A Catalogue of the Effector Secretome of Plant Pathogenic Oomycetes

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Abstract

The oomycetes form a phylogenetically distinct group of eukaryotic microorganisms that includes some of the most notorious pathogens of plants. Oomycetes accomplish parasitic colonization of plants by modulating host cell defenses through an array of disease effector proteins. The biology of effectors is poorly understood but tremendous progress has been made in recent years. This review classifies and catalogues the effector secretome of oomycetes. Two classes of effectors target distinct sites in the host plant: Apoplastic effectors are secreted into the plant extracellular space, and cytoplasmic effectors are translocated inside the plant cell, where they target different subcellular compartments. Considering that five species are undergoing genome sequencing and annotation, we are rapidly moving toward genome-wide catalogues of oomycete effectors. Already, it is evident that the effector secretome of pathogenic oomycetes is more complex than expected, with perhaps several hundred proteins dedicated to manipulating host cell structure and function.

INTRODUCTION

The oomycetes form a phylogenetically distinct group of eukaryotic microorganisms that includes some of the most notorious pathogens of plants (41). Among these, members of the genus *Phytophthora* cause enormous economic losses on crop species as well as environmental damage in natural ecosystems (25). Some *Phytophthora* species, such as the potato and tomato late blight agent *Phytophthora infestans* (11, 45, 90), and the soybean root and stem rot agent *Phytophthora sojae* (85), have caused longstanding problems for agriculture, whereas others, such as the sudden oak death pathogen *Phytophthora ramorum*, have surfaced in recent epidemics (80). Other significant oomycetes include the downy mildews, a heterogeneous and diverse group of obligate parasites (3). Some downy mildews infect economically important hosts such as grapevines (35). *Hyaloperonospora parasitica* is a natural pathogen of *Arabidopsis thaliana* and figures prominently in research on disease resistance in this model plant (89).

Phytophthora and downy mildews establish intimate associations with plants and typically require living host cells to complete their infection cycle, a process known as biotrophy (31, 68). An emerging view on oomycete pathogenesis is that the oomycetes accomplish parasitic colonization of plants by reprogramming the defense circuitry of host cells through an array of disease effector proteins (38). Two classes of effectors target distinct sites in the host plant: apoplastic effectors are secreted into the plant extracellular space, where they interact with extracellular targets and surface receptors; and cytoplasmic effectors are translocated inside the plant cell presumably through specialized structures like infection vesicles and haustoria that invaginate inside living host cells (**Figure 1**).

This review attempts to catalogue the effector secretome of oomycetes. Because of our fragmentary knowledge of the mechanisms of pathogenicity of oomycetes, we retain a flexible definition of the term "effectors" that is consistent with previous publications (38, 41, 99). Effectors are defined as molecules that manipulate host cell structure and function, thereby facilitating infection (virulence factors or toxins) and/or triggering defense responses (avirulence factors or elicitors). This dual (and conflicting) activity of effectors has been broadly reported in plant-microbial pathosystems, such as bacterial and fungal diseases (4, 49, 51, 103). The majority of the oomycete effectors described here, including the avirulence (AVR) proteins, are so far known only by their ability to activate defense responses and innate immunity. An underlying assumption of this review is that these AVR effectors and some of the other defense elicitors have virulence functions of unknown nature. Indeed, one crucial question in the study of oomycete pathogenicity is how effectors disrupt the activation and execution of plant defenses. Future functional studies will undoubtedly reveal a complex array of virulence functions for oomycete effectors.

Effectors: pathogen molecules that manipulate host cell structure and function thereby facilitating infection and/or triggering defense responses. Effectors can be elicitors and/or toxins. Unlike these, the term effector is neutral and does not imply a negative or positive impact on the outcome of the disease interaction

Haustoria: specialized infection structures of biotrophic oomycete and fungal pathogens that invaginate into the plant cells but remain enveloped by a modified host cell membrane.

AVR: avirulence

Toxins: pathogen molecules that cause plant cell death thereby facilitating colonization by necrotrophic pathogens

Elicitors: pathogen molecules that trigger defense responses resulting in enhanced resistance to the invading pathogen

IDENTIFICATION OF OOMYCETE EFFECTORS

Biochemical, genetic, and bioinformatic strategies, often in combinations, have been applied to the identification of effector genes from oomycetes. Traditionally, effectors have been identified by biochemical purification and genetic analyses. With the advent of genomics, novel strategies have emerged. The implementation of functional genomics pipelines to identify effectors from sequence data sets has proved particularly successful. A typical pipeline consists of two major steps: use of data mining tools to identify candidate genes that fulfill a list of specific criteria, followed by analysis and validation of these candidate genes by functional assays, such as expression in planta and evaluation for effector-like activities. In **Figure 2**, we illustrate two examples of functional genomics

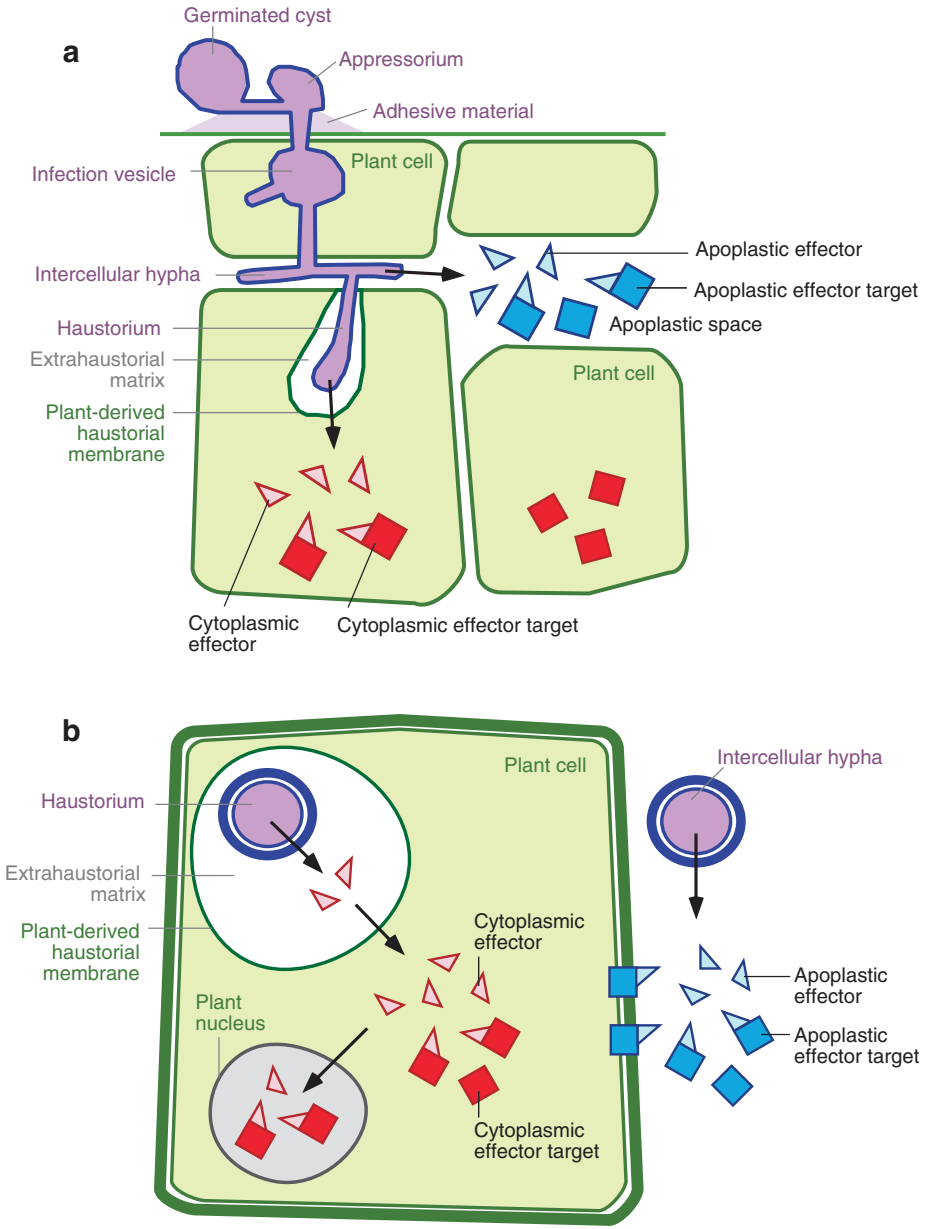

Figure 1

Plant pathogenic oomycetes secrete apoplastic effectors into the plant extracellular space and cytoplasmic effectors inside the plant cell. (*a*) Schematic view of the early stages of infection by *Phytophthora infestans* of a host plant illustrating the sites of action of the apoplastic and cytoplasmic effectors. (*b*) Cross-section of the view in panel A illustrating the delivery of apoplastic and cytoplasmic effectors to their cellular target. One class of cytoplasmic effectors is known to target the plant nucleus (T. D. Kanneganti, J. Win & S. Kamoun, unpublished). Apoplastic effectors interact with extracellular targets or surface receptors. Pathogen structures are shown in *purple* and plant structures in *green*. Apoplastic effectors and effector targets are in *blue*, whereas cytoplasmic effectors and their targets are in *red*. Note that for clarity the figure components were not drawn to scale relative to each other.

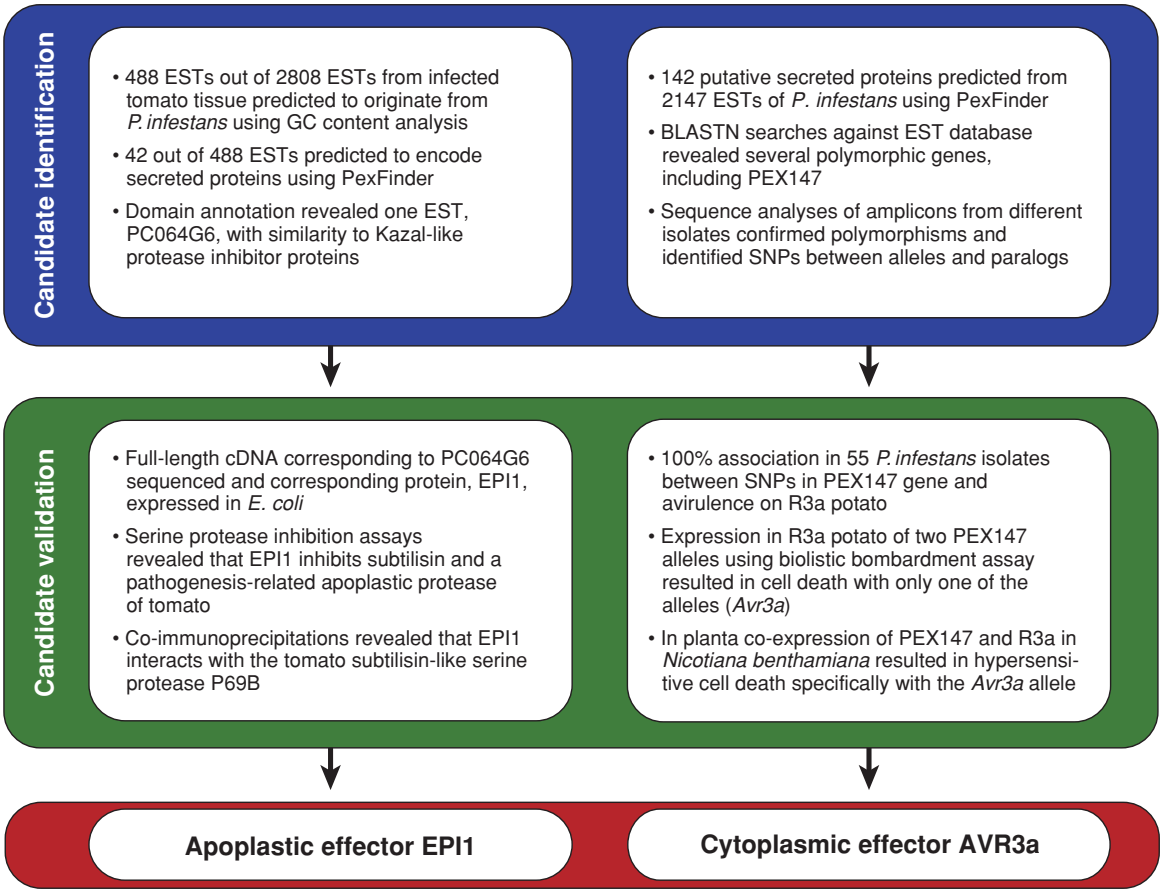

Figure 2

Examples of high-throughput functional genomics pipelines for the identification of effector genes. The left panels illustrate the strategy of Tian et al. (96) for the discovery of the apoplastic effector EPI1. The right panels depict the strategy of Bos et al. (16) as implemented by Armstrong et al. (6) for the cloning of *Avr3a* of *Phytophthora infestans*.

Signal peptides: amino acid sequences that direct the posttranslational targeting of proteins to the general secretory pathway

pipelines that led to the discovery in *P. infestans* of the protease inhibitor EPI1 (96) and the avirulence protein AVR3a (6).

A critical milestone in the identification of oomycete effector genes was the validation of the concept that effector proteins must be secreted in order to reach their cellular targets at the intercellular interface between the plant and pathogen or inside the host cell (99). Identification of candidate secreted proteins was facilitated by the fact that in oomycetes as in other eukaryotes, most secreted proteins are exported through the general secretory pathway via short, N-terminal, amino-acid sequences known as signal peptides (99). Signal peptides are highly degenerate and cannot be identified using DNA hybridization or PCR-based techniques. Nonetheless, computational tools, particularly the SignalP program that was developed using machine learning methods (62), can assign signal peptide prediction scores and cleavage sites to unknown amino acid sequences with a high degree of accuracy (57, 86). Therefore, with the accumulation of oomycete cDNA and genome sequences, lists of candidate secreted

proteins could be readily generated using bioinformatics tools. For instance, Torto et al. (99) developed PexFinder (with Pex standing for *Phytophthora* extracellular protein), an algorithm based on SignalP v2.0 (62) to identify proteins containing putative signal peptides from expressed sequence tags (ESTs). PexFinder was then applied to ESTs in *P. infestans*. Proteomic identification of secreted proteins collected from culture filtrates of *P. infestans* matched PexFinder predictions, convincingly validating the algorithm performance (99). Similar high degrees of accuracy of SignalP were also reported (57, 86).

Computational signal peptide predictions help to identify secreted proteins with no significant matches to known sequences. Annotation of cDNA and gene sequences is routinely done by similarity searches to sequences in public databases. However, this approach is limited in that it identifies only proteins with known functions and thus a large number of proteins with unknown functions are ignored. Computational predictions single out unknown secreted proteins that might otherwise be ignored. This results in a manageable list of candidates that can be further processed by sequence annotation and functional analyses.

Building up an annotated collection of computationally predicted candidate proteins that feed into a functional analysis pipeline is by no means a one-directional process. Rather, criteria for annotating and prioritizing candidate genes are continuously refined as new findings emerge and novel sequence-to-function links are established. One illustrative example is the discovery that all known avirulence proteins of oomycetes carry a conserved motif (RXLR) closely following a signal peptide (see below for more details) (78). Straightforward sequence pattern search tools can thus be used to discover an additional number of candidate effectors of the RXLR family from several *Phytophthora* species.

Identification of genes under diversifying selection (especially when coupled with selection criteria such as presence of secretory signals and in planta expression) can also help to select candidate effector genes (53). Based on the "arms race" model, adaptation and counteradaptation between pathogen and host is likely to drive their antagonistic coevolution and generate the evolutionary forces that shape effectors and their host targets (24, 92). For instance, diversifying selection (also known as positive selection) can be an indicator of functionally important loci that contribute to the fitness of the organism. Indeed, some oomycete effector genes, e.g., *ATR13* and *scr74*, exhibit extreme levels of amino acid polymorphism with evidence of diversifying selection (5, 53). In the future, evolutionary analyses will have an even larger role in the identification of effector genes as genome sequences from additional oomycete species become available.

Table 1 lists major effectors of oomycetes and the corresponding strategies that led to their identification. These effectors are described in more detail in the remainder of this review.

Pex: *Phytophthora* extracellular protein

Diversifying selection: (a.k.a. positive selection), natural selection that favors the fixation of advantageous mutations resulting in adaptive molecular evolution

PR: pathogenesis-related

GIP: glucanase inhibitor protein

APOPLASTIC EFFECTORS

Enzyme Inhibitors

Several plant pathogenesis-related (PR) proteins, such as glucanases, chitinases, and proteases, are hydrolytic enzymes. Fungal and bacterial plant pathogens have evolved diverse mechanisms for protection against the activities of these PR proteins (2, 75). Similar to these pathogens, plant pathogenic oomycetes, such as *Phytophthora*, have also evolved mechanisms to escape the enzymatic activity of PR proteins. Oomycetes contain little chitin in their cell wall and are therefore resistant to plant chitinases (41). *Phytophthora* also evolved active counterdefense mechanisms by secreting inhibitory proteins that target host glucanases and proteases.

Glucanase inhibitors GIP1 and GIP2. The glucanase inhibitors GIP1 and GIP2 are secreted proteins of *P. sojae* that inhibit

Table 1 Disease effectors of plant pathogenic oomycetes

Effector	Species	Identification strategy	Reference
Apoplastic effectors			
Enzyme inhibitors			
Glucanase inhibitors GIP1 and GIP2	*P. sojae*	Biochemical purification	(81)
Serine protease inhibitors EPI1 and EPI10	*P. infestans*	Bioinformatic prediction of secreted and in planta induced proteins coupled with protein domain annotation and followed by biochemical analyses	(65, 66)
Cysteine protease inhibitors EPIC1 and EPIC2	*P. infestans*	Bioinformatic prediction of secreted and in planta induced proteins coupled with protein domain annotation	(M. Tian & S. Kamoun, unpublished)
Small cysteine-rich proteins			
INF1 elicitin	*P. infestans*	Biochemical purification	(79, 46)
INF2A and INF2B elicitins	*P. infestans*	Sequence similarity to INF1	(44)
PcF	*P. cactorum*	Biochemical purification	(65)
PcF-like SCR74 and SCR91	*P. infestans*	Bioinformatic prediction of secreted and in planta induced proteins coupled with analyses of polymorphic diversity	(16, 53)
Ppat12, 14, 23, and 24	*H. parasitica*	Suppression subtractive hybridization	(13)
Nep1-like (NLP) family			
PaNie	*Pythium aphanidermatum*	Biochemical purification	(105)
NPP1	*P. parasitica*	Biochemical purification	(27)
PsojNIP	*P. sojae*	Bioinformatic prediction of secreted proteins coupled with in planta expression	(76)
PiNPP1	*P. infestans*	Bioinformatic prediction of secreted proteins coupled with in planta expression	(T. D. Kannegenati & S. Kamoun, unpublished)
GP42 (PEP13) Transglutaminase	*P. sojae*	Biochemical purification	(63, 83)
CBEL	*P. parasitica*	Biochemical purification	(87, 106)
Cytoplasmic effectors			
RXLR protein family			
$ATR1^{NdWsB}$	*H. parasitica*	Positional cloning	(78)
ATR13	*H. parasitica*	Suppression subtractive hybridization combined with positional cloning	(5)
Avr1b-1	*P. sojae*	Positional cloning and candidate gene approach	(88)
AVR3a	*P. infestans*	Bioinformatic prediction of secreted and polymorphic proteins coupled with association genetics	(6, 16)
CRN protein family (CRN1 and CRN2)	*P. infestans*	Bioinformatic prediction of secreted proteins coupled with in planta expression	(99)

the soybean endo-β-1,3 glucanase EGaseA (81). These inhibitor proteins share significant structural similarity with the trypsin class of serine proteases, but bear mutated catalytic residues and are proteolytically nonfunctional. GIPs are thought to function as counterdefensive molecules that inhibit the degradation of β-1,3/1,6 glucans in the pathogen cell wall and/or the release of defense-eliciting oligosaccharides by host β-1,3 endoglucanases. There is some degree of specificity in inhibition because GIP1 does not inhibit another soybean endoglucanase, EGaseB. Positive selection has acted on β-1,3 endoglucanases in the plant legume genus *Glycine* and may have been driven by coevolution with glucanase inhibitors in *P. sojae* (12). Four genes with similarity to GIPs have been identified in *P. infestans*, and their ability to inhibit tomato endoglucanases is under investigation (23).

Serine protease inhibitors EPI1 and EPI10. EPI1 and EPI10 are multidomain secreted serine protease inhibitors of the Kazal family (MEROPS family I1) (77) that are thought to function in counterdefense (95–97) (**Figure 3*a***). They inhibit and interact with the PR protein P69B, a subtilisin-like serine protease of tomato that is thought to function in defense (95, 96). The *epi1*, *epi10*, and *P69B* genes are concurrently expressed and up-regulated during infection of tomato by *P. infestans*. The mechanism by which inhibition of P69B by EPI1 and EPI10 may affect the late blight disease is not yet elucidated. Recent findings indicate that EPI1 protects several secreted proteins in *P. infestans* from degradation by P69B thereby directly contributing to virulence (M. Tian & S. Kamoun, manuscript submitted).

Inhibition of host proteases by Kazal-like proteins could be a common virulence strategy between plant and mammalian parasites (96). Kazal-like proteins have also been implicated in virulence of the apicomplexans *Toxoplasma gondii* and *Neospora caninum*, a group of mammalian parasites that transit through the digestive tract (22, 52, 60, 74). Four putative proteins with Kazal-like domains were also identified in the genome sequence of another apicomplexan animal parasite, *Cryptosporidium parvum* (1). In oomycetes, secreted Kazal-like proteins form a diverse family of at least 35 members from five plant pathogenic oomycetes including the downy mildew *Plasmopara halstedii* (96). Future biochemical studies of these proteins will address the extent to which inhibition of host proteases is a widespread virulence function in oomycetes.

Cysteine protease inhibitors EPIC1 and EPIC2. The cystatin-like cysteine protease inhibitors EPIC1 and EPIC2 (InterPro IPR000010, MEROPS family I25) (77) form another class of secreted protease inhibitors of *P. infestans* (M. Tian & S. Kamoun, submitted). Recent findings suggest that these two inhibitors target an apoplastic papain-like cysteine protease of tomato (M. Tian & S. Kamoun, submitted). This suggests multifaceted inhibitions of tomato proteases by secreted effectors of *P. infestans*.

Small Cysteine-Rich Proteins

Many eukaryotic *Avr* genes, such as *Cladosporium fulvum Avr2*, *Avr4*, and *Avr9*, *Rhynchosporium secalis nip1*, and *Phytophthora* elicitins, encode small (<150 amino acids) secreted proteins with an even number of cysteine residues, which can induce defense responses when infiltrated into plant tissues (51, 103). Several of these common structural features, most notably secretion and the disulfide bridges formed by the pairs of cysteines, are essential for defense induction and avirulence function (40, 42, 50, 51, 54, 103). The disulfide bridges could enhance stability in the plant apoplast, which is rich in degradative proteases (40, 54).

A number of oomycete effectors are small cysteine-rich proteins. Although quite divergent in primary sequence, in this review these proteins are grouped as a distinct class of oomycete effectors.

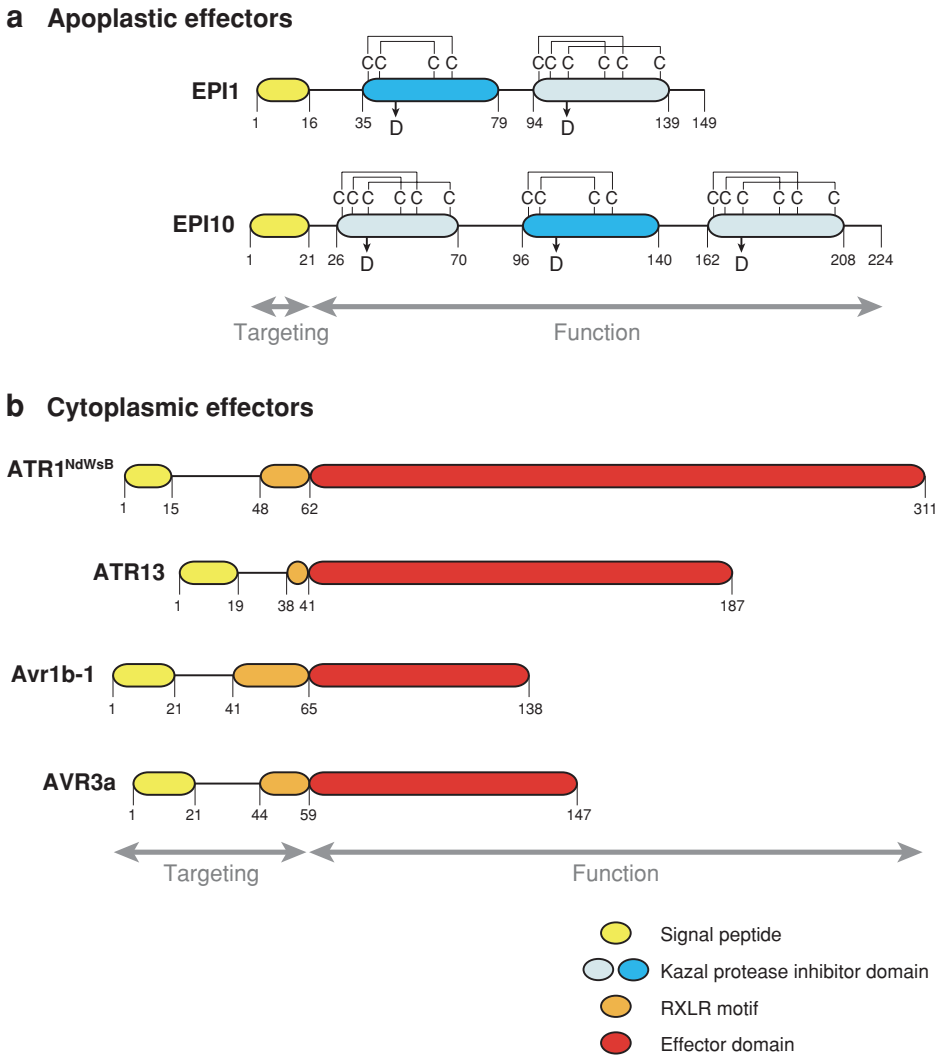

Figure 3

Domain organization of apoplastic and cytoplasmic effectors. (*a*) Schematic drawings of the apoplastic effectors EPI1 and EPI10 of *Phytophthora infestans* (95–96). The two-disulfide bridge Kazal domains of EPI1 and EPI10 that target the tomato protease P69B (95, 97) are shown in *dark blue*. P1 residues that are critical for the inhibitor specificity are marked by an arrow. (*b*) Schematic drawings of the cytoplasmic RXLR effectors ATR1^{NdWsB} and ATR13 of *Hyaloperonsopora parasitica* (5, 78), Avr1b-1 of *Phytophthora sojae* (88), and AVR3a of *P. infestans* (6). In both panels, the numbers under the sequences indicate amino-acid positions. The *gray arrows* distinguish the regions of the effector proteins that are involved in secretion and targeting from those involved in effector activity.

Elicitins: INF1 and others. Elicitins are a family of structurally related extracellular proteins that induce hypersensitive cell death and other biochemical changes associated with defense responses in *Nicotiana* spp. (46, 48, 73, 79, 84). Strains of *P. infestans* deficient in the elicitin INF1 cause disease lesions in *Nicotiana benthamiana*, suggesting that INF1 conditions avirulence in this species (47). In *P. infestans*, a complex set of elicitin-like genes was

identified using PCR amplification with degenerate primers, low-stringency hybridizations, and sequencing of cDNAs and BAC clones (26, 43, 44, 46). All elicitin genes encode putative extracellular proteins that share a 98-amino-acid elicitin domain with a core of 6 conserved cysteines (InterPro IPR002200). Some *inf* genes (*inf2A*, *inf2B*, *inf5*, *inf6*, *inf7*, and *M-25*) encode predicted proteins with a C-terminal domain in addition to the N-terminal elicitin domain. Sequence analysis of these C-terminal domains revealed a high frequency of serine, threonine, alanine, and proline. The amino-acid composition and the distribution of these four residues suggest the presence of clusters of *O*-linked glycosylation sites (44). These proteins may form a "lollipop on a stick" structure in which the O-glycosylated domain forms an extended rod that anchors the protein to the cell wall leaving the extracellular N-terminal domain exposed on the cell surface (39). Therefore, these atypical INF proteins may be surface- or cell wall–associated glycoproteins that could interact with plant cells during infection.

The intrinsic biological function of elicitins in *Phytophthora* has long been subject to investigation. Conclusive evidence finally emerged when it was demonstrated that class I elicitins can bind sterols, such as ergosterol, and function as sterol-carrier proteins (15, 58, 59, 104). Consequently, elicitins were hypothesized as having a biological function of essential importance to *Phytophthora* spp. because they cannot synthesize sterols and must assimilate them from external sources (34). In addition, phospholipase activity was assigned to elicitin-like proteins from *Phytophthora capsici* with significant similarity to INF5 and INF6 (61), suggesting a general lipid binding/processing role for the various members of the elicitin family (66). Other work by Osman et al. (67) using elicitin mutants altered in sterol binding suggests that sterol loading is important for specific binding to a plasma membrane receptor and induction of the hypersensitive response (HR) in tobacco. However, the exact relationship between sterol binding and cell death induction remains to be clarified. More recently, another gene with similarity to elicitins, *M-25*, has been reported to be induced during mating in *P. infestans* (26).

PcF. PcF is a secreted 52-amino-acid peptide of *Phytophthora cactorum* that contains 6 cysteines and a 4-hydroxyproline at residue 49 (65). PcF triggers necrosis in tomato and strawberry, an economically important host for *P. cactorum*. Recombinant expression of PcF in *Pichia pastoris* resulted in a functional protein that induces necrosis and elicits activity of the defense enzyme phenylalanine ammonia lyase (64). PcF was proposed to function as a toxin considering that it triggers responses on host plants that are similar to disease symptoms (65). However, its exact mode of action is unclear.

PcF-like SCR74 and SCR91. Two classes of PcF-like genes, *scr74* (53) and *scr91* (16), are known in *P. infestans*. The expression of *scr74* is up-regulated approximately 60-fold two to four days after inoculation of tomato and is also significantly induced during early stages of colonization of potato (53). *scr74* belongs to a highly polymorphic gene family within *P. infestans* with 21 different sequences identified. Using the approximate and maximum likelihood (ML) methods, Liu et al. (53) showed that diversifying selection likely caused the extensive polymorphism observed within the *scr74* gene family. These results led to an evolutionary model that involves gene duplication and recombination, followed by functional divergence of *scr74* genes in *P. infestans*. Based on the expression pattern of *scr74* and its similarity to a necrosis inducing protein, the selective forces that shaped the evolution of this gene could well be related to host-pathogen coevolution (53).

PPAT12, 14, 23, and 24. Bittner-Eddy et al. (13) used suppression subtractive hybridization to identify genes from the obligate pathogen *Hyaloperonospora parasitica* that are

highly expressed during infection of *Arabidopsis thaliana*. Of the 25 genes identified, four, *Ppat12*, *14*, *23*, and *24*, encode cysteine-rich peptides ranging in size from 70 to 127 amino acids. All four peptides carry predicted signal peptides which suggests that they are likely to be secreted. They share no sequence similarity to other known cysteine-rich proteins and their role in virulence remains unknown.

NEP1-Like Family (PaNie, NPP1, PsojNIP, PiNPP1)

Nep1-like proteins (NLPs) are circa 25-kDa proteins that are widely distributed in bacteria, fungi, and oomycetes, particularly in plant-associated species (70). The canonical 24-kDa necrosis- and ethylene-inducing protein (Nep1) was originally purified from culture filtrates of the fungus *Fusarium oxysporum* f. sp. *erythroxyli* (7). NLPs have subsequently been described in species as diverse as *Bacillus* (94), *Erwinia* (9, 71), *Verticillium* (107), *Pythium* (105), and *Phytophthora* (27, 76). Despite their diverse phylogenetic distribution, NLPs share a high degree of sequence similarity and several members of the family have the remarkable ability to induce cell death in as many as 20 dicotyledonous plants (70). The wide phylogenetic conservation and broad-spectrum activity of NLPs distinguish them from the majority of cell death elicitors and suggest that the necrosis-inducing activity is functionally important.

In oomycetes, NLPs are also broadly distributed in *Phytophthora* and *Pythium*. PaNIe of *Pythium aphanidermatum* (105), NPP1 of *Phytophthora parasitica* (27), PsojNIP of *P. sojae* (76), and PiNPP1 of *P. infestans* (T. D. Kanneganti & S. Kamoun, unpublished) have been studied in most detail. The contribution of these NLPs to disease remains unclear. NLPs induce defense responses in both susceptible and resistant plants. In *P. sojae* and *P. infestans*, the *NLP* genes were expressed late during host infection and thus may function in triggering the host tissue necrosis observed during the necrotrophic phase of infection thereby facilitating colonization (76; T.D. Kanneganti & S. Kamoun, unpublished). A role of NLPs in virulence is supported by the recent finding that disruptions of the NIP_{ecc} and NIP_{eca} genes in *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*, respectively, resulted in reduced virulence phenotypes on potato tubers (71).

GP42 (PEP13) Transglutaminase

GP42 is an abundant cell wall glycoprotein of *P. sojae* that triggers defense gene expression and synthesis of antimicrobial phytoalexins in parsley through binding to a plasma membrane receptor (63, 83). A 13-amino-acid peptide fragment (Pep-13) is necessary and sufficient for activation of defenses in parsley and also triggers cell death in potato (32). Biochemical analyses indicated that GP42 is a Ca^{2+}-dependent transglutaminase (TGase) that is highly conserved in *Phytophthora* (21). The Pep-13 motif was invariant in all examined *Phytophthora* TGases. Mutational analyses indicated that the same residues within the Pep-13 motif are important for activation of plant defenses and TGase activity. This suggests that plants evolved receptors to recognize an essential "epitope" within the TGase proteins and that GP42 functions as a pathogen-associated molecular pattern (PAMP) (21). However, it is not known whether *Phytophthora* GP42 TGases play an essential role in virulence or fitness of the pathogen.

CBEL

CBEL (Cellulose Binding, Elicitor, and Lectin-like) is a 34-kDa cell wall protein that was first isolated from *Phytophthora parasitica* var. *nicotianae*. CBEL has a dual function: (*a*) it elicits necrosis and defense gene expression in tobacco plants; (*b*) it is required for attachment to cellulosic substrates such as plant surfaces (87, 106). Strains of *P. parasitica* silenced for the *CBEL* gene were impaired in their ability to attach to cellophane

membranes, although they remained able to infect tobacco plants (28). CBEL contains two regions with similarity to the PAN module (InterPro: IPR000177), a conserved domain that includes the Apple domain and functions in protein-protein or protein-carbohydrate interactions. PAN module proteins have been implicated in attachment of apicomplexan parasites to host tissues (18–20). The CBEL-like PAN module is particularly diverse in oomycetes, with 52 different sequences identified in 5 species, including the fish pathogen *Saprolegnia parasitica* (100). The role of the PAN/cellulose binding domain of CBEL in induction of defense responses is under investigation (29).

CYTOPLASMIC EFFECTORS

RXLR Protein Family

Race-specific resistance to *Phytophthora* spp. follows the gene-for-gene model, which implies that *Avr* genes from the pathogen are perceived directly or indirectly by matching resistance (*R*) genes from the plant (33, 93). Numerous *R* genes that function against the oomycetes *H. parasitica*, *P. infestans*, and *P. sojae* have been cloned (8, 10, 14, 17, 37, 56, 69, 91, 98, 101, 102). They belong to three different classes of cytoplasmic and extracellular leucine-rich repeat (LRR) disease-resistance proteins, although the great majority belong to the cytoplasmic class of NBS-LRR (nucleotide binding site, and leucine-rich repeat domain) proteins. Race-specific *Avr* genes from oomycetes have been cloned only recently (5, 6, 78, 88). All four oomycete Avr proteins (ATR1, ATR13, AVR3a, and Avr1b) carry a signal peptide followed by a conserved motif (RXLR) that occurs in a large number of secreted oomycete proteins (78) (**Figure 3*b***). The RXLR motif is similar to a host-targeting signal that is required for translocation of proteins from malaria parasites (*Plasmodium* species) into the cytoplasm of host cells (36, 55), leading to the hypothesis that RXLR functions as a signal that mediates trafficking into host cells (78). This finding raises the possibility that plant and animal eukaryotic pathogens share similar mechanisms for effector delivery into host cells.

NBS-LRR: nucleotide binding site and leucine-rich repeat

ATR1^{NdWsB}. *ATR1^{NdWsB}* was identified by map-based cloning as a single dominant gene that segregates in a cross between the isolate Emoy2 (avirulent on *Arabidopsis* ecotype Niederzenz carrying the *RPP1-Nd* resistance gene) and isolate Maks9 (virulent) of *H. parasitica* (78). *ATR1^{NdWsB}* encodes a 311-amino-acid protein that is detected not only by *RPP1-Nd* but also by the *RPP1-WsB* gene in accession Wassilewskija. A construct derived from *ATR1^{NdWsB}* triggered cell death when expressed in the cytoplasm of *Arabidopsis* cells following transient expression using particle bombardment. *ATR1^{NdWsB}* is highly polymorphic among isolates of *H. parasitica*. Six alleles from eight isolates were polymorphic in about one third of all residues. Diversifying selection and recombination apparently drove the evolution of the *ATR1^{NdWsB}* locus. Although the region encompassing the signal peptide and the RXLR motif show nonsynonymous sequence variation, the remainder of the protein is under positive selection, suggesting that this C-terminal region is involved in coevolution with plant factors. Complex patterns of perception by the R proteins were identified. Whereas *RPP1-Nd* recognizes only a single allele of *ATR1^{NdWsB}*, *RPP1-WsB* recognizes four different alleles and confers resistance to a broader range of isolates.

ATR13. *Ppat17*, one of the genes identified in *H. parasitica* by Bittner-Eddy et al. (13) as highly expressed during infection of *Arabidopsis*, was shown to be the *Avr* gene *ATR13* based on cosegregation and in planta expression analyses (5). *ATR13* encodes a 187-amino-acid protein that triggers RPP13-dependent cell death following transient biolistic expression or induced expression in transgenic *Arabidopsis* plants (5). Besides the signal sequence and RXLR motif, ATR13 has a heptad leucine/isoleucine repeat motif followed by

an imperfect direct repeat of 4 × 11 residues. Similar to its cognate resistance gene *RPP13* (82), *ATR13* exhibits extreme levels of polymorphism with evidence of diversifying selection. Six different alleles were identified in isolates of *H. parasitica*. The alleles from isolates Aswa1, Emco5, and Goco1 carry a single 11-amino-acid repeat unit but still trigger RPP13 response. As with ATR1, evidence of recombination and an uneven distribution of nucleotide variation throughout the coding sequence suggest that the effector activity is localized to the C-terminal region of ATR13.

Avr1b-1. Fine structure genetic mapping of avirulence of *P. sojae* on soybean plants carrying the resistance gene *Rps1b* identified a complex locus with at least two functional genes, *Avr1b-1* and *Avr1b-2* (88). The identity of *Avr1b-2* is not known, although it was proposed to be required for the accumulation of *Avr1b-1* mRNA. *Avr1b-1* encodes a 138-amino-acid protein that belongs to a complex gene family in *P. sojae*. Polymorphic alleles or paralogs of *Avr1b-1* occur and exhibit high rates of nonsynonymous substitutions indicative of diversifying selection. Virulent isolates such as P6497 and P9073 contain an intact *Avr1b-1* gene but fail to accumulate *Avr1b-1* mRNA, in contrast to avirulent isolates. Infilitration into soybean leaves of recombinant Avr1b-1 protein produced in the yeast *Pichia pastoris* resulted in *Rps1b*-specific cell death. Avr1b-1 also induced limited cell death responses on *Rps1k* plants, suggesting weak recognition by this resistance gene. Because the *Rps1* gene cluster encodes cytoplasmic NBS-LRR proteins (10), Avr1b-1 is thought to function in host cytoplasm (88). However, still unclear is whether the infiltrated Avr1b-1 protein acts extracellularly or can enter soybean cells in the absence of the pathogen.

AVR3a. *Avr3a* was identified using association analyses of polymorphisms in candidate effector genes with avirulence on potato *R* genes (6, 16). *Avr3a* encodes at least two polymorphic secreted proteins of 147 amino acids that differ in only three residues, two of which are in the mature protein (6). Isolates of *P. infestans* that are avirulent on *R3a* potato carry the avirulence gene $Avr3a\_C^{19}K^{80}I^{103}$, whereas virulent isolates carry only the virulence allele $avr3a\_S^{19}E^{80}M^{103}$. Transient biolistic and agroinfiltration coexpression assays showed that *R3a*-mediated cell death was specifically induced by the *Avr3a* allele, confirming interaction between the gene pair. The *avr3a* allele is conserved among all isolates examined in *P. infestans*, suggesting that it plays an important function in the pathogen. On the other hand, the lack of allelic diversity in the *Avr3a* gene may reflect genetic bottlenecks that populations of *P. infestans* experienced as they spread out worldwide (30). AVR3a does not require a signal peptide sequence for triggering R3a-mediated HR in transient assays and is therefore recognized inside the plant cytoplasm (6). Remarkably, *Avr3a* of *P. infestans* and $ATR1^{NdWsB}$ of *H. parasitica* reside in syntenic regions, suggesting an ancestral effector locus in these oomycete pathogens.

Other RXLR proteins. Bioinformatic analyses indicated that the RXLR motif is frequent among secreted proteins of *P. infestans*, *P. sojae*, and *P. ramorum* with at least 100 proteins identified in each genome (J. Win, C. Young & S. Kamoun, unpublished). This suggests that these pathogens deliver a large set of effectors to the host cytoplasm. Some of the RXLR proteins, such as IPIO (*In Planta* Induced) and several Pexs, have been identified as candidate effectors prior to the discovery of the RXLR motif (72, 76, 99). One RXLR protein of *P. infestans* carries a functional nuclear localization signal and may therefore accumulate in host nuclei (T. D. Kanneganti, J. Win & S. Kamoun, unpublished).

CRN Protein Family (CRN1, CRN2. . .)

CRN1 and CRN2 were identified following an in planta functional expression screen of

candidate secreted proteins of *P. infestans* (Pex) based on a vector derived from *Potato virus X* (99). Expression of both genes in *Nicotiana* spp. and in the host plant tomato results in a leaf-crinkling and cell-death phenotype accompanied by an induction of defense-related genes. Torto et al. (99) proposed that CRN1 and CRN2 function as effectors that perturb host cellular processes based on analogy to bacterial effectors, which typically cause macroscopic phenotypes such as cell death, chlorosis, and tissue browning when expressed in host cells (49). The two *crn* genes are expressed in *P. infestans* during colonization of the host plant tomato. *In planta* expression of a collection of deletion mutants of *crn2* indicate that this protein activates defense responses in the plant cytoplasm (T. Torto-Alalibo & S. Kamoun, unpublished).

Database searches revealed that the CRNs form a complex family of relatively large proteins (about 400–850 amino acids) in *Phytophthora* (99). Sequence analyses revealed evidence of gene conversion and/or recombination in the *crn* gene family of *P. infestans* (Z. Liu & S. Kamoun, unpublished). One of the *crn* genes, *crn8*, encodes a secreted protein with a predicted RD kinase domain that may target host factors (C. Cakir & S. Kamoun, unpublished), whereas most *crn* genes have no similarity to known sequences.

CONCLUSION

Our understanding of the biology of effectors is incomplete despite the tremendous progress made in recent years. The discovery of diverse apoplastic and cytoplasmic effectors facilitated an initial attempt at classifying and cataloguing these molecules. As five species, *H. parasitica*, *P. capsici*, *P. infestans*, *P. ramorum*, and *P. sojae*, undergo genome sequencing and annotation, we are moving rapidly toward genome-wide catalogues of oomycete effectors. Already it is evident that the effector secretome of plant pathogenic oomycetes is much more complex than expected, with perhaps several hundred proteins dedicated to reprogramming host cells. Comprehensive knowledge of the structure and function of pathogen effectors and the perturbations they cause in plants is a precondition for understanding the molecular basis of pathogenesis and disease. As molecular analyses become more detailed, novel strategies for manipulating plants toward resistance to oomycete pathogens will undoubtedly become apparent.

SUMMARY POINTS

1. Plant pathogenic oomycetes modulate host cell defenses through a complex array of effector proteins.
2. Functional genomics pipelines have greatly facilitated the identification of effectors from cDNA and genomic sequences.
3. Apoplastic effectors are secreted into the plant extracellular space where they interact with their host targets.
4. Several apoplastic effectors function as inhibitors of the enzymatic activities of plant PR proteins, such as glucanases and proteases.
5. Cytoplasmic effectors are translocated inside the plant cell where they target different subcellular compartments.
6. Several cytoplasmic effectors carry the RXLR motif, which is similar to a signal required for translocation of proteins from malaria parasites to host cells.

FUTURE ISSUES TO BE RESOLVED

1. What are the biochemical activities of oomycete effectors? How do they operate to modulate host defenses? What are their host substrates?
2. How does the RXLR motif function in translocation of effectors inside host cells? What is the translocation machinery? Is it derived from the pathogen or the host?
3. To what extent are effector secretion systems conserved in pathogenic eukaryotes?

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