here.

The Effector Armory of Biotrophs

Fungal effectors have been functionally analyzed most extensively in the biotrophs. To downregulate PTI induced by fungal contact with the plant surface, effectors are needed during penetration. Pep1 is a secreted effector of *U. maydis* and related smut fungi that accumulates in the apoplast (**Figure 7**). The deletion of *pep1* results in a complete block of pathogenic development at the onset of penetration of maize epidermal cells and negatively affects cell-to-cell passages of intracellular hyphae. *pep1* mutants induce strong plant defense responses (27). Pep1 suppresses plant

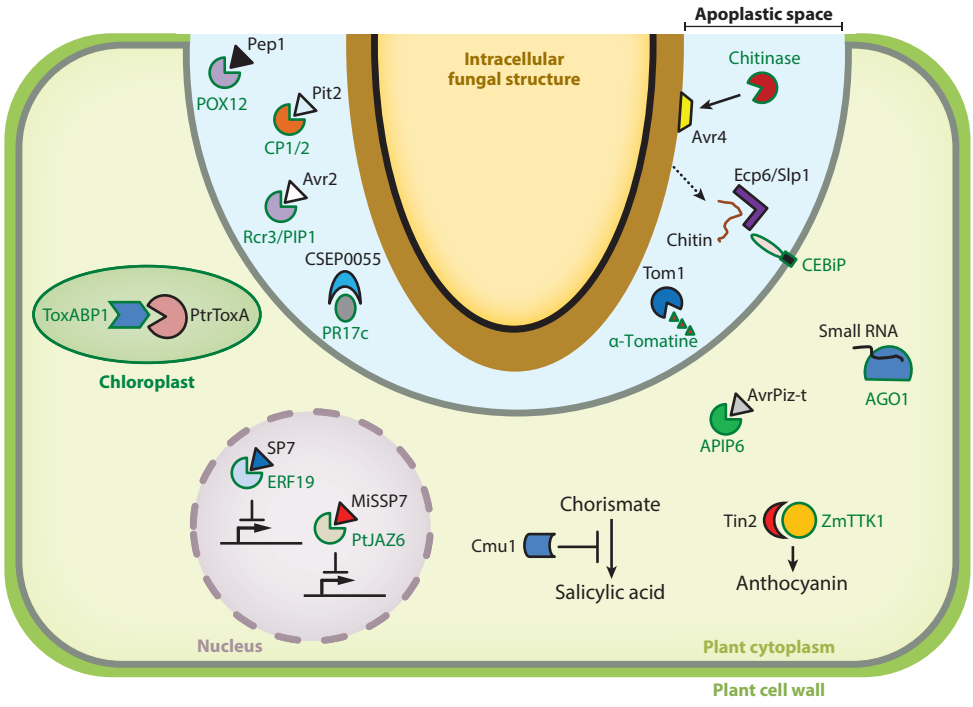


Figure 7

Mode and site of action of fungal effectors. An intracellular fungal structure that secretes effectors is shown in yellow. This structure could be the tip of a biotrophic hyphae, part of a haustorium, or part of an arbuscule. Plant plasma membranes are shown in gray, the fungal plasma membrane is shown in black, the fungal cytoplasm is shown in brown, the plant cytoplasm is shown in light green, and the plant cell wall is shown in dark green. The plant membrane surrounding the arbuscules is also called the periarbuscular membrane or extrahaustorial membrane (in fungi that form haustoria). The apoplastic space between the fungal hypha and the plant plasma membrane has been widened and is shown as a light blue area; in reality, these membranes tightly encompass fungal structures. Fungal effectors and targeted plant proteins are shown in various colors and are surrounded by black lines and dark green lines, respectively. Effectors with a known mode of function are depicted here with their plant proteins or plant-derived substances as interaction partners. Note that the PtrToxA-ToxABP1 interaction may not directly induce plant cell death (108).

immunity by inhibiting POX12, a secreted maize peroxidase that is a conserved component of the plant reactive oxygen species (ROS)–generating system (50) (**Figure 7**).

Pit2, a secreted effector of *U. maydis*, is required for virulence. *pit2* mutants can still colonize maize plants but are severely attenuated in tumor induction (26). Pit2 directly inhibits a set of apoplastic maize cysteine proteases whose activity promotes salicylic acid–associated plant defenses (96) (**Figure 7**).

Phytohormones are key signaling molecules in plants that elicit defense reactions against various pathogens. Salicylic acid has a role in plant development and in responses to abiotic stresses but also induces defense reactions that culminate in cell death, which are considered to be effective mainly against biotrophic pathogens (41, 168). *U. maydis* secretes high amounts of the chorismate mutase Cmu1 during plant colonization to counteract salicylic acid–induced immune responses. Immunoelectron microscopy revealed that Cmu1 is translocated into the cytoplasm of plant cells. The cytoplasmic activity of Cmu1 reduces the levels of chorismate, which can serve as a precursor

for the synthesis of salicylic acid in plastids, thereby promoting virulence (**Figure 7**). In addition, Cmu1 spreads to neighboring cells, priming the surrounding tissue for the upcoming infection (23).

Recent findings have demonstrated that effectors can also target plant secondary metabolite pathways. The *U. maydis* effector Tin2 is specifically induced during biotrophic development and is required for both virulence and the biosynthesis of anthocyanin, a red pigment that accumulates in infected tissue (9, 156). Tin2 functions in the cytosol of plant cells, where it interacts with and stabilizes the maize cytoplasmic protein kinase ZmTTK1 (**Figure 7**). ZmTTK1 stabilized by Tin2 likely promotes anthocyanin biosynthesis (156). The resulting increase in anthocyanin production negatively affects the lignin biosynthetic pathway by reducing the levels of the common precursor *p*-coumaric acid. This strategy prevents lignification of plant cell walls, which would impose a physical barrier to pathogen spread (156).

Fungal pathogens can overcome PTI induced by chitin via different mechanisms. Avr4 of *C. fulvum* induces a hypersensitive response in resistant plants but has a virulence function in susceptible tomato plants. Avr4 binds to chitin in the fungal cell wall, thereby protecting against hydrolysis by plant chitinases (163) (**Figure 7**). Expression of secreted Avr4 in *A. thaliana* and tomato increased virulence in the necrotrophic fungi *B. cinerea* and *Plectosphaerella cucumerina* but did not do so in bacterial or oomycete pathogens lacking chitin in their cell walls (164). Ecp6 of *C. fulvum* sequesters chitin oligosaccharides that are released from the cell walls of invading hyphae to prevent the elicitation of host immunity (18) (**Figure 7**). Structural analysis revealed that two of the three LysM domains in Ecp6 undergo ligand-induced intrachain dimerization, forming an intramolecular chitin-binding groove that binds chitin with ultrahigh affinity. Interestingly, the third LysM domain binds chitin with low affinity but nevertheless contributes to the suppression of chitin-induced PTI, possibly by interfering with dimerization of the plant chitin receptor (135) (**Figure 7**).

C. fulvum also targets secreted cysteine proteases that play a key role in plant immunity through the action of Avr2, an effector that was initially identified as an Avr protein in resistant plants but was later shown to be a genuine virulence factor of *C. fulvum* in susceptible plants (165). Avr2 selectively inhibits the apoplastic proteases PIP1 and Rcr3 (130, 140) (**Figure 7**). In addition, *C. fulvum* secretes Tom1—a tomatinase that degrades α-tomatine into the less toxic compounds β-tomatine and tomatidine—into the apoplast (**Figure 7**). α-Tomatine is an antifungal glycoalkaloid that provides a basal defense against *C. fulvum* in tomato. *tom1* mutants are more sensitive to α-tomatine and display reduced virulence on tomato (106).

Zhang et al. (176) used host-induced gene silencing to study the effector candidate CSEP0055 of the obligate biotrophic barley powdery mildew fungus (*B. graminis* f. sp. *hordei*). This study revealed that fungal entry, particularly at secondary penetration sites, was lower when *CSEP0055* was silenced. CSEP0055 was shown to interact with members of the plant pathogenesis-related 1 (PR1) and PR17 protein families, which are secreted upon biotic stress, suggesting a role in suppressing defense (176) (**Figure 7**).

In the obligate biotroph *B. graminis* f. sp. *hordei*, several *Blumeria* effector candidate (BEC) transcripts are upregulated during the haustorial stage. Through host-induced gene silencing, BEC1011 and BEC1054 proteins were implicated in virulence (120). These effectors contain a ribonuclease scaffold but are unlikely to be active ribonucleases because they lack critical residues in the active site. Complementation experiments indicated that both proteins may function inside the plant cell and that BEC1011 may interfere with pathogen-induced host cell death (120).

BEC4 is a putative *B. graminis* f. sp. *hordei* effector interacting with ADP ribosylation factor–GTPase-activating protein (ARF-GAP). This supports the speculation that BEC4 interferes with defense-associated host vesicle trafficking (138).

RTP1p, a conserved effector in the rust fungi *Uromyces viciae-fabae* and *Uromyces striatus*, was the first fungal protein for which localization inside infected plant cells could be shown directly

by immunoelectron microscopy (64). RTP1 has been proposed to play a structural and stabilizing role in the host cell by forming amyloid-like filamentous structures (63). Whether this function is relevant for plant colonization by rust fungi remains to be shown.

Necrotrophic effector: a host-selective protein toxin that induces host cell death when recognized by the product of a cognate plant sensitivity gene

Effector-triggered sensitivity: plant sensitivity to necrotrophs, triggered upon recognition of a necrotrophic effector by the product of the cognate sensitivity gene

The Effector Armory of Necrotrophs

Effectors of necrotrophic fungi induce plant cell death. Their armory consists of secondary metabolites, polyketide toxins, nonribosomal peptide toxins, necrosis- and ethylene-inducing peptide 1 (Nep1), and Nep1-like proteins, all of which have been reviewed extensively elsewhere (121, 148), as well as protein toxins covered here. The elucidation of the mechanism of action of protein toxins led Oliver et al. (108) to propose an inverse gene-for-gene model, in which the establishment of compatibility is based on the recognition of the effector by the R protein, which in this context is defined as a sensitivity protein.

The necrotrophic wheat pathogens *S. nodorum* and *P. tritici-repentis* produce several necrotrophic effectors that induce severe necrosis in wheat harboring dominant sensitivity genes (108). In all combinations (SnToxA-Tsn1, SnTox1-Snn1, SnTox2-Snn2, SnTox3-Snn3, and SnTox4-Snn4), both the effector and the host sensitivity protein are required for a compatible interaction (effector-triggered sensitivity), i.e., the opposite of ETI (108). Intriguingly, the wheat protein Tsn1, which provides sensitivity to ToxA, displays typical features of R proteins, including NB-LRR domains, indicating that necrotrophs also transfer effectors and have hijacked the mechanism conferring resistance to biotrophs in order to establish a compatible interaction (33). The ToxA effector is internalized exclusively in sensitive, Tsn1-expressing wheat genotypes (86). ToxA localizes to the chloroplast and interacts with ToxABP1 (**Figure 7**), which is a protein likely involved in thylakoid formation. This interaction is suspected to result in perturbation of the photosystem, leading to cell death (87). The expression of the PtrToxA effector in nonpathogenic isolates confers virulence in toxin-sensitive wheat cultivars, illustrating that PtrToxA is sufficient to confer fungal virulence (14).

More recently, small RNAs have also been identified as pathogen effectors. *B. cinerea* small RNAs, which are produced from repetitive elements (170), hijack the host RNA interference machinery by binding to *A. thaliana* ARGONAUTE 1 (AGO1) (**Figure 7**) and selectively silence host immunity genes that show complementarity to these RNAs.

The Effector Armory of Hemibiotrophs

Hemibiotrophic fungi combine a biotrophic with a necrotrophic lifestyle—i.e., they initially need effectors suppressing plant defenses and later need effectors that kill plant cells. In *M. oryzae*, Slp1 (a LysM protein that is closely related to Ecp6 in *C. fulvum*) specifically accumulates at the plant-fungus interface during the early stages of rice blast infections. Slp1 specifically binds chitin (**Figure 7**) and is able to suppress chitin-triggered PTI. In addition to binding soluble chitin oligosaccharides, Slp1 competes for chitin binding with CEBiP, the rice PRR chitin elicitor–binding protein that together with OsCERK plays a key role in the perception and transduction of the chitin oligosaccharide signal (58, 92) (**Figure 7**). *M. oryzae Δslp1* strains were affected in virulence, but silencing of CEBiP allowed them to regain the ability to cause rice blast disease (92). *Z. tritici* has three Ecp6 homologs; of these, Mg3LysM and Mg1LysM are upregulated during the biotrophic phase, but only Mg3LysM blocks the elicitation of chitin-induced plant defenses, and virulence was attenuated when it was deleted. However, similarly to Avr4, both proteins could protect fungal hyphae against plant-derived chitinases (88). This demonstrates the

broad involvement of LysM effectors in suppressing defense but also illustrates that the activity of LysM-containing proteins is far from understood.

In addition to the apoplastic components of plant resistance, hemibiotroph effectors also interfere with cytoplasmic plant immunity. An example is the *M. oryzae* avirulence gene *AvrPiz-t*, which targets the cytosolic rice *R* gene *Piz-t* (78, 178). In plants lacking Piz-t, AvrPiz-t contributes to *M. oryzae* virulence. Expression of AvrPiz-t in transgenic rice suppressed PAMP-triggered generation of ROS and enhanced susceptibility to *M. oryzae* (111). AvrPiz-t interacts with and inhibits the rice RING E3 ubiquitin ligase APIP6 (**Figure 7**), which can ubiquitinate AvrPiz-t. The silencing of APIP6 in transgenic rice also suppressed ROS generation and enhanced susceptibility to *M. oryzae*, demonstrating that AvrPiz-t promotes *M. oryzae* virulence by suppressing PTI in rice (111).

In *M. oryzae*, the avirulence gene *ACE1* (encoding a polyketide synthase) is specifically upregulated during penetration. Ace1 is not predicted to be secreted and localizes in the cytoplasm of the appressorium, suggesting that unknown secondary metabolites synthesized by Ace1 might function as effectors recognized by the cognate R protein (5). So far, it has not been possible to ascribe a virulence function to Ace1 (5). An additional example implying a role of secondary metabolite effectors is likely to exist in *C. higginsianum*. In this fungus, 12 secondary metabolism gene clusters are induced before penetration and during biotrophy (102). At these stages, plant cells are still alive, prompting speculation that the metabolites produced may function in host manipulation, similarly to protein effectors.

Orbach et al. (109) cloned the *M. oryzae Avr-Pita* gene as an avirulence factor recognized by the *R* gene *Pi-ta* in rice, and the predicted protein has typical features of metalloproteases but is not required for virulence. Avr-Pita was able to elicit a Pi-ta-dependent resistance response when expressed in rice cells without a signal peptide and prodomain (54). In *V. dahliae*, the Avr protein Ave1 is recognized on the cell surface by the receptor-like protein Ve1, triggering a defense response (19). Ave1 is homologous to a widespread family of peptides present in plants and some fungi that are related to cell wall–loosening expansins and plant natriuretic peptides, the latter of which are mobile signaling molecules that accumulate in the apoplast after biotic stress. Ave1 also has a virulence function, but the mechanism underlying the virulence function is unknown (19).

Fusarium oxysporum f. sp. *lycopersici* secretes effector proteins into the xylem [secreted-in-xylem (SIX) proteins]. Most of the genes encoding the 11 SIX effectors reside on chromosome 14, a lineage-specific chromosome required for infection of tomato (83). Avr2/SIX3, Avr3/SIX1, and SIX6 contribute to virulence in susceptible tomato lines (154). Coexpression of the *I-2 R* gene and the *AVR2/SIX3* gene without a signal peptide triggered a hypersensitive response in *Nicotiana benthamiana*, indicating that Avr2 is recognized by I-2 inside the plant cell. Moreover, SIX6 specifically suppressed Avr2/I-2-mediated cell death when coexpressed (154).

In *C. higginsianum*, the Nep1-like protein ChNLP1 is specifically expressed during a switch to the necrotrophic lifestyle and can induce plant cell death in *N. benthamiana*. Interestingly, this plant cell death can be suppressed by coexpressing several other ChEC effectors expressed during the biotrophic phase. A related phenomenon has been observed in *Colletotrichum orbiculare* (175). Although mutants lacking these effectors were not altered in virulence, these reports suggest that the balance between effector proteins that induce cell death and those that suppress it may control the switch from biotrophy to necrotrophic development in hemibiotrophic fungi.

The Effector Armory of Symbionts

SP7, a repetitive effector from the AM fungus *Glomus intraradices*, interacts with ERF19, a host ethylene-responsive transcription factor that regulates the expression of several defense-related

genes in *Medicago truncatula* (69) (**Figure 7**). When constitutively expressed without its signal peptide in *M. truncatula*, SP7 localized to the nucleus and increased mycorrhization. By contrast, overexpression of ERF19 resulted in an impaired mycorrhizal symbiosis, and downregulation of ERF19 promoted mycorrhizal symbiosis (69). This suggests that SP7 modulates the activity of the ERF19 transcription factor, leading to a downregulation of PTI.

The *L. bicolor* MiSSP7 effector protein is highly induced upon perception of diffusible signals from plant roots and is necessary for the mutualistic symbiotic relationship with host roots (119). *L. bicolor* transformants with reduced MiSSP7 expression were unable to enter into symbiosis with poplar roots, and this defect could be complemented by expressing MiSSP7 in the cytosol of transgenic poplar plants. A recent report found that MiSSP7 localizes to the plant nucleus and interacts with PtJAZ6 (**Figure 7**), a negative regulator of jasmonic acid–induced gene regulation in *Populus trichocarpa* (118, 119). MiSSP7 prevented jasmonic acid–dependent degradation of PtJAZ6, resulting in the repression of jasmonic acid–induced genes. These genes include those with a predicted function in cell wall modification (118), which could facilitate hyphal entry into the root and establishment of the Hartig net (118). Intriguingly, this contrasts with AM and biotrophic pathogens that induce jasmonic acid responses during host colonization (28, 81) and suggests that ECM fungi may have evolved unique colonization strategies.

COMMON PLANT NODES TARGETED BY FUNGAL, OOMYCETE, AND BACTERIAL EFFECTORS

As discussed above, fungal effectors target plant defense components, signaling, and metabolic pathways to promote host plant colonization. Knowing the extent to which such routes are also targeted by other pathogen effectors and not by symbiont effectors would be useful in developing new strategies for combating plant diseases.

Protease inhibitor activity is a common effector function in fungal and oomycete effectors. As with Avr2 in *C. fulvum* (130) and Pit2 in *U. maydis* (96), the oomycete pathogen *P. infestans* secretes EPIC1 and EPIC2B effector proteins, which inhibit tomato apoplastic PIP1 and Rcr3 cysteine proteases (145, 158). This indicates that the inhibition of apoplastic proteases is a key function for establishing compatibility. However, which proteins are targeted by these apoplastic proteases and how they trigger plant defense responses remain to be elucidated.

The ubiquitin-proteasome system is another conserved target. The *M. oryzae* AvrPiz-t effector suppresses the RING E3 ubiquitin ligase APIP6 (111); the GALA effector of *Ralstonia solanacearum* interacts with the *A. thaliana* SKP1-like protein, a component of the SCF-type E3 ubiquitin ligase, presumably to interfere with the ubiquitin-proteasome pathway and promote disease (4); and the *P. infestans* effector Avr3a interacts with and stabilizes the U-box E3 ubiquitin ligase CMPG1, which is required for INF1-triggered cell death (7, 44).

A third common target is plant immune receptors, which play crucial roles in the perception of pathogens. Bacterial effectors directly target receptor proteins to circumvent the activation of defense signaling pathways (43). The proposed interference of the plant chitin receptor with dimerization by the LysM domain effector Ecp6 of *C. fulvum* could also represent such an example (135).

Salicylic acid is a key phytohormone for inducing cell death, and its manipulation by effectors should therefore be an attractive node for establishing successful infection. Metabolic priming by the translocated fungal effector Cmu1 currently serves as the fungal example for downregulating salicylic acid levels (23). Several bacterial effectors secreted via the type III secretion apparatus are known to target the salicylic acid biosynthesis pathway (22, 100). HopI1 of *Pseudomonas syringae* targets the chloroplast and suppresses accumulation of salicylic acid. HopI1 directly interacts with

Hsp70 to stimulate its ATP hydrolysis activity. Hsp70 has been suggested to promote defense, which is subverted by HopI1 (53). The HaRxL44 effector from the downy mildew *Hyaloperonospora arabidopsidis* localizes to the nucleus in infected *A. thaliana* plants and interacts with the mediator subunit protein MED19a (11). HaRxL44 interaction with MED19a results in proteasomal degradation of MED19a, which reduces the expression of salicylic acid–related defense responses. These examples illustrate that, depending on the system, different strategies are used to downregulate salicylic acid levels.

JAZ proteins, which function as transcriptional repressors for the jasmonic acid signaling pathway, are targeted by the fungal effector MiSSP7 from *L. bicolor* (116). JAZ proteins are also targeted by bacterial effectors like the type III effector HopZ1a of *P. syringae* (55). The interaction with HopZ1a triggers the degradation of AtJAZ1 and activates the jasmonic acid signaling pathway, resulting in the promotion of bacterial growth (55). Another type III effector, HopX1 of *P. syringae* pv. *tabaci*, associates with several JAZ proteins and promotes their degradation (38). These studies indicate that jasmonate signaling is targeted by both pathogens and symbionts. Because of their universal role in plant defense and physiology, phytohormone pathways are manipulated by many other effectors (for a recent review, see 62). Many more common nodes will likely become apparent once the mechanisms of action of more effectors are elucidated.

UPTAKE OF SECRETED FUNGAL EFFECTORS BY HOST CELLS

The first evidence for fungal effector translocation inside host cells came from the observation that Avr proteins trigger cell death upon coexpression with their cognate intracellular host R proteins, implying a function inside host cells (150). However, the molecular basis of uptake is still poorly understood (123). Bioinformatic analysis has failed so far to identify conserved motifs in fungal effectors that may mediate uptake. The apparent lack of a common sequence motif could suggest that there is no universal entry mechanism and that different fungal plant pathogens may have evolved uptake strategies as diverse as the infection structures they develop during plant colonization (**Figure 1**). Alternatively, uptake motifs may exist but may not be recognizable through conservation on the primary amino acid sequence level.

Powdery mildew fungi represent one of the few exceptions, where 80% of the candidate-secreted effector proteins share the N-terminal tripeptide motif Y/F/WxC downstream of the signal peptide (42). The other exception comes from the root endophyte *P. indica*, where a group of 25 small secreted effectors (termed DELD effectors) share the C-terminal RSIDELD motif (179). However, evidence for an involvement of these motifs in translocation is missing.

On the basis of its ability to trigger cell death in the presence of the host R protein M, the effector AvrM of the flax rust *M. lini* was shown to translocate into the host cell in a pathogen-independent manner via an exposed hydrophobic patch that mediates binding to the plant plasma membrane (167). Whether membrane-bound AvrM is then internalized via endocytosis requires further investigation. Internalization via an integrin-like receptor has been postulated for PtrToxA secreted by *P. tritici-repentis* (86). Internalization depends on an RGD vitronectin-like motif in PtrToxA (85, 86, 91). Host-mediated internalization has also been shown for the effector MiSSP7 secreted by the ECM fungus *L. bicolor*. Endocytosis might in this case depend on the ability of MiSSP7 to bind phospholipids (117). Further investigation is essential to clarify whether exosomes may be involved in the secretion and uptake of a subset of effectors such as unconventionally secreted proteins (48, 128) or small interfering RNAs that have been recently identified as translocated effectors in the necrotrophic fungus *B. cinerea* (171).

Progress in understanding fungal effector uptake has been hampered by the lack of a reliable uptake assay. The first attempts to demonstrate translocation of non-Avr fungal effectors exploited

the ability of Avr effectors to induce cell death in resistant plants (124, 150). In such uptake assays, the putative effector is fused to an Avr protein and transiently expressed as a secreted protein (i.e., with the signal peptide) in plant cells along with the corresponding cytoplasmic plant *R* gene. The Avr-effector fusion protein is able to trigger cell death only if, after secretion, it reenters the host cell, and this is then interpreted as pathogen-independent effector uptake (124). The main flaw of this assay is the impossibility of proving that the effector is first secreted by the plant and that what is measured is reentry rather than cytoplasmic leakage along the secretory path. To address this issue, an alternative assay has been developed that is based on the incubation/infiltration of plant root tips or leaves in a solution containing microgram amounts of heterologously expressed and purified effectors. Localization is then assessed by fluorescence microscopy using effector-specific antibodies, tagging the effectors with a fluorescent protein (FP) or conjugating them to a fluorophore (30, 59, 86, 117). However, concerns have been expressed about this assay, which seems to lack specificity and reproducibility (169, 174). The avirulence activity and, hence, the cytoplasmic function of candidate effectors have been more recently assayed by delivering the fungal proteins into the plant cell via the bacterial type III secretion system (142, 162). This assay has proven to be effective for delivery into rice, wheat, and barley, but whether it is applicable to other pathosystems remains to be investigated. In addition, this type of delivery might prove ineffective if the effector needs to be posttranslationally modified. The most reliable and powerful tool so far used for in planta localization of effectors is electron microscopy (23, 64, 124). Unfortunately, this technique may generate artifacts, is time consuming, requires highly specific antibodies, and cannot easily be used to screen the localization of hundreds of effectors.

Live cell imaging techniques have been introduced as a powerful tool to detect in planta secretion and uptake of FP-tagged effectors. Unfortunately, they have proven so far to be effective only for translocated effectors secreted by *M. oryzae* (40). In this hemibiotrophic fungus, FP-tagged effectors displayed two distinct localization patterns (40): The apoplastic effectors were conventionally secreted in the matrix surrounding the hyphae, whereas the translocated effectors were detected in the BIC structure (**Figure 1**) and plant cytosol/nucleus (39). The secretion targeted to the BIC differed from the conventional secretion in that it required expression of the effectors from the native promoter and was brefeldin A insensitive. Hence, it is likely to bypass Golgi bodies but still depends on components of the exocyst complex and soluble NSF attachment protein receptors (SNAREs) (39). Because effector accumulation in the BIC may not always coincide with translocation and vice versa (126, 134), further work is needed to clarify the nature of the BIC and whether the BIC is the organelle-mediating effector uptake in this system.

In *C. higginsianum*, sequential effector secretion has been detected by tagging candidate effectors with FPs (68). Before penetration, effectors accumulated at the appressorium pore from which they were focally secreted. After penetration, they displayed two different patterns of localization: Some accumulated in discrete foci scattered along the surface of the biotrophic hyphae at the interface between the fungal cell wall and the host plasma membrane, and others displayed a more uniform, less punctuated distribution in the biotrophic interface. Whether this relates to where these effectors ultimately function needs to be established.

During the early biotrophic stage of *C. orbiculare*, FP-tagged effectors localized to a ring-like structure around the neck of the biotrophic hyphae between the fungal cell wall and the plant plasma membrane (51). Effector localization to this interface depended on expression during the early biotrophic stage as well as on the conventional secretion pathway and occurred irrespective of where the effector is presumed to function. Neither in *Colletotrichum* spp. nor in *U. maydis* could translocation of FP-tagged effectors into the host cell be observed, even though Cmu1 and Tin2 of *U. maydis* were shown to have a cytoplasmic localization and function, respectively (23, 156). The failure of FP-tagged effectors to enter the host cytoplasm suggests

that the translocation mechanism may require the partial unfolding of the protein. FP tags may hinder translocation or, alternatively, may be unfolded during the translocation process and fail to refold in the cytoplasm. There is also the possibility that the fluorescent signal, which is proportional to the amount of the intracellular effector, is too weak to be detected with respect to plant cell autofluorescence. To facilitate detection of cytoplasmic effectors, a nuclear localization signal has been fused to FP-tagged effectors in order to concentrate the fluorescent signal in the plant nucleus. Although this strategy has proven to be effective for *M. oryzae* translocated effectors (40), the possibility cannot be excluded that the addition of a nuclear localization signal (i.e., a stretch of positively charged amino acids) to an effector could alter its uptake properties in other systems. Overcoming the difficulties of using FPs as tags in uptake assays for several fungal effectors will require new strategies to unequivocally prove uptake by host cells and to enable the study of the translocation process.

POSTTRANSLATIONAL MODIFICATION AND PROCESSING OF EFFECTORS

Proteins secreted via the classical endoplasmic reticulum–Golgi apparatus route are cotranslationally inserted into the endoplasmatic reticulum, where they can be N-glycosylated and/or O-glycosylated. Chen et al. (12) recently showed that *M. oryzae ALG3*, encoding an endoplasmic reticulum–localized α-1,3-mannosyltransferase involved in N-glycosylation, is required for virulence. In *alg3* mutants, secondary hyphae were arrested and massive production of ROS was observed. This defect could be linked to missing N-glycosylation of Slp1, which altered its chitin-binding activity. In addition, significantly smaller amounts of Slp1 were detected in the *alg3* mutant, suggesting that glycosylation may also affect protein stability. The apoplastic *M. oryzae* effector Bas4 was also shown to be N-glycosylated, but in this case the functional relevance is unknown (12).

N-glycosylation is also implicated in the pathogenicity of *U. maydis* and *Z. tritici* (34, 95, 136). *U. maydis gas1* mutants lacking glucosidase II as well as *gls1* mutants lacking glucosidase I both arrested shortly after penetration and elicited massive production of ROS, suggesting an altered perception by the plant (34, 136). However, in these cases it is not clear whether N-glycosylated effectors are the critical targets necessary to establish the biotrophic interaction.

Several effectors, such as Avr4 and Avr9 from *C. fulvum* and SnToxA from *S. nodorum*, must be processed to become active. These proteins are synthesized as precursor proteins, and after removal of the signal peptide, further N-terminal processing occurs by unknown fungal and/or plant proteases (150). In addition, Avr-Pita of *M. oryzae*, proposed to encode a transferred metalloprotease interacting with the cytoplasmic receptor Pi-ta of rice, must be N-terminally processed to trigger a hypersensitive response (54). In *U. maydis*, several secreted effectors (e.g., Rep1, Hum3, and Rsp1) are repetitive proteins that are processed by the subtilisin protease Kex2 (97, 129, 173), and a *hum3 rsp1* double mutant was severely compromised in virulence.

Collectively, these studies indicate that effectors can undergo significant posttranslational modifications, which may be needed to convert them to the active form. This understanding has far-reaching consequences, as it means, for example, that nonglycosylated effectors produced heterologously in *Escherichia coli* may not be biologically active or may be unstable when infiltrated into plant tissue. Alternatively, effector proteins expressed transiently in the cytosol of plants may not be equivalent to effectors that are delivered by the fungus in a modified and processed form. Finally, expressing an effector that requires glycosylation in yeast two-hybrid screens may not allow identification of true interaction partners.

FUTURE CHALLENGES

Compared with the avirulence function of effectors, a molecular understanding of the virulence- and symbiosis-promoting activities of fungal effectors is still in its infancy. In particular, no effectors have been identified that redirect plant metabolism to meet the nutritional demands of the invading fungi. In addition, the plant signals that modulate effector gene expression temporally and spatially are largely unknown. Advances in the molecular understanding of effector function will very much depend on more efficient assays to study the functions of individual effectors and those of large families that may have partially redundant or overlapping functions. Another question not widely addressed in present studies is how changes in plant physiology and immunity by mutualistic and pathogenic fungi affect plant colonization by other microbes. It is conceivable that fungal effectors may also be used to combat other microbes rather than being exclusively addressed toward plant targets.

We also consider it a challenge to elucidate why highly specialized haustoria are needed in some systems, whereas other fungi thrive without developing such structures and proliferate without even entering plant cells. Moreover, what is the primary difference that determines whether the outcome of an infection is disease or a mutualistic/asymptomatic interaction? Can we generalize two recent observations showing that a fungal endophyte can become a pathogen by switching from restricted to proliferative growth and, vice versa, that a biotrophic pathogen can be turned into an endophyte by deleting a large cluster of effector genes that contribute to its virulence (9, 155)? Another issue deserving priority is how biotrophic mycorrhizal fungi can have such a broad host range, given that PTI provides highly efficient protection against colonization by biotrophic pathogens. In addition, addressing the unresolved issues of fungal effector uptake and whether there are common or distinct mechanisms for uptake by plant cells will require more reliable and generally accepted assays, knowledge about what directs effector secretion to specific sites or organelles, structural information about the relevant effector domains, and molecular studies of the uptake mechanisms.

SUMMARY POINTS

1. From comparative analysis of the secretomes of 84 plant-colonizing fungi, lifestyle-specific patterns are emerging.
2. Biotrophy is accompanied by a reduction of plant cell wall–degrading enzymes whose action could damage the host and/or trigger plant defense responses.
3. Effector proteins target common processes in different pathosystems.
4. Modulation of phytohormone levels is a common strategy employed by both pathogenic and symbiotic fungi.
5. Apoplastic effectors function as enzyme inhibitors, protect fungal hyphae from recognition, or scavenge molecules that trigger immune responses.
6. Effectors that are translocated to the host cells appear to lack a conserved motif, suggesting different modes of uptake.
7. Effector genes are under strong selection pressure, and accelerated evolution is accomplished by the location of effector genes in species-specific, flexible genome compartments.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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