9 The Secretome of Plant-Associated Fungi and Oomycetes

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I. Introduction

The secretome of plant-associated fungi and oomycetes has been the subject of much research since the publication about 10 years ago of the first edition of "*The Mycota: Plant Relationships*". The concept that filamentous microbes require secreted proteins to alter their environment and the organisms they colonize is not particularly novel, but technology has matured to the point where it is nowadays possible to generate catalogs of the complete set of secreted proteins (the secretome) for a given organism. This has been driven by the coming of age of genome sequencing coupled with robust computational predictions of secretion signals. This chapter surveys some of the key concepts and findings that recently emerged from the study of the fungal and oomycete secretome, with an emphasis on effector proteins.

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Filamentous microorganisms, such as fungi and oomycetes, include highly developed plant pathogens that are intimately associated with their host plants and cause a variety of disease pathologies in natural and agricultural plant communities. Until recently, our knowledge of fungal and oomycete pathogenicity was mainly limited to the development of specialized infection structures, secretion of hydrolytic enzymes, and production of toxins (see Chaps. 10, 11). New findings, however, broadened our view of pathogenicity and suggested that filamentous pathogens are sophisticated manipulators of plant cells. It is now well accepted that similar to bacterial pathogens, eukaryotic pathogens secrete an arsenal of effector proteins that modulate plant innate immunity and enable parasitic infection (Birch et al. 2006; Chisholm et al. 2006; Kamoun 2006; O'Connell and Panstruga 2006). Deciphering the biochemical activities of effectors to understand how pathogens successfully colonize and reproduce on their host plants became a driving paradigm in the field of fungal and oomycete pathology.

II. Definition of Effectors

As in previous publications (Kamoun 2003; Torto et al. 2003; Huitema et al. 2004), I continue to use a flexible definition of the term "effectors". I define effectors as molecules that alter host cell structure and function, thereby facilitating infection (virulence factors or toxins) and/or triggering defense responses (avirulence factors or elicitors). The concept of "extended phenotype" (i.e. genes "whose effects reach beyond the cells where they reside") put forward by Richard Dawkins in a classic 1982 book (Dawkins 1999) sums up perfectly this view of effectors. Effectors can be viewed as "parasite genes having phenotypic expression in host bodies and behavior" (Dawkins 1999). Indeed, effectors are the products of genes residing in pathogen

Plant Relationships, 2nd Edition
The Mycota V
H. Deising (Ed.)

genomes but actually functioning at the interface with the host plant or even inside plant cells, providing a vivid example of Dawkins "extended phenotype" (Kamoun 2006, 2007).

III. Identification of Secreted Proteins and Effectors

Biochemical, genetic, and bioinformatic strategies, often in combination, have been applied to the identification of secreted proteins from filamentous pathogens. Traditionally, secreted proteins were identified by biochemical purification followed by genetic analysis. With the advent of genomics, novel strategies emerged. Identification of candidate secreted proteins was facilitated by the fact that in fungi and 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 (Torto et al. 2003). Signal peptides are highly degenerate and cannot be identified using DNA hybridization or PCR-based techniques. Nonetheless, computational tools, particularly the SignalP program

Table 9.1. Filamentous pathogen effectors discussed in this chapter. *NA* Not applicable

Pathogen species	Effector	Localization in plant tissue[a]	Signal peptide length[b]	Virulence activities
Blumeria graminis f. sp. *hordei*	AVRa10	Cytoplasmic	NA	Enhances infection in susceptible barley plants[c]
	AVRk1	Cytoplasmic	NA	Enhances infection in susceptible barley plants[c]
Cladosporium fulvum	Avr2	Apoplastic	20	Cysteine protease inhibitor, inhibits tomato Rcr3[c]
	Avr4	Apoplastic	18	Contains CBM14 chitin binding domain, protects fungal cell walls from hydrolysis by plant chitinases[c]
	Avr9	Apoplastic	23	Structural similarity to cystine knot carboxypeptidase inhibitor[d]
Fusarium oxysporum f. sp. *lycopersici*	SIX1	Apoplastic (xylem)	21	
Hyaloperonospora parasitica	ATR1	Cytoplasmic	15	
	ATR13	Cytoplasmic	18	
Leptosphaeria maculans	AvrLm1	Probably cytoplasmic	22	
Melampsora lini	AvrL567	Cytoplasmic	23	
	AvrM	Cytoplasmic	28	
	AvrP123	Cytoplasmic	23	Kazal-like protease inhibitor[d]
	AvrP4	Cytoplasmic	28	
Phytophthora infestans	Avr3a	Cytoplasmic	21	Cell death suppressor[c]
	CRN1	Cytoplasmic	17	Elicits cell death in host plants[c]
	CRN2	Cytoplasmic	22	Elicits cell death in host plants[c]
	CRN8	Cytoplasmic	17	Similarity to RD kinase[d], elicits cell death in host plants[d]
	EPI1	Apoplastic	16	Kazal-like serine protease inhibitor, inhibits tomato P69B[c]
	EPI10	Apoplastic	21	Kazal-like serine protease inhibitor, inhibits tomato P69B[c]
	EPIC1	Apoplastic	21	Cystatin-like cysteine protease inhibitor[d]
	EPIC2B	Apoplastic	21	Cystatin-like cysteine protease inhibitor, inhibits tomato PIP1[c]
Phytophthora sojae	Avr1b-1	Cytoplasmic	21	
Uromyces fabae	*Uf*-RTP1	Cytoplasmic	19	Localizes to host nucleus[c]

[a] Cytoplasmic versus apoplastic effectors based on the classification described in the text.
[b] Length in amino acids, based on SignalP ver. 2.0-NN (www.cbs.dtu.dk/services/SignalP-2.0).
[c] Evidence is based on wet laboratory experimental data.
[d] Evidence is based on computational analyses

that was developed using machine learning methods (Nielsen et al. 1999), can assign signal peptide prediction scores and cleavage sites to unknown amino acid sequences with a high degree of accuracy (Menne et al. 2000; Schneider and Fechner 2004). Therefore, with the accumulation of cDNA and genome sequences, lists of candidate secreted proteins can be readily generated using bioinformatics tools (Table 9.1). For instance, Torto et al. (2003) developed PexFinder (with Pex standing for *Phytophthora* extracellular protein), an algorithm based on SignalP ver. 2.0 (Nielsen et al. 1999) to identify proteins containing putative signal peptides from expressed sequence tags (ESTs). PexFinder was then applied to ESTs in *P. infestans* to identify candidate secreted proteins, ultimately leading to the discovery of novel effectors of the RXLR and Crinkler families (for more information about these effectors, see Sect. VI.E, F). Variations on the Torto et al. (2003) approach have been successfully implemented, resulting in the identification of a number of important effectors from fungal pathogens (Dodds et al. 2004; Kemen et al. 2005; Catanzariti et al. 2006).

The overwhelming majority of filamentous pathogen effectors identified to date carry typical signal peptides that can be predicted using SignalP (Kamoun 2007). SignalP ver. 2.0 predictions were also convincingly validated for filamentous pathogens using proteomics (Torto et al. 2003) and a yeast secretion assay (Lee et al. 2006). Similar high degrees of accuracy of SignalP were also reported for other eukaryotes (Menne et al. 2000; Schneider and Fechner 2004). However, it is evident that many secreted proteins do not carry signal peptides and, therefore, cannot be identified using SignalP. One future challenge is to identify alternative secretory pathways, to complete cataloguing the secretomes of filamentous pathogens.

IV. Classes of Effectors

Effectors can be classified in two classes based on their target sites in the host plant (Kamoun 2006, 2007). Apoplastic effectors are secreted into the plant extracellular space, where they interact with extracellular targets and surface receptors. Cytoplasmic effectors are translocated inside the plant cell presumably through specialized structures like infection vesicles and haustoria that invaginate inside living host cells. In the following sections, we review a selection of examples of apoplastic and cytoplasmic effectors from plant pathogenic fungi and oomycetes.

V. Apoplastic Effectors

A. *Cladosporium fulvum* and *Phytophthora infestans* Protease Inhibitors

Effector proteins with inhibitory activities for protection against host proteases have been reported in fungi and oomycetes (Rooney et al. 2005; Kamoun 2006; van den Burg et al. 2006). Among these, *P. infestans* EPI1 and EPI10 are multidomain secreted serine protease inhibitors of the Kazal family that bind and inhibit the pathogenesis-related (PR) protein P69B, a subtilisin-like serine protease of tomato that functions in defense (Tian et al. 2004, 2005). *P. infestans* also secretes the cystatin-like cysteine protease inhibitors EPIC1 and EPIC2B that target PIP1 and other apoplastic cysteine proteases of tomato (Tian et al. 2007). PIP1 is closely related to tomato Rcr3, another apoplastic cysteine protease that is required for Cf-2 mediated resistance to the fungus *Cladosporium fulvm* and is inhibited by the fungal Avr2 protein (Kruger et al. 2002; Rooney et al. 2005). Unlike *P. infestans* cystatin-like EPICs, Avr2 does not have any obvious similarity to other cysteine protease inhibitors.

Other effectors that might function as protease inhibitors include *C. fulvum* Avr9 (van Kan et al. 1991). This protein shows structural similarity to cystine knot carboxypeptidase inhibitors but so far no biochemical data has been reported to support the observed similarity (van den Hooven et al. 2001). The flax rust avirulence protein AvrP123 is a 117-amino-acid protein with similarity to Kazal serine protease inhibitors and might, therefore, also target host proteases (Catanzariti et al. 2006).

B. *Phytophthora* Glucanase Inhibitors

Other secreted proteins with inhibitory activities against host hydrolytic enzymes are *Phytophthora* glucanase inhibitors. The glucanase inhibitors GIP1 and GIP2 are secreted proteins of *P. sojae* that inhibit the soybean endo-β-1,3 glucanase EGaseA (Rose et al. 2002). 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* (Bishop et al. 2004). Four genes with similarity to GIPs have been identified in *P. infestans*, and their ability to inhibit tomato endoglucanases is under investigation (C. Damasceno and J. Rose, personal communication).

C. *Cladosporium fulvum* Avr4

The *Cladosporum fulvum* secreted protein Avr4 is a cysteine-rich protein that contributes to fungal virulence on tomato plants that lack the resistance protein Cf-4 (Joosten et al. 1997). The Avr4 protein has similarity to the chitin-binding domain CBM14 and was shown to bind chitin (van den Burg et al. 2004). Natural variants of Avr4 that are disrupted in their disulfide bridges retain the ability to bind chitin but evade recognition by Cf-4 (van den Burg et al. 2004). Avr4 appears to contribute to virulence by protecting fungal cell walls from hydrolysis by plant chitinases (van den Burg et al. 2006).

D. *Fusarium oxysporum* Six1

Six1 is a 32-kDa cysteine-rich protein secreted by the fungus *Fusarium oxysporum* f. sp. *lycopersici*, a vascular pathogen of tomato (Rep et al. 2004). Within infected tomato plants, Six1 accumulates in the xylem where it is processed by either fungal or plant proteases into a 12-kDa protein (Rep et al. 2004). Six1 mediates avirulence to tomato plants carrying the resistance gene *I-3*, as shown by complementation and knockout experiments (Rep et al. 2004). However, a direct cell death elicitor activity has not been reported for Six1. Presumably, Six1 interacts with a receptor at the surface of the xylem parenchyma cells to trigger hypersensitive cell death, but cloning and functional characterization of the *I-3* gene is needed to help clarify this issue (Rep et al. 2004).

VI. Cytoplasmic Effectors

A. Flax Rust Effectors AvrL567, AvrM, AvrP123, and AvrP4

The haustoria of the flax rust fungus *Melampsora lini* mediate the delivery of effector proteins inside host cells (Dodds et al. 2004; Catanzariti et al. 2006). Four flax rust effectors, AvrL567, AvrM, AvrP123, and AvrP4, were identified among 21 haustorially expressed secreted proteins (HESPs) as having a hypersensitive elicitor activity towards their cognate flax resistance genes (Catanzariti et al. 2006). This work indicated that haustoria are highly enriched in secreted effector proteins and most likely play a role in mediating effector translocation into host cells.

How the flax rust effectors enhance virulence is unknown. As mentioned above, AvrP123 has similarity to Kazal serine protease inhibitors and might target host proteases (Catanzariti et al. 2006). AvrL567 is a highly polymorphic effector that binds flax L5, L6, and L7 resistance proteins in the plant cytoplasm to activate hypersensitivity and defense (Dodds et al. 2004). Diversifying selection has acted on the *AvrL567* gene, and the positively selected sites were shown to alter binding to plant resistance protein receptors, providing evidence that natural selection has acted on modifying binding affinity between pathogen and plant proteins (Dodds et al. 2006).

B. *Uromyces fabae Uf*-RTP1

Uf-RTP1 is a 24-kDa haustorial protein secreted by the rust fungus *Uromyces fabae* (Kemen et al. 2005). This protein is exceptional in possessing, in addition to a classic signal peptide, a bipartite nuclear localization signal (NLS) RQHHKR[X9]HRRHK. Expression of a green fluorescent protein (GFP) and *Uf*-RTP1 fusion protein in tobacco protoplasts demonstrated that the NLS mediates protein accumulation in plant cell nuclei (Kemen et al. 2005). Most interestingly, *Uf*-RTP1 was detected inside infected plant cells, including host nuclei, by immunofluorescence and electron microscopy, providing direct evidence that this protein translocates into host cells during colonization of broad bean plants by the rust fungus (Kemen et al. 2005). This suggests that, similar to some bacterial effectors like *Xanthomonas* AvrBs3 (Lahaye and Bonas 2001), filamentous pathogen effectors also target the host nucleus where they possibly alter host gene expression.

Besides *Uf*-RTP1, *P. infestans* candidate effectors Nuk6, Nuk7, Nuk10 and Nuk12 were recently shown to carry a combination of signal peptide and NLS and to accumulate in plant nuclei in transient expression assays (Kamoun 2006; Kanneganti et al. 2007). Nuclear localization of Nuk6, Nuk7, and Nuk10 was

dependent on the plant protein importin-α, suggesting that these effectors exploit the host machinery to localize in the nucleus (Kanneganti et al. 2007). These findings are particularly interesting in view of the emerging evidence that nucleo-cytoplasmic trafficking is important for plant disease resistance response and resistance protein activity (Palma et al. 2005; Shen et al. 2006).

C. *Leptosphaeria maculans* AvrLm1

AvrLm1, a 205-amino-acid protein secreted by the fungal pathogen *Leptosphaeria maculans*, mediates avirulence on *Brassica napus* plants carrying the resistance gene *Rlm1* (Gout et al. 2006). A laborious 10-year positional cloning project was recently capped by the identification of *AvrLm1* in a gene-poor heterochromatin-like region of the *L. maculans* genome (Gout et al. 2006). This 260-kb region is composed essentially of nested long tandem repeat (LTR) retrotransposons and is surrounded by isochores, high GC gene-rich islands. *AvrLm1* was the only predicted gene in the 260-kb region (Gout et al. 2006). The occurrence of *AvrLm1* in such a distinct genome environment is reminiscent of the location of *Magnaporthe oryzae* avirulence gene *Avr-Pita* in a highly unstable telomeric region (Orbach et al. 2000). Also, in the oomycete *P. infestans*, the *Avr3b-Avr10-Avr11* locus displays copy number variation resulting in amplification of multiple truncated copies of a transcription factor-like gene in avirulent strains (Jiang et al. 2006). Localization of effector genes in regions with high genome plasticity is likely to increase genetic and epigenetic variation, perhaps resulting in accelerated evolution (Orbach et al. 2000). In *Plasmodium*, genes for host-translocated effectors are often found near chromosomal ends, possibly because this position favors rapid adaptation to the host (Freitas-Junior et al. 2000; Marti et al. 2004).

The origin of *AvrLm1* remains mysterious. This gene has a lower GC content compared to linked genes, and is absent in virulent races of *L. maculans* as well as isolates of related species (Gout et al. 2006). Although the function of *AvrLm1* was confirmed by transformation into a virulent strain, Rlm1-specific elicitor activity has not been demonstrated yet (Gout et al. 2006). Furthermore, it remains unclear whether AvrLm1 functions in the apoplast or inside plant cells. However, this protein carries only a single cysteine residue and thus differs from apoplastic effectors, which are typically cysteine-rich.

D. Barley Powdery Mildew Fungus $AVRa_{10}$ and $AVRk_1$

The AVR_{a10} and AVR_{k1} genes of the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* were first identified as candidate genes by positional cloning, and then shown to trigger hypersensitive cell death when expressed in the cytoplasm of barley cells carrying the *Mla10* and *Mlk1* resistance genes, respectively (Ridout et al. 2006; Chaps. 3, 18). The predicted AVR_{a10} and AVR_{k1} proteins are remarkable among filamentous pathogen effectors in being the only proteins to lack a typical secretion signal peptide (Ridout et al. 2006). How these proteins are secreted by the fungus is unclear but most likely involves alternative secretory pathways. Transient expression experiments of AVR_{a10} and AVR_{k1} in susceptible barley cells indicated that these effectors increase the number of successful infections sites, suggesting a virulence function of an unknown nature (Ridout et al. 2006).

E. Oomycete RXLR Effectors

The oomycete effectors ATR1, ATR13, AVR3a, and AVR1b are defined by an N-terminal motif (arginine, any amino acid, leucine, arginine; RXLR) and are thought to be delivered inside plant cells where they alter host defenses (Rehmany et al. 2005; Birch et al. 2006; Kamoun 2006; Whisson et al. 2007). Genome-wide catalogs of RXLR effectors, generated using computational approaches, unraveled a remarkably complex and divergent set of hundreds of candidate genes. Tyler et al. (2006) reported 350 RXLR effectors each in the genomes of *Phytophthora ramorum* and *P. sojae* using iterated similarity searches. Win et al. (2007) used combinations of motif and hidden Markov model searches to uncover at least 50 candidates in the downy mildew *Hyaloperonaspora parasitica* and more than 200 each in *P. capsici*, *P. infestans*, *P. ramorum*, and *P. sojae*. These large numbers of effectors suggest that oomycetes extensively modulate host processes during infection. They also raise technical challenges for studying RXLR effectors and call for the implementation of high-throughput functional analyses (Torto et al. 2003; Huitema et al. 2004).

RXLR effectors are modular proteins with two main functional domains (Bos et al. 2006; Kamoun 2006). While the N-terminal domain encompassing the signal peptide and conserved RXLR region functions in secretion and targeting, the remaining C-terminal domain carries the effector activity and operates inside plant cells. The RXLR motif defines a domain that functions in delivery of the effector proteins into the host cell (Whisson et al. 2007). Interestingly, the RXLR motif is similar in sequence and position to the plasmodial host translocation (HT)/Pexel motif that functions in delivery of parasite proteins into the red blood cells of mammalian hosts (Hiller et al. 2004; Marti et al. 2004). The RXLR domains of *P. infestans* AVR3a and another RXLR protein PH001D5 are able to mediate the export of the GFP from the *Plasmodium falciparum* parasite to red blood cells (Bhattacharjee et al. 2006), indicating that plant and animal eukaryotic pathogens share similar secretory signals for effector delivery into host cells (Haldar et al. 2006).

The *Phytophthora infestans* RXLR effector Avr3a is able to suppress hypersensitive cell death induced by another *P. infestans* protein, INF1 elicitin, pointing to a possible virulence function (Bos et al. 2006). The occurrence of cell death suppressing effectors has been hypothesized for biotrophic fungal and oomycete pathogens (Panstruga 2003), based on cytological observations of susceptible interactions and the prevalence of cell death suppressors among bacterial type III secretion system (TTSS) effectors (Jamir et al. 2004; Janjusevic et al. 2006). However, to date Avr3a is the only cell death suppressor described in filamentous pathogens. The extent to which other effectors can suppress cell death is a subject of intense investigation.

F. Oomycete Crinklers

The "Crinkler" proteins (CRNs) form a distinct class of secreted proteins that alter host responses and are thought to play important roles in disease progression (Torto et al. 2003; Kamoun 2007; Win et al. 2007). The CRNs were identified following an in planta functional expression screen of candidate secreted proteins of *P. infestans* based on a vector derived from Potato virus X (Torto et al. 2003). Ectopic expression of both genes in *Nicotiana* spp. and in the host plant tomato resultED in a leaf-crinkling and cell-death phenotype accompanied by an induction of defense-related genes. Torto *et al.* (2003) proposed that the CRNs 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. In planta expression of a collection of deletion mutants of *crn2* indicated that this protein activates defense responses in the plant cytoplasm, suggesting they are cytopalsmic effectors (T. Torto and S. Kamoun, unpublished data).

Computational analyses revealed that the CRNs form a complex family of relatively large proteins (about 400–850 amino acids) in *Phytophthora*. Interestingly, the CRNs are defined by a distinct conserved N-terminal motif characterized by the consensus LXLFLAK (Kamoun 2007; Win et al. 2007). In *H. parasitica*, LXLFLAK overlaps with the RXLR motif, resulting in RXLRLFLAK (Win et al. 2007). This finding, along with the observation that the N-terminal region of the CRNs is dispensable for cell death induction in planta, suggests that the LXLFLAK motif contributes to host targeting analogous to RXLR. Thus, similar to RXLR effectors, the CRNs are modular proteins consisting of distinct N-terminal and C-terminal domains. Importantly, the evolutionary history of the CRN family is fundamentally different from that of the RXLR effectors. The CRNs show high rates of gene conversion and a prominent recombination site is present after the conserved N-terminal motifs (Z. Liu and S. Kamoun, personal communication). Therefore, a number of the *P. infestans* CRNs are chimeras that show unique associations between a conserved N-terminal domain and a variety of divergent C-terminal regions. These findings point to an alternative mode of host adaptation by *P. infestans* and suggest that the CRN proteins may fulfill different functions from the RXLR proteins.

VII. Conclusions

With the recent emergence of novel and cheaper DNA sequencing technologies, the flow of cDNA and genome sequences of plant-associated filamentous pathogens has rapidly accelerated. Genome sequences are becoming available for species that represent the diverse lifestyles and phylogenetic spectrum of plant-associated fungi

and oomycetes. The available sequences will likely further reinforce the importance of secreted proteins in the associations between filamentous microbes and their host plants. For instance, the genome sequence of the mycorrhizal fungus *Laccaria bicolor* revealed an unexpectedly diverse and complex secretome (F. Martin, personal communication). The extent to which secreted proteins in *L. bicolor* and a diverse range of other filamentous microbes function as effectors that impact host plants is therefore poised to continue to be an exciting topic of research.

Acknowledgements: Research in my laboratory is supported by the Gatsby Charitable Foundation. Parts of this review were adapted from previous publications (Kamoun 2006, 2007).

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