

Review

Fungal effectors: past, present, and future

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Fungal effector proteins function at the interfaces of diverse interactions between fungi and their plant and animal hosts, facilitating interactions that are pathogenic or mutualistic. Recent advancements in protein structure prediction have significantly accelerated the identification and functional predictions of these rapidly evolving effector proteins. This development enables scientists to generate testable hypotheses for functional validation using experimental approaches. Research frontiers in effector biology include understanding pathways through which effector proteins are secreted or translocated into host cells, their roles in manipulating host microbiomes, and their contribution to interacting with host immunity. Comparative effector repertoires among different fungal–host interactions can highlight unique adaptations, providing insights for the development of novel antifungal therapies and biocontrol strategies.

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Introduction

Fungi are a vital component of our ecosystem, significantly impacting agricultural productivity and public health as symbionts and pathogens of both plant and animal hosts [1]. Climate change has intensified the pressure caused by fungal diseases due to the adaptability of many fungal pathogens and their resilience in changing environments [2]. Key players involved in host–fungal interactions are effectors, defined as secreted proteins or small molecules that interact with hosts and facilitate colonization [3]. Characterized effectors include proteins, RNAs, and other small molecules. For this review, we focus on effector proteins by summarizing current knowledge of fungal effectors, present tools, and diverse challenges. We will conclude by offering emerging perspectives on differentiating *bona fide* effectors involved in host–fungal interactions from enzymes used to support fungal growth and the importance of recognizing different effector profiles for distinct interactions with different hosts.

Fungal effectors and their involvement in diverse host–fungal interactions

Effector proteins that are involved in plant and mammalian pathogenic and mutualistic interactions can be further categorized to apoplastic effectors that function in the extracellular space and cytoplasmic effectors that translocate into host cells (Figure 1). In addition to interact with host immunity directly, effectors can also manipulate host microbiomes and influence the outcomes of host–fungal interactions.

Effector proteins in plant pathogenic fungi

The most abundant and highly conserved apoplastic effectors among plant pathogenic fungi are plant cell wall–degrading enzymes (CWDEs). Other well-characterized apoplastic effectors in fungi include LysM domain-containing proteins that contribute to fungal evasion of chitin-triggered plant immunity recognition by binding and masking the fungal cell wall chitin [4]. Other examples include alkalinizing peptides produced by the *Fusarium oxysporum* that interact with the plant receptor-like kinase FERONIA in the apoplastic space to promote infection [5] and SnTox3 from *Parastagonospora nodorum* that inhibits the PR1 C-terminal peptide to prevent PR1-mediated defense in *Triticum aestivum* [6].

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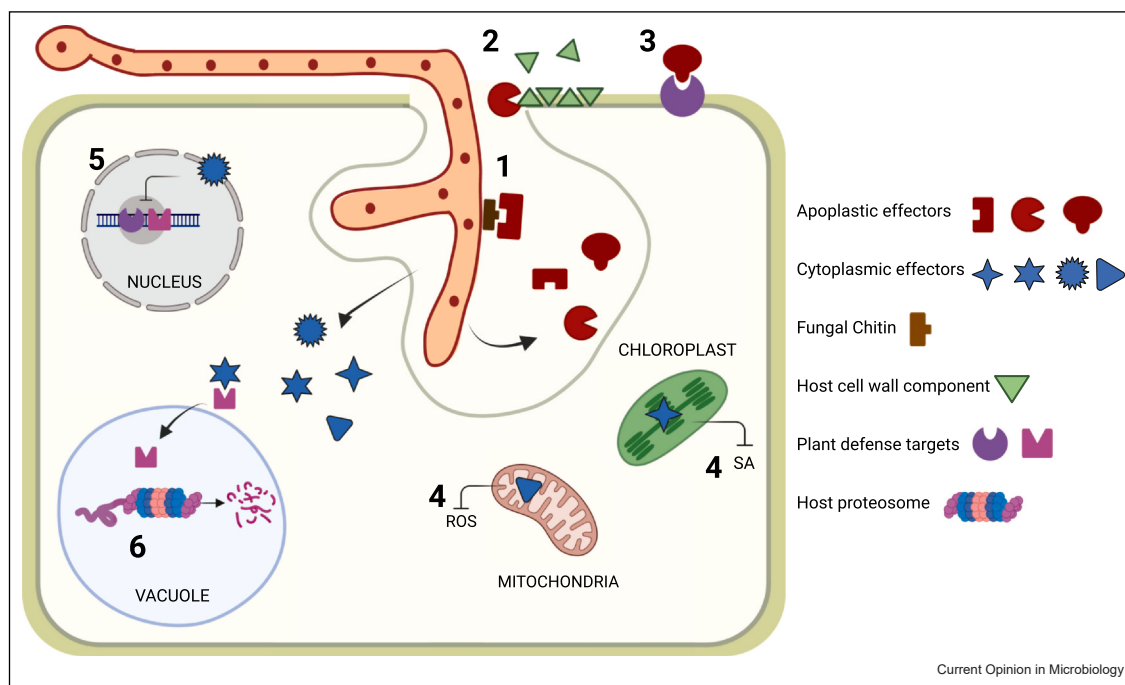
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Figure 1



Fungal conserved mechanisms of host colonization depicted in a plant cell. Depicted is a fungal hypha colonizing the apoplastic space of a plant cell, with examples of apoplastic and cytoplasmic secreted effectors and their host targets. Apoplastic effectors may function to (1) evade chitin-triggered plant immunity recognition, (2) degrade plant cell wall, or (3) bind to host proteins to change microenvironment or alter host defenses and promote colonization. Cytoplasmic effectors may localize to subcellular compartments to (4) perturb defense signaling pathways through mitochondria or chloroplasts, (5) reprogram transcription, or (6) target or mimic host proteasome machinery to regulate plant immune responses.

Functioning within the intracellular space of the host, cytoplasmic effectors commonly contribute to fungal pathogenesis by targeting plant organelles to manipulate diverse cellular processes [7]. For instance, MoHTR1 and MoHTR2 reported in *Magnaporthe oryzae* [8] and Nkd1 described in *Ustilago maydis* [9] can target host nuclei to reprogram host transcription. The host mitochondria and chloroplast, two central organelles in plant cells, are also common targets. The *M. oryzae* effector Avr-Pita suppresses host innate immunity by disrupting Reactive oxygen species (ROS) metabolism in mitochondria [10]. Similarly, the wheat stripe rust fungus *Puccinia striiformis* secretes the haustorium-specific effector (Pst_12806) that can be translocated into host cell and enter plant chloroplasts and interacts with host protein and promote disease [11]. Some effectors, such as Osp24 in *Fusarium graminearum*, can target the host proteasome, interfere with host protein homeostasis and evade host defenses [12].

It is well known that fungi coexist with diverse microbes. Recently, fungal effectors have been found to impact disease outcomes by influencing the composition of the phytobiome. Examples include *Verticillium dahliae* VdAve1, which exerts antimicrobial activity [13], and a Crinkler effector in *Phytophthora* spp. that suppresses

plant-associated actinobacteria [14], manipulating the host microbiota to facilitate host colonization and promote diseases.

Effector proteins in mutualistic plant–fungal interactions

Far less studied compared with those of pathogenic interactions, both apoplastic and cytoplasmic effectors also play essential roles in mutualistic interactions, including mycorrhiza and mutualistic endophytes.

Two well-studied types of mycorrhizal fungi include the arbuscular mycorrhizal fungi (AMF), which colonize plant roots intracellularly, and the ectomycorrhizal fungi (EMF), which maintain a symbiotic relationship extracellularly. Like pathogenic interactions, a LysM-containing apoplastic effector (RiSLM) was reported to bind to fungal chitin to evade plant chitin-triggered immune responses for the AMF symbiosis [15]. The first functionally characterized AMF (*Rhizophagus irregularis*) cytoplasmic effector is SP7, which interacts with a transcription factor ERF19 to attenuate plant immune responses [16]. AMF nucleus-localized Nuclear Localized Effector1 (RiNLE1) [17] and Crinkler effector1 (RiCRN1) [18] were reported to enhance fungal colonization and are essential for arbuscule

development. In EMF, the functionally characterized effector is the Mycorrhiza-induced Small Secreted Protein7 (MiSSP7) in *Laccaria bicolor*. By interacting with the host Jasmonic Acid (JA)-perception protein complex, this cytoplasmic effector alters the JA-signaling network within host nuclei and promotes the symbiotic interaction [19].

Like mycorrhizal fungi, endophytic fungi produce effectors to establish endophytic interactions. For example, the *Epichloë festucae* effector Efe-AfpA was identified as a key player in the mutualistic interaction [20]. The expression of numerous extracellular proteins from a *Trichoderma guizhouense* endophytic strain NJAU strain was upregulated after inoculating cucumber plants, including CWDEs, expansion-like proteins, and peroxidases [21]. A comprehensive comparison of 44 endophytic versus diverse pathogenic *Fusarium oxysporum* strains identified 66 candidate endophytic-encoding effectors [22].

As reported in pathogenic fungi, effectors from an endophyte *Serendipita vermifera* provide interkingdom synergistic beneficial effects by suppressing plant defense and interacting with root-colonizing microbiota through antimicrobial activities [23].

Effector proteins in human pathogenic fungi

Most effectors reported so far are apoplastic, but both apoplastic and cytoplasmic effectors are involved in fungal–human interactions.

Like phytopathogenic fungi, human pathogenic fungi also employ LysM-domain-containing effectors to bind to fungal chitin and avoid host recognition. For instance, LysM1 and LysM2 — two LysM-domain-containing effectors reported in the dermatophyte fungus *Trichophyton rubrum* — can bind to fungal chitin to evade host immunity [24]. Lacking plant CWDEs, human pathogenic fungi often secrete proteases to facilitate host colonization. For instance, the secretion of metalloprotease and cysteine proteases from the opportunistic fungal pathogen *Aspergillus fumigatus* was involved in altering the human airway respiratory epithelial cells and inducing host proinflammatory responses [25]. Another commonly used mechanism between plant and human fungal pathogens is to produce effectors that interact with host receptors. Reported in the human pathogen *Cryptococcus neoformans*, the effector CPL1 interacts with Toll-like receptor4, a key mammalian innate immunity activator, to enhance host macrophage polarization and promote fungal infection [26]. The other interesting observation is that the *A. fumigatus* surface-exposed effector HscA interacts directly with p11, a host calcium-binding EF-hand protein, to redirect fungal-containing phagosomes to a nondegradative pathway, avoiding its phagolysosomal killing [27]. So far, the only cytoplasmic

effector reported among human fungal pathogens is the *Histoplasma capsulatum* calcium-binding protein 1, which forms an effector complex within the cytosol and drives macrophage lysis [28].

Interestingly, as part of the intestinal microbiome, some commensal fungal species also use fungal effectors. For instance, candidalysin, a secreted peptide processed from a secreted protein Ece1, can manipulate the composition of intestinal bacterial and fungal communities and promote the establishment of the commensal colonization of *Candida albican* [29].

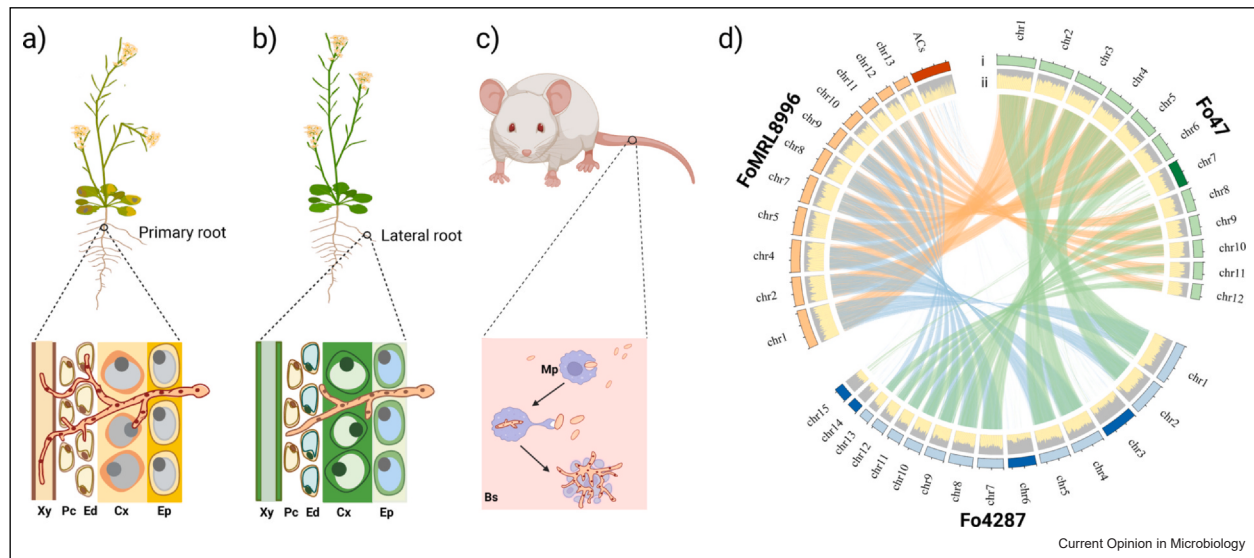
Effector delivery

Another interesting topic in effector biology is the delivery mechanisms through which effector proteins are secreted or translocated into host cells. The discovery of bacterial effector secretion systems, first reported in the late 1990s and now comprising over 12 reported systems [30], foreshadows the potential complexity of fungal effector delivery. An extracellular protein complex related to fungal virulence was identified in *U. maydis* [31]. While apoplastic effectors are secreted through the conventional ER-Golgi secretion pathway, the translocation of cytoplasmic effectors seems to use a different delivery system. Some cytoplasmic effectors have a codon-usage bias, translating -AA over -AG codons via the 2-thiolation of the wobble uridine on transfer RNA anticodon; this codon bias could create ribosome pausing and consequentially sort cytoplasmic effectors into unconventional secretory pathways [32]. It has been documented that cytoplasmic effectors PWL2 from *M. oryzae* [33] and RXLRs *Phytophthora infestans* [34] are packaged into vesicle-like compartments and translocated into host cells by exploiting host clathrin-mediated endocytosis.

Tools for studying fungal effectors

- 1) *Identification*: The identification of effectors and the prediction of their localization is improving with the ongoing refinement of machine learning models trained on experimentally validated apoplastic and cytoplasmic effectors [3]. Functional prediction can be further implemented for sequence unrelated but structurally similar effectors based on shared protein structural folds [35**,36]. Recognizing novel effectors based on their rapid evolution using host and pathogen interaction networks is another powerful approach [37]. The identification of effector pairs that are clustered at the same genomic region, such as SIX8-PSE1 in *F. oxysporum* [38] and the AvrLm10A-AvrLm10B in *Leptosphaeria maculans* [39], illuminates an approach in identifying ‘cooperating proteins’.
- 2) *Functional importance*: Understanding the biological function of an effector requires experimental validation. Reverse genetics tools, such as RNAi silencing and CRISPR knockout, are widely used to test the direct involvement of an effector in a specific

Figure 2



Host–fungal interactions are illustrated using *Fusarium oxysporum* species complex. A cross-kingdom fungal pathogen, members within the *F. oxysporum* species complex include (a) plant pathogens that cause vascular wilt diseases in many economically important plants, as illustrated using the model plant *Arabidopsis thaliana*, (b) the endophytic strains that provide protective advantages to host plants and promote plant growth, and (c) human pathogens that repress mammalian immunity and cause systematic infections. (d) Whole genome comparison among three *F. oxysporum* genomes that represent a plant pathogen (Fo4287 in blue), an endophytic strain (Fo47 in green) and a human pathogen (FoMRL8996 in orange). All three genomes share 11 conserved core chromosomes, while each carries unique set of accessory chromosomes highlighted in darker shades in each genome (i) with low gene density (ii) that contributed to the unique host-specific interactions. The inner circle indicates syntenic alignments using Nucmer. (Xy: Xylem vessel, Pc: Pericycle, Ed: Endodermis, Cx: Cortex, Ep: Epidermis, Mp: Macrophage, Bs: Bloodstream). (a) and (b) were adopted from Martínez-Soto D et al <https://doi.org/2023.10.1094/MPMI-08-22-0166-SC> and (c) was based on <https://doi.org/10.1371/journal.pone.0101999>.

interaction. At the same time, heterologous expression systems that transiently express effectors in non-native hosts have also been established as versatile tools, especially when genetic engineering strategies are not viable or when a rapid and systematic screen of interactions between effector proteins and host is desirable. To study effectors involved in plant–fungal interactions, *Agrobacterium tumefaciens*–mediated heterologous expression in *Nicotiana* spp. is commonly used. The induction or suppression of characteristic plant immune responses indicate the potential involvement of the candidate effector in the host–fungi interaction, and the presence or absence of the signal peptide can purposely direct the effectors into either apoplastic or cytoplasmic space, respectively [40]. In addition, fungal effectors can be delivered into host cells through a bacterial type III secretion system, as illustrated by the co-expression of effectors in the *E. coli* SHuffle strain with enhanced ability to express cysteine-rich, disulfide-bonded proteins [41].

- 3) *Interactive partners*: Many effectors reprogram host processes by interacting with host proteins, and there are increasing tools to identify effector targets. Split-reporter protein constructs, including GFP, RFP, and tdTomato, are utilized to confirm the subcellular localization of cytoplasmic effectors [42]. Yeast two

hybridization and co-immunoprecipitation followed by liquid chromatography–mass spectrometry are proven tools to identify effector–partner complexes [43]. The turbo biotin ligase tag (TurboID) enables *in vivo* proximity labeling and co-immunoprecipitation [44]. Using crystallization, the structure of effector–receptor complexes can be resolved, as demonstrated by the AVR–Pii–OsExo70F2 complex in *M. oryzae* [45].

Challenges and potential solutions

Identifying bona fide effector proteins

The heterotrophic and absorptive lifestyle of fungi creates a heavy dependency on secreted enzymes to obtain nutrients through their environments by depolymerizing complex natural products. To identify *bona fide* effectors involved in host–fungal interactions, it is crucial to separate effectors from enzymes used to support fungal physiology. A few approaches, such as using expression to filter candidate effectors directly involved in the interaction and identifying proteins from apoplastic spaces [46] and extracellular vesicles [47], are adopted to circumvent this challenge. Moreover, a comprehensive pan-genome analysis that defines the ancestral state of each effector will add an evolutionary perspective to this puzzle.

Uncovering underground fungal–plant interactions

Functional characterization of effectors among mycorrhizal fungi and soil-borne pathogens lags foliar pathosystems due to an inherent difficulty in observing underground interactions. In addition to testing heterologous expression systems, particularly with the co-expression of effector and plant partners [48], several hairy root transgene expression systems were established to study mycorrhizal symbiosis and soil-borne pathogens [49].

Differentiating effector profiles for different interactions

There are notable differences in effector profiles between beneficial and pathogenic fungi. For example, EMFs lost most cellulose-degrading enzymes, and AMFs contain few plant cell wall modification enzymes [16]. Endophytic fungi often contain plant CWDEs [50], but in much smaller numbers than pathogenic fungi. A cross-kingdom fungal pathogen, the *F. oxysporum* species complex includes plant pathogens causing devastating vascular wilt diseases, endophytes used as biocontrol agents and plant-fitness promoters, as well as human pathogens responsible for disseminated fusariosis and blinding corneal infections in humans. With a conserved core among these species, their accessory chromosomes helped define distinct functions, making them an excellent model to establish a good understanding among these different interactions (Figure 2).

Conclusion

Fungal effector biology will continue to be an important topic for understanding diverse fungal–host interactions that contribute to the health of our ecosystem. Such knowledge will have practical implications, such as effector-mediated resistance breeding. The interplay between fungal effectors and the host microbiomes will guide the potential design of healthy phytobiomes to combat plant diseases or potential supplements to control human diseases. Still, many questions must be explored, including how are effectors co-ordinated to facilitate host colonization and infection? Are effectors from different fungal species or evolutionarily distant micro-organisms antagonistic or synergistic during co-colonization of the same host? What is the significance of effectors conserved in plant pathogenic fungi and human pathogenic fungi? These questions drive scientists to producing innovative and high-quality research. Bringing fungal effector biology from the laboratory to the forefront of applied health solutions drives advancement in basic and translational discoveries.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Cytoplasmic effectors of *Magnaporthe oryzae* are secreted into the biotrophic interfacial complex (BIC) and were found to be translocated across plant cytoplasm via clathrin-mediated endocytosis (CME). Using live cell imaging in rice cells, the authors observed fluorescently labeled cytoplasmic effectors congregating in a puncta, or membranous effector compartment, and demonstrated the reliance of these structures and cytoplasmic effector localization in the plant endocytosis pathway. Clathrin-mediated and clathrin-independent endocytosis mechanisms were examined using transgenic rice lines and viral-induced gene silencing, and CME was found to play a significant role in effector translocation from the BIC, as well as appressorial penetration. This study is an exciting new breakthrough in our understanding of how a fungal pathogen co-opts plant function and facilitates infection during the early stages.
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The evolution of effector proteins can be difficult to study as many of them lack sequence similarity with other effectors. This study approaches this setback by looking at the protein structure using AlphaFold2, specifically focusing on sequence-unrelated structurally similar (SUSS) effectors and common folds that are conserved across the fungal species. Comparing the secretome and effectome composition and clustering of 20 different fungal species, and one oomycete, they proposed that the effectors have undergone divergent evolution and have lost sequence similarities, making this hard to discover using tools such as BLAST. This paper is an exciting approach into what is the beginning of further understanding these effectors.

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