Unraveling the entry mechanism of oomycete and fungal effector proteins into host cells

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Abstract Oomycetes and fungi facilitate pathogenesis via secretion of effector proteins that have apoplastic and intracellular localizations. These effector proteins have a diverse array of functions that aid in pathogenesis, including modification of defense responses. In the oomycetes, well characterized effector proteins that can translocate into the host cells share a pair of conserved N-terminal motifs known as RXLR and dEER. The RXLR motif has been shown to mediate translocation of the oomycete avirulence proteins Avr1b and Avr3a into host cells. Detailed mutagenesis of the RXLR motif of Avr1b revealed that the motif is tolerant to several amino acid substitutions while retaining functional translocation activity, resulting in the definition of a broadened RXLR-like motif, [R,K,H]X[L/M/I/F/Y/W]X. This motif has been used to identify functional translocation motifs in several fungal effector proteins, AvrL567, Avr2, and AvrLm6. Effectors with both RXLR and RXLR-like motifs bind phosphatidylinositol-3-phosphate (PI-3-P) to mediate translocation via lipid raft mediated endocytosis. Mutations in RXLR or RXLR-like motifs result in loss of phospholipid binding and translocation by effectors. Effector entry into plant cells can be blocked by proteins and inositides that disrupt binding to PI-3-P, suggesting effector-blocking technologies that could be used in agriculturally important plant species.

Keywords

Avirulence gene, biotechnology development, effectors, endocytosis, RXLR-dEER motif, fungi, lipid raft, phosphatidylinositol-3-phosphate (PtdIns-3-P), phospholipids

Phytophthora

Phytophthora comprises a genus in the stramenopiles notorious for their devastating destruction of important crops (Erwin and Ribiera 1996). Though more closely related to diatoms and algae, oomycetes share similar mechanisms of pathogenesis to fungi (Meng et al. 2009). *Phytophthora sojae* accounts for US$1-2 billion of losses to soybean (*Glycine max*) production each year (Tyler 2007). *P. infestans*, the causative agent of the Irish potato famine, is responsible for losses to potato (*Solanum tuberosum*) exceeding US$6 billion dollars yearly (Haverkort et al. 2008). *P. palmivora* is responsible for 10-30% of loss in production of cacao beans annually (Guest 2007). Other oomycetes, such as *P. ramorum*, which causes sudden oak death syndrome on live oaks, are considered a significant threat to California's coastal forests (Rizzo et al. 2002). The genus contains over 106 species that cause significant damage to a large number of diverse plants (Erwin and Ribiero 1996).

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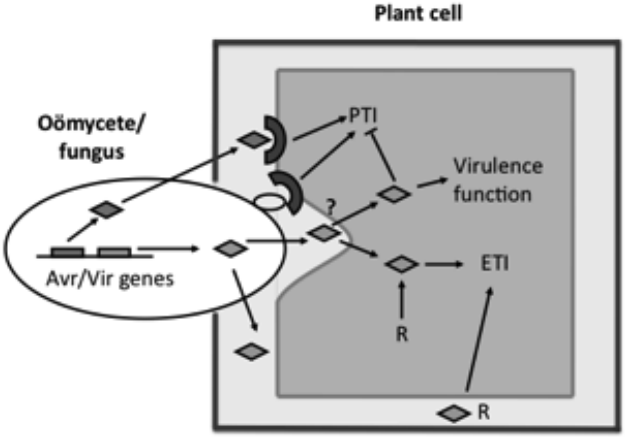
Pathogenesis

The outcome of pathogenesis by *P. sojae* on soybean is often decided in the first 24 hours of infection. In a compatible interaction, oomycete zoospores are attracted to soybean roots via isoflavones (Tyler et al. 1996). Encystment occurs on a root and penetration commences immediately. The germ tube initially transverses between root epidermal cells, into the root cortex (Tyler 2007). By 4 hours, haustoria are abundant along the hyphae, which have generally reached the 4th layer of the cortex (Tyler 2007). By 15 hours hyphae have generally reached the vascular tissue and cells are being penetrated directly (Tyler 2007). After approximately 16-24 hours of biotrophy *P. sojae* switches to a necrotrophic mode (Tyler 2007).

Haustoria form an intimate site of interaction between host plant cells and fungal or oomycete pathogens. Nutrient acquisition from host to pathogen occurs through the haustoria. Haustoria also mediate the delivery of some, possibly many, pathogen-encoded secreted proteins, known as effectors.

Effectors are diverse proteins secreted by many pathogens that modify host physiology to promote infection (Fig. 1). Some effectors remain in the apoplast, whereas others are targeted to the cytoplasm of host cells. Many effectors from bacteria are injected into host cells via the type III secretion machinery (Cornelis 2006). The mechanisms by which fungal and oomycete effectors enter host cells are less well characterized; recent progress in this area will be discussed below. Intracellular effectors such as AvrPtoB from the bacterium *Pseudomonas syringae* pv. *tomato* and ATR1 from the oomycete *Hyaloperonospora arabidopsidis* have the ability to suppress host immune responses

Fig. 1 ***Roles of effector proteins in pathogenesis. Pathogen Associated Molecular Patterns (PAMPs) trigger a defense response that mediates immunity (PAMP triggered immunity, PTI). Effectors, intracellular or extracellular, can be "recognized" directly or indirectly by R gene products to trigger a robust defense response that mediates immunity (effector triggered immunity, ETI). A subset of effectors can suppress PTI and ETI. Others have different functions in virulence***



(Abramovitch et al. 2006; Sohn et al. 2007). Some effectors such as Avr4 and Ecp6 from the fungus *Cladosporum fulvum* play functional roles in the apoplast (van den Burg et al. 2006; de Jonge et al. 2010). Plant defenses can be triggered by commonly occurring microbial molecules (Pathogen Associated Molecules Patterns; PAMPs) such as bacterial flagellin or fungal chitin (Felix et al. 1999; Boller et al. 1995). Defenses can also be triggered by pathogen effectors though the action of NBS-LRR resistance proteins (DeYoung and Innes 2006). In turn, pathogens have evolved effectors that can suppress both PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). The resulting interplay, or arms race, between pathogen and host to silence or amplify a defense response causes rapid evolution of the pathogen and host molecules involved.

RXLR effector reservoirs

Oomycete effectors are characterized by a conserved N-terminal motif, RXLR-dEER. The RXLR-dEER motif was identified after the sequencing of the genomes of *P. sojae* and *P. ramorum,* and ESTs of *P. infestans,* together with the cloning of the first oomycete avirulence genes, *P. sojae Avr1b-1*, *Hyaloperonospora arabidopsidis ATR13* and *ATR1*, and *P. infestans Avr3a* (Allen et al. 2004; Shan et al. 2004; Armstrong et al. 2005; Rehmany et al. 2005; Randall et al. 2005; Birch et al. 2006; Tyler et al. 2006). Comparison amongst these avirulence proteins and with predicted proteins that shared similarity to Avr1b resulted in the identification of the RXLR and dEER motifs (Rehmany et al. 2005; Birch et al. 2006; Tyler et al. 2006). A Hidden Markov Model (HMM) targeted to the 24 amino acids spanning the RXLR motif was utilized to identify large reservoirs of putative effector proteins in oomycete genomes: 396 and 374 RXLR proteins in the genomes of *P. sojae* and *P. ramorum*, respectively, and 550 and 134 in the recently sequenced *P. infestans* and *H. arabidopsidis* genomes, respectively (Jiang et al. 2008; Haas et al. 2009; Baxter et al. 2010). Subsequently, the RXLR motif was used as an aid to clone additional avirulence genes including *P. sojae Avr3a, Avr3c, Avr1a,* and *Avr4/6*, and *P. infestans Avr2, Avr4, AvrBlb1,* and *AvrBlb2* (Qutob et al. 2009; Dong et al. 2009; Dou et al. 2010; Lokossou et al. 2009; van Poppel et al. 2008; Vleeshouwers et al. 2008; Oh et al. 2009). The dEER (also referred to as EER) motif is also present in these avirulence proteins (except for ATR13) though the sequence, length, and position vary in every avirulence protein.

Oomycete effector protein translocation

The RXLR motif was initially postulated to be a protein translocation motif based on the fact that several avirulence proteins containing this motif, interacted with known intracellular resistance (*R*) gene products (Rehmany et al. 2005; Birch et al. 2006), and that the related *Plasmodium falciparum* PEXEL motif, RXLX[E/D/Q], facilitated translocation of *Plasmodium* effectors into erythrocytes (Marti et al. 2004).

In vivo assays using *P. sojae* Avr1b and *P. infestans* Avr3a demonstrated the physiological relevance of the RXLR and dEER motifs in protein translocation (Whisson et al. 2007; Dou et al. 2008). Alanine substitution mutations of the Avr1b and Avr3a RXLR and dEER motifs resulted in a loss of protein translocation inferred by a loss of the avirulence phenotype conferred by the genes in pathogen transformants (Whisson et al. 2007; Dou et al. 2008). Expression of these RXLR and/or dEER mutant proteins in the cytoplasm of host cells resulted in cell death associated with avirulence implying that the mutations do not affect the interaction of the Avr and R proteins (Whisson et al. 2007; Dou et al. 2008). The N-terminus of Avr3a was sufficient to translocate β-glucuronidase from *P. infestans* transformants into potato cells (Whisson et al. 2008). Even subtle mutations of the RXLR and dEER motifs of Avr3a to KMIK-DDK resulted in loss of translocation (Whisson et al. 2008).

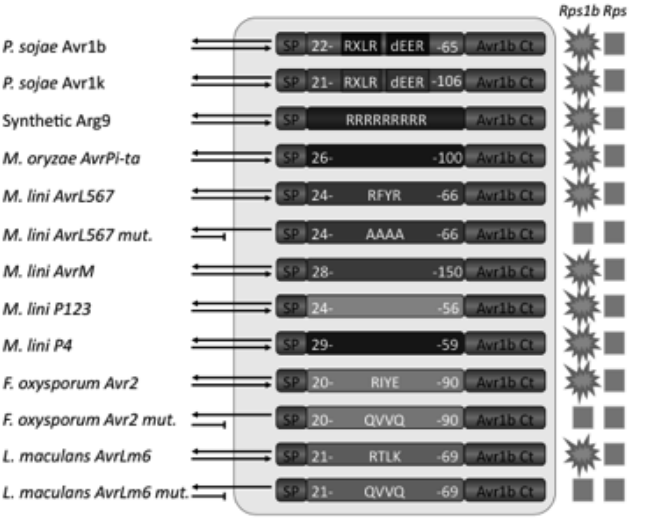
The validation of the RXLR-dEER motif in protein translocation *in vivo* was the first step to understanding the translocation of effector proteins. As a next step, purified Avr1b -GFP fusion proteins expressed in *E.coli,* added to soybean roots exogenously, were found to enter the root cells, revealing that no pathogen-encoded machinery was needed for entry (Dou et al. 2008; Kale et al. 2010). Similar findings were obtained for the oomycete effector proteins Avh331 and Avh5. Mutating the RXLR and/or dEER motifs of these proteins resulted in a loss of translocation of the GFP fusions into soybean cells (Kale et al. 2010). A novel double barrel particle bombardment assay was used to show that pathogen-independent translocation also occurs in soybean leaf cells and onion epidermal cells. Furthermore, full length Avr1k and Avr1b proteins, when applied exogenously, had the ability to enter soybean leaf cells and produce a cell death response in the presence of Rps1k and Rps1b, respectively (Shan et al. 2004; Kale et al. 2010). Interestingly, Avr1b contains two RXLR motifs, one predicted to be functional by HMM and the other not (Dou et al. 2008). Mutation of the non-canonical RXLR motif of Avr1b still resulted in translocation of GFP implying that the surrounding sequences are important for the correct function of the RXLR motif (Dou et al. 2008). Oomycete effector proteins also could translocate into other cell types such as human airway epithelial cells (Kale et al. 2010). That entry was also reliant on the RXLR motif.

Fungal effector protein translocation

In fungi there are no obvious conserved translocation motifs among intracellular effectors comparable to the oomycete RXLR and dEER motifs, except for effectors from powdery mildew fungi (Godrey et al. 2010). Using the double barrel gene gun assay, detailed mutagenesis of the RXLR motif of oomycete effector Avr1b was performed to identify which amino acid substitutions in the motif resulted in loss of translocation activity (Kale et al. 2010). Activity was retained when the first arginine was mutated to lysine or histidine, but not glutamine. The leucine could be substituted with a wide variety of large hydrophobic amino acids, but not alanine or glycine. The second arginine could be substituted with a wide array of amino acids ranging from glycine to glutamine. Based on this analysis of Avr1b, a much broader "RXLR-like" motif, [R,K,H]X[L/M/I/F/Y/W]X, was defined (Kale et al. 2010). Concurrently the N-termini of several known fungal intracellular effectors were fused to the C-terminus of Avr1b and tested using the soybean bombardment re-entry assay (Fig. 2) (Kale et al. 2010). In many cases the N-termini of the fungal effectors possessed cell entry activity. Each of these N-terminal cell entry domains contained RXLR-like motif(s). Mutation of the RXLR-like motifs resulted in identification of functional motifs required for translocation in the bombardment re-entry assay. In the case of *Melampsora lini* AvrL567 the N-terminal motif RFYR was required for translocation (Kale et al. 2010; Rafiqi et al. 2010). The N terminus of *M. lini* AvrM containing several RXLR-like motifs was shown to mediate translocation (Kale et al. 2010; Rafiqi et al. 2010). Rafiqi et al. (2010) were able to narrow down the translocation domain to a region containing three RXLR-like sequences. *Fusarium oxysporum* Avr2 contained one functional motif (RIYER) and one non-functional motif (RMLH) (Kale et al. 2010). *Leptosphaeria maculans* AvrLm6 also contained an RXLR-like motif (RYWT) that mediated translocation (Kale et al. 2010).

A drawback of the high sequence redundancy of the RXLR-like motifs is the large number of false positives produced when the motif is used for bioinformatic searches. Thus experimental assays are essential for identifying which motif (if any) is actually functional in a potential effector protein.

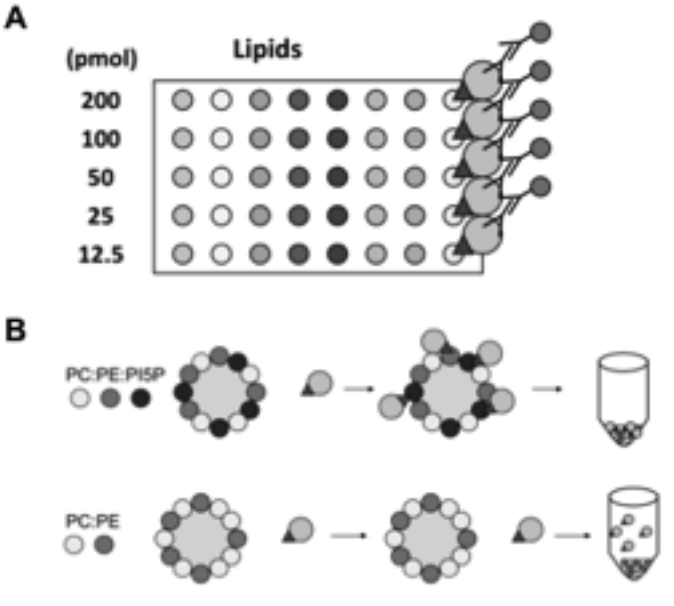
Fig. 2 ***Oomycete and fungal cell entry domains identified by the soybean bombardment re-entry assay. Arrows indicate translocation across the membrane. Cell death occurs in leaf tissue containing Rps1b only when the C-terminal domain of Avr1b re-enters the cells after secretion. When Rps1b is absent (Rps) no cell death occurs. Explosion symbol indicates cell death due to hypersensitive response (HR), blank symbol signifies a lack of cell death***



Phospholipid binding

As described above, several oomycete and fungal effectors can translocate into a variety of cell types without any pathogen-encoded machinery via RXLR or RXLR-like motifs. Phosphatidylinositol-3-phosphate (PtdIns-3-P) has been shown to mediate the entry of these effectors into host cells (Kale et al. 2010). So far a total of 3 oomycete and 3 fungal effector proteins have been found to bind PtdIns-3-P in a lipid filter assay and a liposome-binding assay (Kale et al. 2010) (Fig. 3). In each case, mutation of the functional RXLR or RXLR-like motif required for cell entry also resulted in a loss of PtdIns-3-P binding (Kale e al. 2010). A strong correlation thus exists between cell entry activity and binding of PtdIns-3-P for these effectors.

Fig. 3 ***Assaying lipid binding. A) The lipid filter assay is a useful tool to perform an initial screen against many different lipids. If used appropriately, the assay can provide a semi-quantitative measure of the strength of protein-lipid binding. Protein bound to a lipid(s) is detected through HRP-linked antibodies. B) The liposome binding assay is used to validate a potential interaction. The assay provides greater physiological relevance and confidence for an interaction. When an interaction occurs the bound protein pellets with the liposomes after high speed centrifugation. Protein that does not interact with liposomes stays in the supernatant. Binding to liposomes can be quantitated to obtain dissociation constants***



The presence of PtdIns-3-P on the outer leaflet of plant cells was demonstrated using three specific PtdIns-3-P-binding proteins, 2xFYVE, VAM7p-PX domain, and PEPP1-PH domain fused to a fluorescent protein (GFP and mCherry) (referred to from here as biosensors) (Kutateladze et al. 2001; Lee et al. 2006; Dowler et al. 2000). Incubation of these probes with soybean cells at 4°C, to prevent endocytosis, resulted in binding of the PtdIns-3-P biosensors to the outer leaflet the plasma membranes of the cells (Kale et al. 2010). In contrast, a PtdIns-4-P biosensor did not bind the membranes. The same experiments demonstrated that PtdIns-3-P was also on the surface of human lung epithelial cells, albeit in punctate patterns that differ from the uniform labeling of the plant membranes.

Mechanism of entry

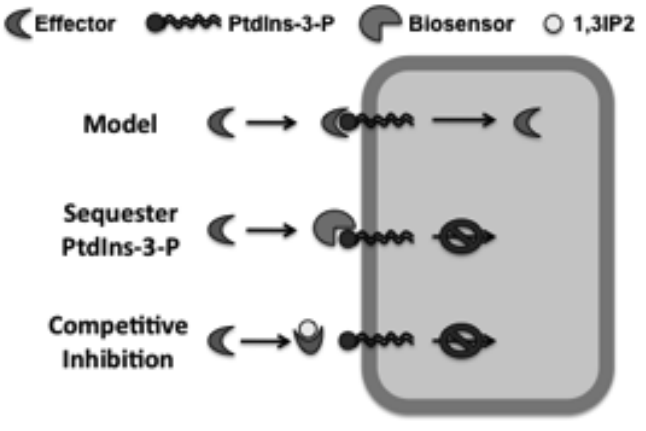
When RXLR effector-GFP fusions are rapidly internalized in human airway epithelial cells, the proteins can be visualized in small vesicle structures (Kale et al. 2010). Similar vesicle structures can be visualized when effector-GFP fusion proteins are incubated with soybean suspension culture cells (Kale et al. 2010). Filipin and nystatin, that disrupt the formation of lipid rafts and thus raft-mediated endocytosis, block entry of the effector-GFP fusions (Kale et al. 2010). Inhibitors of clathrin-mediated endocytosis, macropinocytosis, and flippases did not inhibit internalization. These results indicate that the RXLR and RXLR-like effectors likely bind to PtdIns-3-P found in lipid rafts and this binding results in endocytosis of the effectors.

Blocking effector entry

Effector entry mediated by PtdIns-3-P can be blocked by two strategies (Fig. 4). The first strategy utilizes the biosensor proteins that have a strong affinity to PtdIns-3-P. These biosensor proteins, when pre-incubated with soybean root cells or human airway epithelial cells, prevented entry of Avr1b-GFP and AvrL567-GFP, presumably by sequestering PtdIns-3-P (Kale et al. 2010). The second strategy is based on competitive inhibition of the binding of Avr1b-GFP and AvrL567-GFP to PtdIns-3-P by 1,3-inositol-bisphosphate (1,3IP2). Incubation of Avr1b-GFP and AvrL567-GFP with soybean root cells or human airway epithelial cells in the presence of 500 µM 1,3-inositol-bisphosphate (1,3IP2) prevented internalization of effector proteins (Kale et al. 2010). Liposome binding assays validated that 1,3IP2 could block the binding

of Avr1b-GFP and AvrL567-GFP to PtdIns-3-P (Kale et al. 2010). These experiments confirm that PtdIns-3-P binding is required for cell entry and suggest strategies for blocking the infection of crop plants by fungal and oomycete pathogens that depend on RXLR-like effectors.

Fig. 4 ***Simplified model of effector entry and effector-blocking strategies. Certain oomycete and fungal effectors bind PtdIns-3-P in lipid raft regions on the outer leaflet of plant and animal cells to mediate translocation into host cells. Entry mechanism involves endocytosis. The mechanism of escape from these endosomes is currently unknown. Access to PtdIns-3-P may be blocked through the use of Ptd-3-P-binding biosensor proteins. The binding pocket of the effectors may be occupied by a small molecule such as 1,3IP2, thereby competing against binding by PtdIns-3-P on the membrane***



Cereal rusts perspectives

Although dissimilar phylogenetically, oomycetes and cereal rust pathogens share some remarkably similar mechanisms of pathogenesis as a result of convergent evolution. Both oomycetes and cereal rust pathogens utilize haustoria for nutrient acquisition and, presumably, as a site for effector delivery. At least one effector from a rust fungus, and likely many more, translocate into host plant cells via RXLR-like sequences and PI-3-P. We speculate that inhibiting translocation of effector proteins by targeting PI-3-P and/or RXLR-like sequence motifs may provide broad spectrum protection against diverse fungal and oomycete pathogens, including multiple species of rust fungi. For example, wheat might be protected simultaneously against *P. striiformis*, *P. graminis* f. sp. *tritici* and *P. triticina*.

Acknowledgements

This work was supported by the Agriculture and Food Research Initiative of the USDA National Institute of Food and Agriculture, grant number #2007-35319-18100 and by the U.S. National Science Foundation, grant number IOS-0924861. S.D.K. was supported in part by a U.S. National Science Foundation predoctoral fellowship.

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