RXLR effectors of plant pathogenic oomycetes
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Oomycetes are a phylogenetically distinct group of organisms that include some of the most devastating plant pathogens. Recent characterization of four oomycete Avr genes revealed that they encode effector proteins with a common modular structure, including a N-terminal conserved RXLR motif. Several lines of evidence initially indicated, with support from more recent works, that these Avr proteins are secreted by the pathogen and then translocated into the host cell during infection. In addition to elucidating the machinery required for host-cell transport, future works remain to determine the myriad virulence functions of oomycete RXLR effector proteins.

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Current Opinion in Microbiology 2007, 10:332–338

This review comes from a themed issue on Fungi
Edited by Jim Kronstad
Available online 17th August 2007
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DOI 10.1016/j.mib.2007.04.005

Introduction
Prokaryotic and eukaryotic pathogens of plants secrete effector proteins to different cellular compartments of their hosts to modulate plant defense circuitry and enable parasitic colonization [1–4]. The current paradigm in the study of plant–microbe interactions is that unravelling the molecular function of effectors is central to a mechanistic understanding of pathogenicity. Indeed, significant progress has been made in elucidating the virulence functions of bacterial effectors [2], and research with eukaryotic plant pathogens is progressing rapidly as illustrated by the recent identification of effectors from the flax rust and barley powdery mildew fungi [5–7], the oomycetes Phytophthora and Hyaloperonospora [8,9,10**,11], as well as root-knot nematodes [12,13].

Oomycetes form a distinct group of eukaryotic microorganisms that includes some of the most notorious pathogens of plants [14]. Research on oomycete effectors has accelerated in recent years partly because of the blossoming of genomic resources. Oomycetes are now thought to secrete hundreds of effector proteins that target two distinct sites in the host plant [1,3,15**].

Apoplastic effectors are secreted into the plant extracellular space, whereas cytoplasmic effectors are translocated into the plant cell, where they target different subcellular compartments [1,3]. Several apoplastic effectors contribute to counter-defense by inhibiting host enzymes, such as proteases and glucanases, that accumulate in response to pathogen infection [16–18]. By contrast, the biochemical activities of cytoplasmic effectors remain poorly understood. Oomycete cytoplasmic effectors have been discovered through their avirulence (Avr) function, that is, their ability to trigger hypersensitive cell death on host genotypes with corresponding disease resistance (R) genes [8,9,10**,11], but their function in plants lacking cognate R genes remains largely unknown [3].

This review summarizes recent findings on the structure and function of the RXLR class of oomycete cytoplasmic effectors [1,3]. These effectors function inside host cells and are characterized by a highly conserved region defined by the invariant sequence RXLR. This review will cover two main topics of RXLR effector research: trafficking and virulence function.

The RXLR sequence defines a conserved domain of oomycete Avr proteins
Four oomycete Avr genes have been described in the past three years: ATR1NDSWr and ATR13 from the downy mildew Hyaloperonospora parasitica [8,10**], Acr1b-1 from the soybean pathogen Phytophthora sojae [11], and Acr3a from Phytophthora infestans [8,9,10**,11]. All four Avr proteins contain within the N-terminal 60 amino acids a secretory signal peptide and a conserved domain featuring the motif RXLR, flanked by a high frequency of acidic (D/E) residues (Figure 1).

Oomycete RXLR proteins interact with intracellular host proteins
Interestingly, the R proteins that target each of these four Avr proteins belong to the intracellular class of NBS-LRR (nucleotide binding site and leucine-rich repeat domain) proteins [19–21]. The intracellular location of these R proteins suggests that these Avr proteins are detected inside the plant cytoplasm [8,9,10**]. Consistent with this idea, AVR3aK1, ATR1NdsWr, and ATR13 do not require a signal peptide sequence to trigger hypersensitivity when directly expressed in planta [8,9,10**].

The Phytophthora RXLR domain mediates host targeting in Plasmodium
The oomycete RXLR motif is similar in sequence and position to the host cell targeting signal (HT/Pexel motif)
required for translocation of proteins from malaria parasites (Plasmodium species) into host red blood cells [22,23]. The discovery that host-targeted proteins from Phytophthora and Plasmodium share a positionally conserved sequence begged the examination of functional conservation. Bhattacharjee et al. [24**] demonstrated that the RXLR leader sequences of P. infestans Avr3a and another candidate effector is sufficient to mediate the export of the green fluorescent protein (GFP) from the Plasmodium falciparum parasite to the host red blood cell (erythrocyte) (Figure 2). Mutations in the RXLR consensus abolished export. Consistent with the observed sequence biases flanking the RXLR motif (see above), regions upstream and downstream of the RXLR motif were required for host targeting, thereby defining a ca. 30 amino acid targeting domain. In summary, these findings suggest that plant and animal eukaryotic pathogens use similar signals for targeting effectors into host cells [24**]. However, it is presently unclear whether this functional similarity reflects conserved transport machinery between these divergent eukaryotes (see below for further discussion on this topic).

The RXLR domain is required for host cell targeting in Phytophthora
Recent evidence indicates that the RXLR motif is indeed required for targeting these oomycete effectors into host plant cells. It was recently found that the RXLR motifs of P. infestans Avr3a and P. sojae Avr1b-1 are needed to confer avirulence on resistant plants when expressed in

(a) Domain organization of cytoplasmic RXLR effectors. Schematic drawings of ATR1\textsuperscript{NBD\_Mut} and ATR13 of Hyaaloperonospora parasitica, Avr1b-1 of Phytophthora sojae, and AVR3a of Phytophthora infestans. The numbers under the sequences indicate amino acid positions. The highlighted RXLR domain includes the RXLR sequence itself and the downstream dEER sequence. The gray arrows distinguish the regions of the effector proteins that are involved in secretion and targeting from those involved in effector activity. (b) Similarity between the RXLR motif of oomycetes and the HT/Pexel motif of Plasmodium falciparum. Sequence logos were derived from P. infestans and P. falciparum effector proteins. Adapted from Bhattacharjee et al. [24**].
The pathogen, but not when transiently expressed in planta (Whisson et al. abstract 549; D Dou et al., abstract 552, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007). Furthermore, an Avr3a fluorescent fusion protein was specifically secreted from haustorial projections, potentially accumulating inside host cells (S Whisson et al. abstract 549, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007). By contrast, an RXLR mutant version of this fusion protein accumulated in the apoplastic space. These results suggest that the RXLR domain directs translocation of effector proteins into the plant host cell, following signal peptide-mediated secretion from the pathogen.

The RXLR domain is not required for effector activities

The emerging view then is that oomycete RXLR effectors, analogous to bacterial effectors of the type III secretion system, are modular proteins organized into two main functional domains [3] (Figure 1). The N-terminal domain, encompassing the signal peptide and RXLR region, functions in secretion and targeting, while the remaining C-terminal domain possesses the effector activity. This model predicts that the RXLR region should not be required for activity when the effector is expressed inside host cells. Indeed, Bos et al. [25**] recently showed that mutation of the P. infestans AVR3a Kl RXLR sequence did not interfere with induction of R3a hypersensitivity when the protein is directly expressed in Nicotiana benthamiana leaves. In fact, deletion analyses of AVR3aKl showed that the C-terminal 75 amino acids, which excludes the RXLR region but includes the two polymorphic amino acids K80 and I103 that are mutated in the nonfunctional allele, was sufficient for avirulence function when expressed directly inside plant cells [25**]. These findings are consistent with the view that the N-terminal region of oomycete RXLR effectors is involved in secretion and targeting but is not required for effector activity.

The C-terminal domain of RXLR effectors is typically under positive selection

Direct interaction between an Avr protein and its cognate R protein can lead to a coevolutionary arms race [26]. As a result, the effector domains recognized by the R protein will be under selection to diversify. Consistent with a role in effector activity, the C-terminal regions of H. parasitica ATR1 and ATR13 have higher levels of polymorphisms, particularly non-synonymous substitutions, than the signal peptide and RXLR region [8,10**]. Furthermore, two out of the three polymorphic residues between the two Avr3a alleles of P. infestans, amino acids 80 and 103, are located in the C-terminal effector domain [9]. Recent genome-wide analyses of RXLR effectors showed that positive selection has for the most part targeted the C-terminal effector domain rather than the signal peptide and the RXLR regions (S Kamoun et al. abstract 188, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007). In summary, the preponderance of non-synonymous polymorphisms in the C-terminal domain of the RXLR proteins is consistent with a role in effector activities, while the conservation of the N-terminal domains is consistent with a role in protein targeting.
Can RXLR effectors enter host plant cells in the absence of the pathogen?

Machinery for host cell transport of RXLR proteins could be encoded either by the pathogen, the host, or both. In our own work with AVR3aKI, we noted that *in planta* expression of the full-length protein resulted in R3a-dependent hypersensitivity [25**]. Catanzariti et al. [5] reported similar results with the rust fungus effector AvrM. However, these experiments are not particularly informative since they could be equally explained by (1) secretion of the effector followed by its re-entry into the plant cell or (2) mis-targeting of the protein to the cytoplasm through the well-established retrograde transport pathway [27,28].

Shan et al. [11] reported that infiltration of *P. sojae* RXLR effector Avr1b-1, expressed in *Pichia pastoris*, into Rps1b soybean leaves resulted in cell death. The ability of the infiltrated protein to trigger cell death via its intracellular R protein, Rps1b, argues that pathogen-encoded machinery and structures (i.e. haustoria) are not required for host cell transport. Surprisingly, the ability of recombinant Avr1b-1 to trigger cell death presumably by entering plant cells has not been exploited to study the contribution of the RXLR domain for instance by infiltration of mutant forms of Avr1b-1. This is possibly explained by the poor reproducibility of these experiments. Indeed, recombinant Avr1b-1 produced in *Escherichia coli* or *Sf9* insect cells failed to exhibit any biological activity on Rps1b soybean plants (K Valer-Saldana, PhD thesis, Ludwig-Maximilians-University of Munich, 2006).

A model for RXLR effector delivery into the host

Many key questions remain about how the RXLR domain functions in host targeting of effectors. What is the transport machinery of RXLR effectors? Is it derived from the pathogen or are the effectors exploiting host transport systems? Is similar machinery used to deliver oomycete and *Plasmodium* effectors? Despite these persisting questions, some reasonable assumptions about the translocation process can be made. For instance, it seems sensible to break down the transport process into two steps [24**]. First, the effectors are secreted outside the pathogen cell through the general secretory pathway using endoplasmic reticulum (ER) type signal peptides. Then, the secreted effectors are transported across a host-derived membrane, most probably the haustorial membrane, via the RXLR leader. In the GFP export experiments of Bhattacharjee et al. [24**], constructs with a mutated RXLR sequence accumulated GFP outside the malarial parasite but within the parasitophorous vacuole suggesting that the main function of the RXLR leader consists of transport across a host-derived membrane.

The structural similarity of the RXLR domain to the leader peptides of other protein transport systems suggests that comparable components may be required for the transport of RXLR effectors. Here, we suggest a model for effector delivery (illustrated in Figure 3) on the basis of the recurrent themes seen in these diverse protein targeting systems [29]. We propose that host translocation of RXLR effectors involves at least a RXLR leader binding protein, one or more additional chaperones, and a translocon, which could be of either pathogen or plant origin. Translocation into host cells initiates with the RXLR-binding protein recruiting mature effectors secreted via the general secretory pathway. In coordination with chaperones, the effector cargo is then transferred to a translocon embedded in the extrahaustorial membrane, and is then released across the membrane into the plant cytosol. The chaperones are important for maintaining the folding state of the transported effectors both before and after transit through the translocon.

At this point, this model is highly speculative, but this outline provides a useful hypothesis generator to help guide future research. Indeed, the model suggests immediate research avenues that would shed light on the translocation process, for instance the identification of RXLR binding proteins and effector chaperones.

Virulence functions of RXLR effectors

Although the RXLR effectors were identified by virtue of their avirulence activity on plant cultivars with cognate *R* genes, presumably these effectors confer a selective advantage to the pathogen when infecting susceptible hosts. Consistent with this idea, overexpressing *Acr1b-1* increased pathogen virulence on a compatible host (D Dou et al. abstract 552, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007). Unfortunately, the virulence function of RXLR effectors remains in great part unknown. Several RXLR effectors possess nuclear localization signals (NLS), suggesting that they manipulate host gene expression to secure an appropriate environment for infection [3,30].

Bos et al. [25**], in an effort to assign virulence-related functions to AVR3a, discovered that AVR3aKI suppresses the hypersensitive cell death induced by the major *P. infestans* elicitor INF1 in *N. benthamiana*. The cell death suppression activity of AVR3aKI exhibited some level of specificity. AVR3aKI did not suppress the cell death induced by other *P. infestans* effectors, like PiNPP1 and CRN2, which elicit distinct and antagonistic cell death signaling pathways compared with INF1 [31]. The biological relevance of this activity of AVR3aKI could be significant considering that suppression of innate immunity is a widespread function of biotrophic pathogen effectors, particularly the type III secretion system (TTSS) effectors of bacterial phytopathogens [32]. AVR3aKI could interfere with the avirulence activity of INF1 or other unidentified effectors that trigger hyper-
sensitivity using similar pathways as INF1 [25**]. Future works are needed to clarify these issues and determine whether cell death suppression is a common function among RXLR effectors.

**Outlook: too many effectors, too little time**

Considering that genome sequencing and annotation is nearing completion for five oomycete species *H. parasitica*, *P. capsici*, *P. infestans*, *P. ramorum*, and *P. sojae*, we are moving rapidly toward genome-wide catalogs of RXLR effectors. Already it is evident that the RXLR effector secretome of plant pathogenic oomycetes is much more complex than expected, with perhaps several hundred proteins dedicated to manipulating host cells [3,15**]. Tyler et al. [15**] reported 350 RXLR effectors each in the genomes of *P. ramorum* and *P. sojae* using iterated similarity searches. Analyses in our own laboratories using combinations of motif and hidden Markov model searches uncovered at least 50 candidates in the downy mildew *H. parasitica* and more than 200 each in *P. capsici*, *P. infestans*, *P. ramorum*, and *P. sojae* (S Kamoun et al. abstract 188, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007).

The task of tackling the study of so many effectors is daunting. One of the challenges is to establish “effectoromics” approaches, or global studies of effector function and activity. In addition to inferring function on the basis of homology and high throughput biochemical assays, other promising approaches include screens for suppressors of programmed cell death, phenotypic analysis of pathogen gene knockdowns, host transcriptional profiling in response to individual effectors, and three-dimensional structural determination of effectors (reviewed in [33,34]). Ultimately, comprehensive understanding of RXLR effector activities and the perturbations they cause in plants is crucial for understanding the molecular basis of oomycete pathogenesis and disease.

**Acknowledgements**

Research in SK’s laboratory is supported by NSF Plant Genome grant DBI-0211659 and State and Federal Funds appropriated to OARDC, the Ohio State University.
References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


