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Filamentous pathogen effector functions: of pathogens, hosts and microbiomes

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Microorganisms play essential roles in almost every environment on earth. For instance, microbes decompose organic material, or establish symbiotic relationships that range from pathogenic to mutualistic. Symbiotic relationships have been particularly well studied for microbial plant pathogens and have emphasized the role of effectors; secreted molecules that support host colonization. Most effectors characterized thus far play roles in deregulation of host immunity. Arguably, however, pathogens not only deal with immune responses during host colonization, but also encounter other microbes including competitors, (myco)parasites and even potential co-operators. Thus, part of the effector catalog may target microbiome co-inhabitants rather than host physiology.

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Introduction

During early microbial colonization stages, plant cell surface-localized pattern recognition receptors (PRRs) recognize microbe-associated molecular patterns (MAMPs), such as fungal chitin, to activate immune responses [1,2]. In order to establish themselves, adapted pathogens secrete effector molecules that deregulate immune responses and facilitate host colonization. Simultaneously, hosts evolve effector recognition by novel receptors that reinstall immunity [1,2]. Consequently, effectors are subject to various selective forces that drive their evolution, leading to diversified effector repertoires between pathogen lineages. Functional characterization of effectors and determination of their contribution to the microbial lifestyle provides insight in relevant processes for host colonization.

Plant pathogen effectors deregulate host immunity in various subcellular compartments

Many pathogens initially enter the plant apoplast, which contains enzymes that hamper microbial colonization. For example, chitinases target fungal cell walls to release chitin fragments that activate immune receptors, leading to further chitinase accumulation to induce hyphal lysis. In turn, fungal pathogens secrete chitin-binding effectors to protect their cell walls and interfere with immune receptor activation [3–6]. The LysM domain-containing Ecp6 effector of the leaf mold fungus *Cladosporium fulvum* can outcompete host receptors through chitin binding with unprecedented ultrahigh (pM) affinity by intramolecular LysM domain dimerization [7••]. Additionally, LysM effectors likely interfere with receptor dimerization that is required to activate immune signaling [7••,8•,9].

Although effectors that directly target chitinases have not yet been identified, some effectors target other apoplastic hydrolytic enzymes, such as proteases. For example, sequence-unrelated effectors of *C. fulvum*, the oomycete *Phytophthora infestans*, and the parasitic nematode *Globodera rostochiensis* inhibit tomato cysteine proteases including Rcr3 [10,11,12•]. The closely related oomycetes *P. infestans* and *P. mirabilis* express an orthologous pair of host protease inhibitor effectors that are subject to positive selection, which was implicated in adaptation to unique protease targets in their respective host plants [13••]. Besides protease inhibitors, *P. infestans* secretes the Avrblb2 effector that interferes with protease secretion [14]. The smut fungus *Ustilago maydis* inhibits apoplastic proteases via multiple effectors. While Pit2 directly inhibits cysteine proteases [15], Pep1 induces the maize cystatin CC9 that inhibits apoplastic proteases in turn [16]. Pep1 furthermore inhibits the maize peroxidase POX12 to perturb reactive oxygen species balances [17]. Thus, the plant apoplast is a dynamic battlefield for plant pathogens.

In addition to apoplastic effectors, many pathogens deliver effectors that act inside host cells, although mechanisms that govern their uptake remain controversial [18]. The rice blast fungus *Magnaporthe oryzae* was shown to secrete various effectors that enter rice cells, and even move to non-infected neighboring cells, presumably to prepare these for infection [19]. The AvrPiz-t effector targets proteasome activity through interaction with the RING E3 ubiquitin ligase APIP6, leading to their mutual degradation and suppression of PRR-mediated immunity

[20]. Effector diffusion from infected cells into neighboring cells was similarly observed for the *U. maydis* chorismate mutase Cmu1 that targets the shikimate pathway to channel chorismate into the phenylpropanoid pathway, thus adversely affecting salicylic acid (SA) biosynthesis [21•]. *U. maydis* furthermore secretes the Tin2 effector to stabilize the maize ZmTTK1 kinase that controls anthocyanin biosynthesis, possibly to suppress tissue lignification [22••]. Also the oomycete *Hyaloperonospora arabidopsidis* targets SA signaling by secreting a nuclear-localized effector that interacts with the mediator complex that controls interactions between transcriptional regulators and RNA polymerase [23]. Host transcription is furthermore perturbed by effectors that inhibit transcription factor translocation to the nucleus [24]. Additionally, nuclear-localized effectors may affect host immunity post-transcriptionally by suppressing the biogenesis of small RNAs in the host [25•]. Interestingly, *Botrytis cinerea* was recently suggested to deliver even small RNAs into host cells to affect immune responses [26••].

Finally, several effectors target host cell death mechanisms, such as *P. infestans* Avr3a and PexRD2. While Avr3a suppresses INF1-triggered cell death by stabilizing the U-box E3 ligase CMPG1 during biotrophic growth, PexRD2 targets the kinase domain of the cell death regulator MAPKKKε [27,28]. During later stages of infection, however, *P. infestans* relies on induction of host cell death as it switches to a necrotrophic lifestyle. Necrotrophic pathogens evolved effectors that actually induce cell death. An elegant example is provided by the *Cochliobolus victoriae* effector victorin that binds to thioredoxins including TRXh5, which is required for redox control of the transcriptional immune regulator NPR1. TRXh5 binding activates the NB-LRR-type immune receptor LOV1, facilitating necrotrophic exploitation of host cell death by *C. victoriae* [29•].

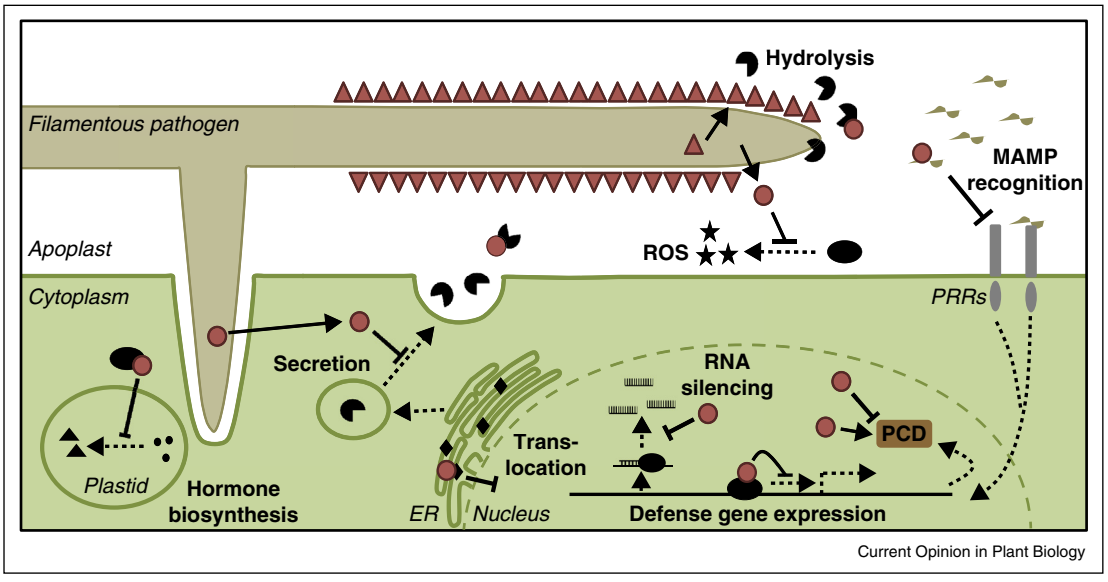
In conclusion, although information for the vast majority of pathogen effectors, particularly of filamentous pathogens, is still lacking, effector molecules are highly versatile. Clearly, recently uncovered functions revealed that virulence effectors, despite the finding that they converge onto pivotal elements of the plant immune system [30], can deregulate any step of immunity in any cellular compartment (Figure 1 and Table 1).

Endophytes and mutualists use effectors to suppress host immunity too

Like pathogens, commensalistic endophytes and mutualists develop intimate host–plant associations. During initiation of such symbioses, PRRs continue to perceive MAMPs. Consequently, similar to pathogens, endophytes and mutualists are recipients of immune responses. However, the precise role and fate of host immunity in the establishment of symbiosis have remained enigmatic.

The root endophyte *Piriformospora indica* has a wide host range and induces enhanced growth and stress resistance in colonized hosts. Rather than evading host detection, the fungus actively suppresses immunity [31]. During early biotrophic growth at the onset of symbiosis, about

Figure 1



Filamentous pathogen effectors deregulate host immunity in various host subcellular compartments. Pathogens secrete effectors (red symbols) to deregulate plant immunity (see text for details). Whereas one group of effectors (red circles) interacts with host targets that act in immunity (black shapes), another group of effectors (red triangles) acts in self-defense to protect the pathogen from host-derived antimicrobials.

Table 1

Effectors of filamentous plant-associated microbes for which molecular virulence targets were identified

Effector	Origin	Target	Function	Refs
BEC4	*Blumeria graminis* f.sp. *hordei*	ARF-GAP proteins	Interference with host vesicle trafficking	[65]
Avr2	*Cladosporium fulvum*	Cysteine proteases	Cysteine protease inhibition	[66,10]
Avr4	*Cladosporium fulvum*	Chitin	Hyphal protection	[67]
Ecp6	*Cladosporium fulvum*	Chitin	Perturbation of chitin-triggered immunity	[3]
CfTom1	*Cladosporium fulvum*	α-Tomatine	Detoxification	[68]
Victorin	*Cochliobolus victoria*	TRX-h5	Induction of LOV1-mediated cell death	[29•]
SP7	*Rhizophagus irregularis*	ERF19	Deregulation of host gene expression	[40•]
HaRxL44	*Hyaloperonospora arabidopsidis*	MED19a	Interference with SA-triggered immunity	[23]
MiSSP7	*Laccaria bicolor*	JAZ6	Deregulation of host gene expression	[36]
AvrPiz-t	*Magnaporthe oryzae*	RING E3 ubiquitin ligase APIP6	Suppression of MAMP-triggered immunity	[20]
Slp1	*Magnaporthe oryzae*	Chitin	Perturbation of chitin-triggered immunity	[6]
MfAvr4	*Mycosphaerella fijiensis*	Chitin	Hyphal protection	[69]
Mg1LysM	*Mycosphaerella graminicola*	Chitin	Hyphal protection	[5]
Mg3LysM	*Mycosphaerella graminicola*	Chitin	Perturbation of chitin-triggered immunity	[5]
Avr3a	*Phytophthora infestans*	CMPG1	E3 ligase stabilization	[27]
Avrblb2	*Phytophthora infestans*	C14 protease	Suppression of protease secretion	[14]
EPI1	*Phytophthora infestans*	Serine proteases	Inhibition of serine proteases	[70]
EPI10	*Phytophthora infestans*	Serine proteases	Inhibition of serine proteases	[71]
EPIC1	*Phytophthora infestans*	Cysteine proteases	Inhibition of cysteine proteases	[72,11]
EPIC2B	*Phytophthora infestans*	Cysteine proteases	Inhibition of cysteine proteases	[72,11]
PexRD2	*Phytophthora infestans*	MAPKKKε	Suppression of host cell death	[28]
Pi03192	*Phytophthora infestans*	NTP1, NTP2	Suppression of transcription factor relocation	[24]
GIP1	*Phytophthora sojae*	β-1,3-Glucanases	Glucanase inhibition	[73]
RTP1p	*Uromyces fabae/U. striatus*	Proteases	Protease inhibition	[74]
Cmu1	*Ustilago maydis*	Cm2	Interference with SA biosynthesis	[21•]
Pep1	*Ustilago maydis*	POX12	Inhibition of peroxidase-mediated ROS production	[17]
Pit2	*Ustilago maydis*	CP2, CP1A/B, XCP2 proteases	Cysteine protease inhibition	[15]
Tin2	*Ustilago maydis*	TmTTK1	Control of anthocyanin biosynthesis	[22••]

10% of the transcriptome encodes putative effector proteins [32]. At later growth stages the fungus requires host cell death for further colonization, thus resembling hemibiotrophic pathogens such as *Mycosphaerella graminicola* and *M. oryzae*. Like *C. fulvum*, these latter species utilize LysM effectors to suppress immune responses [3,5,6]. *P. indica* carries an expanded LysM domain-containing effector repertoire that may similarly act in immune suppression [32].

Effector-like proteins are also encoded by genomes of other mutualists [33–35]. The ectomycorrhiza *Laccaria bicolor* genome encodes hundreds of small secreted proteins, several of which are only expressed in symbiotic tissues. Of these, MiSSP7 was shown to translocate to the nucleus of poplar host cells to stabilize the JAZ6 protein and repress jasmonate signaling [34,36]. Likewise, the ectomycorrhiza *Tuber melanosporum* expresses 125 cysteine-rich small secreted proteins, including a LysM effector, which are highly upregulated during symbiosis [35].

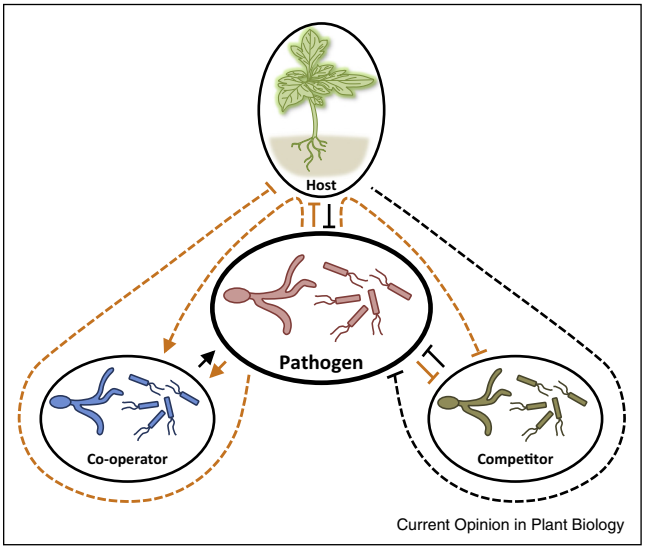
It was recently shown that arbuscular endomycorrhizal fungi produce lipochitooligosaccharide mycorrhizal (Myc) factors that stimulate root growth and branching to initiate symbiosis [37]. Similar to endophytes and ectomycorrhiza, arbuscular endomycorrhiza secrete effector-like proteins during symbiotic interactions [38,39,40•]. The genome of *Rhizophagus irregularis* encodes a family of CRN-like proteins that are abundantly found in plant pathogenic *Phytophthora* spp. [39]. *R. irregularis* was furthermore found to encode an effector that interacts with the pathogenesis-related ethylene-responsive transcription factor 19 (ERF19) in the host nucleus to promote mycorrhization, potentially by counteracting MAMP-induced host defense responses that are regulated by ERF19 [40•].

Collectively, these findings suggest that symbiotic associations that include endophytism, mutualism and parasitism form a continuum in which effectors play essential roles (Table 1).

Effectors act in self-defense and competition

The ability to establish symbiosis evolved multiple times in microbes, presumably from saprotrophism, and many plant pathogens still display saprotrophic life stages. Saprotrophs generally reside within the soil where they feed on decaying organic matter in the presence of a rich microbiota. In this environment, microbial competition as well as co-operation occurs (Figure 2). Threats are posed by (myco)parasites and competitors that produce antibiotics with specific or broad-spectrum activities. Consequently, microbes require molecules for self-defense and interaction with other microbiome partners.

Figure 2



How pathogens influence the local biota by exploiting effector activities. The interaction between microbial pathogens and plant hosts occurs in environments that contain additional microbiome partners that can negatively (competition) or positively (co-operation) impact the pathogen as well as the host. Consequently, the pathogen and host may target each other directly (solid lines) as well as indirectly (dotted lines). Likely, pathogens exploit effector activities (orange lines) to not only directly modulate their hosts, but also to influence the local microbiota that can impact the outcome of the interaction with their hosts.

Similar to infected plants, many mycoparasites secrete hydrolytic enzymes including proteases, chitinases and glucanases to target fungal cell walls. Presumably, chitin-binding effectors that protect hyphal cell walls against plant-derived chitinases similarly protect against mycoparasite-derived chitinases, which may explain abundant LysM effector catalogs of non-pathogenic fungi [41,42]. As LysM domains occur in peptidoglycan-binding proteins of various origins, LysM effector homologs that bind non-chitin substrates likely occur. Indeed, a plant pathogen LysM effector that binds bacterial cell walls was characterized (Kombrink and Thomma, unpublished data), potentially implicating this effector in bacterial competition or protection against bacterial mycoparasites. Genome analyses furthermore revealed that saprotrophic species encode abundant catalogs of small secreted proteins that resemble pathogen effector catalogs [42–45]. Although these potential effectors are poorly studied, one such effector, CipC, was implicated in competition with bacteria in *Aspergillus* spp. [45,46]. The genome of the ubiquitous saprophyte and opportunistic mammalian pathogen *A. fumigatus* encodes several effector proteins [47]. However, since the vast majority of fungi that cause disease in animals are soil saprophytes that opportunistically infect their hosts, to which they are not highly adapted, it has been speculated that infection does not rely on the activity of effectors [48]. Rather, their effectors are thought to be required for saprophytic survival [48]. Nevertheless, effectors that evolved to enable saprophytic survival may be co-opted for opportunistic infection as well.

Likely, competition between plant-associated microbes also occurs within hosts, although perhaps to a lesser extent than in soil due to reduced species diversity. Indeed, the second most abundantly *in planta*-expressed gene of the fungal endophyte *Epichloë festucae* encodes a secreted antifungal protein [49•]. Thus, effector homologs may play crucial roles in microbial competition in a broad spectrum of environments.

Do pathogens shape local microbiomes?

For various types of multicellular organisms it is increasingly recognized that their microbiome, i.e. the community of microbes that thrives in, on, or immediately near the organism, greatly influences its performance [50]. For plants, it has been particularly well documented that the rhizosphere microbiota affects plant growth and stress tolerance. In addition, the importance of the phyllosphere microbiota is increasingly recognized [51]. These microbiota comprise members that provide direct and indirect pathogen protection through antibiosis and induced immunity, respectively. Whereas soil types have a major impact on root inhabiting bacterial community compositions on Arabidopsis, host genotypes were reported to only have a minor impact [52,53]. In contrast, different Arabidopsis accessions were found to harbor different phyllosphere communities and several host genetic mutations were found to perturb the microbiota composition, demonstrating that host genetic factors shape the associated microbiota [54]. It is less clear, however, whether plants evolved to actively recruit phyllosphere communities. Potentially, plants recruit founder species that further shape local microbiomes through inter-microbe interactions [51]. Such interactions may require effectors. Considering that plant factors control the composition of the microbiota, microbiome members may utilize effectors to modulate hosts and control competitors indirectly. Additionally, manipulation of host metabolism could even establish microbial cooperation (Figure 2). Although not immediately addressing inter-microbial interactions, an insect-transmitted phytoplasma was recently shown to utilize an effector to alter floral development of host plants, converting them into vegetative tissues that attract leafhopper vectors [55]. This represents a striking example of the exploitation of effector activity to influence compositions of the local biome. Similarly, the rust fungus *Puccinia monoica* induces floral mimicry in the host *Boechera stricta* to enhance its reproduction and spore dispersal by insects [56].

Considering the importance of the microbiome for the ability of plants to withstand pathogen infection, it is

conceivable that pathogens evolved to affect host microbiomes, possibly through effector activities (Figure 2).

Different mechanisms drive evolution of effector repertoires

Mechanisms underlying genome plasticity and evolution have been intensely studied, especially for plant pathogens. As genomes are structured and not just a random sequence of genes, effector genes are often found in dynamic genomic compartments, such as gene-sparse regions, subtelomeric regions or conditionally dispensable (pathogenicity) chromosomes [57•]. For example, effector localization in gene-sparse regions was recorded for the endophyte *P. indica* [32], while in the saprophyte *N. crassa* genes encoding small secreted proteins are found in subtelomeric regions [43]. Genetic plasticity in such compartments is governed by diverse mechanisms including recombination and activity of transposable elements. A direct implication of genomic rearrangement in the evolution of fungal aggressiveness was shown for the vascular wilt fungus *Verticillium dahliae*, leading to the emergence of lineage-specific regions that are enriched for virulence effectors [58•]. High genetic variability in effector genes enables rapid evolutionary processes. The importance of dynamic genome compartments for accelerated gene evolution was underlined in the specialization of *P. infestans* after the host jump that separated this species from related species. Uneven evolutionary rates across the genome occur, with *in planta*-induced genes residing in fast-evolving compartments [59]. In turn, effector specialization can lead to diversification and speciation in pathogen lineages [13••]. In this manner, effectors can determine microbial niches. Moreover, composition of effector catalogs can dictate microbial lifestyles. For example, the leaf epiphyte and antagonist of powdery mildews *Pseudozyme flocculosa* lost its ability to parasitize plants like its smut fungi relatives due to loss of virulence effectors [60•]. However, the biocontrol agent has acquired other effectors that are not found in the smut relatives that may have shaped its current lifestyle [60•]. These findings suggest that effector catalogs evolve via different mechanisms and that their composition influences a microbe's lifestyle in a given environment.

An experimental way forward

The interaction between pathogenic (filamentous) microbes and the organisms they encounter in their niches, either while colonizing the host or during free-living stages in the environment, is poorly understood. An extensive characterization of the complex microbial communities in such niches may lead to a better understanding of the interactions that take place beyond the direct interaction between pathogen and host. Detailed transcriptome analyses may lead to the identification of particular triggers of effector gene expression derived from microbial co-inhabitants, and may hint toward functions in inter-microbial interactions [61,62] that can subsequently be tested for in targeted analysis to reveal components that either promote or inhibit other microbes [42].

Conclusions

Although a paradigm in plant pathology dictates that existence of disease requires the interaction of a virulent pathogen with a susceptible host in a favorable environment, plant–microbe interactions are mostly studied as one-on-one relationships. However, in addition to host immune responses, pathogenic microbes continuously encounter other microbes that include competitors and mycoparasites that need to be dealt with simultaneously. Importantly, findings for pathogenic microbes can be extrapolated to other types of symbioses as well. After all, irrespective of the type of symbiosis, the interest of the microbial partner is merely to exploit the host for nutrition and shelter. This may also explain the thin line that is regularly observed between the different types of symbioses [32,33,63,64]. In all types of symbioses, the microbial partner needs to suppress host immune responses and ward off microbial antagonists. Using effectors as probes, further critical processes in host colonization will be uncovered, leading to enhanced understanding of the biology of microbes that aim to establish symbioses.

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