

# Structure and Function of RXLR Effectors of Plant Pathogenic Oomycetes

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## 1 Introduction

A diverse number of plant pathogens, including bacteria, fungi, oomycetes and nematodes, secrete effector proteins to different cellular compartments of their hosts to modulate plant defense circuitry and enable parasitic colonization (Birch et al., 2006; Chisholm et al., 2006; Kamoun, 2006; O'Connell and Panstruga, 2006). The current paradigm in the study of plant-microbe interactions is that unraveling 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 and the biochemical activities that enable these proteins to perturb host defense processes and facilitate pathogenicity (Chisholm et al., 2006). In contrast, little is known about the biology of effectors of eukaryotic plant pathogens. Nonetheless, this area of research is progressing rapidly as illustrated by the recent identification of effectors from the flax rust and barley powdery mildew fungi (Catanzariti et al., 2006; Dodds et al., 2004; Ridout et al., 2006), the oomycetes *Phytophthora* and *Hyaloperonospora* (Allen et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005; Shan et al., 2004), as well as root-knot nematodes (Huang et al., 2006a; Huang et al., 2006b).

Oomycetes form a distinct group of eukaryotic microorganisms that includes some of the most notorious pathogens of plants (Kamoun, 2003). Research on oomycete effectors has accelerated in recent years due in great part to the availability of resources stemmed from genomics. The emerging picture is complex and fascinating. Oomycetes are now thought to secrete hundreds of effector proteins belonging to two classes that target distinct sites in the host plant (Birch et al., 2006; Kamoun, 2006; Tyler et al., 2006). Apoplastic effectors are secreted into the plant extracellular space, whereas cytoplasmic effectors are translocated inside the plant cell, where they target different subcellular compartments (Birch et al., 2006; Kamoun, 2006). Several apoplastic effectors have been determined

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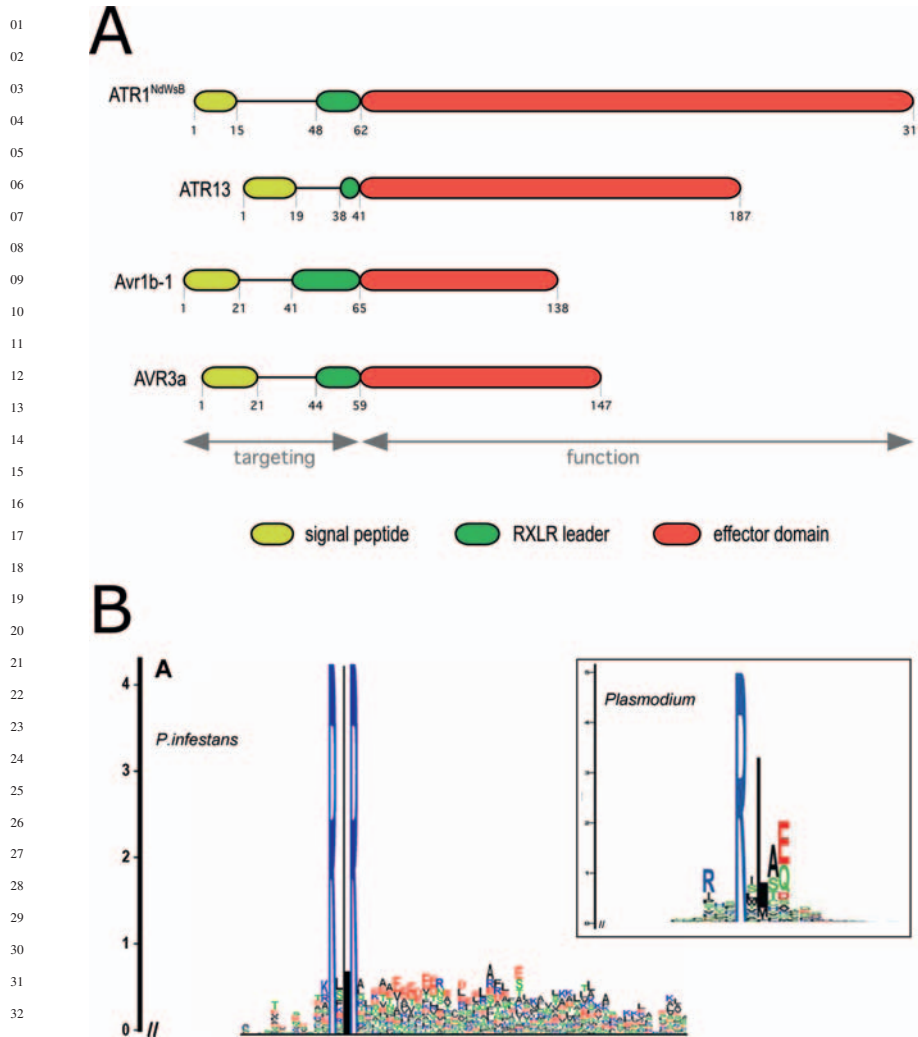
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01 to function as inhibitors of host enzymes, such as proteases and glucanases (Rose  
02 et al., 2002; Tian et al., 2005; 2004). They are thought to contribute to counter-  
03 defense by disabling host enzymes that accumulate in response to pathogen infec-  
04 tion. In contrast, the biochemical activities of cytoplasmic effectors remain poorly  
05 understood. Oomycete cytoplasmic effectors have been discovered first through  
06 their avirulence (Avr) function, i.e. their ability to trigger hypersensitive cell death  
07 on specific host genotypes that carry particular disease resistance (*R*) genes (Allen  
08 et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005; Shan et al., 2004). The  
09 function of these effectors in plants that do not carry the cognate *R* genes remains  
10 largely unknown (Kamoun, 2006).

11 This review summarizes recent findings on the structure and function of the  
12 RXLR class of oomycete effectors (Birch et al., 2006; Kamoun, 2006). These effec-  
13 tors function inside host cells and are characterized by a highly conserved region  
14 defined by the invariant sequence RXLR. This review will cover two main topics of  
15 RXLR effector research: trafficking and function.

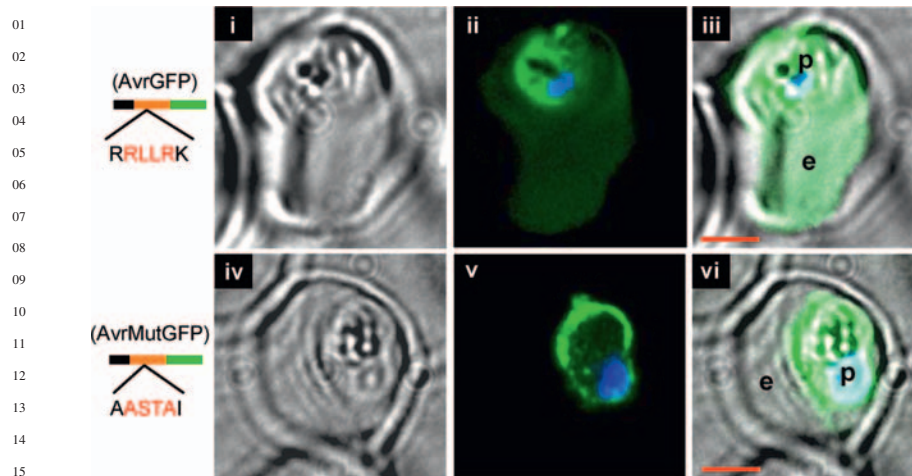
## 18 2 The RXLR Sequence Defines a Conserved Domain 19 of Oomycete Avr Proteins

21 Four oomycete *Avr* genes, *ATR1<sup>NdWsB</sup>* and *ATR13* from the downy mildew  
22 *Hyaloperonospora parasitica*, *Avr1b-1* from the soybean pathogen *Phytophthora*  
23 *sojiae*, and *Avr3a* from *P. infestans*, have been cloned recently (Allen et al., 2004;  
24 Armstrong et al., 2005; Rehmany et al., 2005; Shan et al., 2004). The R proteins  
25 that target *ATR1<sup>NdWsB</sup>*, *ATR13*, and *AVR3a<sup>KI</sup>* belong to the intracellular class  
26 of NBS-LRR (nucleotide binding site and leucine-rich repeat domain) proteins,  
27 suggesting that recognition of these *Avr* proteins occurs inside the plant cytoplasm  
28 (Allen et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005). Indeed, when  
29 directly expressed *in planta* by transient transformation, *AVR3a<sup>KI</sup>*, *ATR1<sup>NdWsB</sup>*, and  
30 *ATR13* did not require a signal peptide sequence to trigger hypersensitivity, and  
31 are therefore recognized inside the plant cytoplasm (Allen et al., 2004; Armstrong  
32 et al., 2005; Rehmany et al., 2005). How these effectors are translocated into host  
33 cells during infection is unknown but a conserved sequence centered on the RXLR  
34 motif might be implicated (Birch et al., 2006; Kamoun, 2006; Rehmany et al., 2005)  
35 (Fig. 1). All four oomycete *Avr* proteins carry a signal peptide followed by the  
36 RXLR region, which occurs within the N-terminal ca. 60 amino acids of these pro-  
37 teins (Rehmany et al., 2005). So far, definite elucidation of the function of the RXLR  
38 region has not been obtained, but the prevailing hypothesis is that RXLR functions  
39 in delivery of the effectors inside host cells (Birch et al., 2006; Kamoun, 2006;  
40 Rehmany et al., 2005). Indeed, this motif is similar in sequence and position to  
41 a host cell-targeting signal (HT/Pexel motif) that is required for translocation of  
42 proteins from malaria parasites (*Plasmodium* species) into the cytoplasm of host  
43 red blood cells (Hiller et al., 2004; Marti et al., 2004). Interestingly, the cellular  
44 biology of *Plasmodium* infection of host blood cells shares some commonalities  
45



34 **Fig. 1** (A) Domain organization of cytoplasmic RXLR effectors. Schematic drawings of  
 35 ATR1<sup>NdwsB</sup> and ATR13 of *Hyaloperonospora parasitica*, Avr1b-1 of *Phytophthora sojae*, and  
 36 AVR3a of *P. infestans*. The numbers under the sequences indicate amino-acid positions. The high-  
 37 lighted RXLR domain includes the RXLR sequence itself and the downstream DEER sequence.  
 38 The *gray arrows* distinguish the regions of the effector proteins that are involved in secretion  
 39 and targeting from those involved in effector activity. (B) Similarity between the RXLR motif of  
 40 oomycetes and the HT/Pexel motif of *Plasmodium falciparum*. Sequence logos were derived from  
 41 *P. infestans* and *P. falciparum* effector proteins. Adapted from Bhattacharjee et al. (2006)

42 with oomycete infection structures like haustoria (Bhattacharjee et al., 2006).  
 43 During the blood stages of infection, *Plasmodium* invades mature erythrocytes and  
 44 develops within a parasitophorous vacuolar membrane (PVM) derived from an  
 45 invagination of the erythrocyte membrane. While residing within the PVM, the



**Fig. 2** The *Phytophthora infestans* AVR3a RXLR leader region mediates the export of the green fluorescent protein (GFP) from the *Plasmodium falciparum* parasite to the host erythrocyte. Erythrocytes expressing wild-type or mutated RXLR region of AVR3a (residues 21 to 69) fused to a *P. falciparum* signal peptide and GFP. Panels ii and v represent fluorescence images, i and iv bright field images, and iii and vi merged images. Parasite (p), erythrocyte (e), Hoechst stained nucleus (blue), scale bar represents 2  $\mu$ m. Adapted from Bhattacharjee et al. (2006)

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parasite exports effector proteins to the erythrocyte resulting in reprogramming of the invaded blood cell. Thus, both *Plasmodium* and oomycetes need to translocate effector proteins across a host-derived membrane during colonization of host cells suggesting that the conserved sequence motif might be required to overcome this comparable hurdle (Bhattacharjee et al., 2006). Although direct demonstration that the oomycete RXLR domain is a host-targeting signal has not been reported, a number of experiments that are consistent with this hypothesis have been performed. The next three sections describe these experiments.

The discovery that host-targeted proteins from *Phytophthora* and *Plasmodium* share a positionally conserved sequence begged the examination of functional conservation. Bhattacharjee et al. (2006) demonstrated that the *P. infestans* AVR3a<sup>K1</sup> RXLR leader sequence is sufficient to mediate the export of the green fluorescent protein (GFP) from the *Plasmodium falciparum* parasite to the host red blood cell (erythrocyte) (Fig. 2).

### 3 The *Phytophthora* RXLR Domain Mediates Host Targeting in *Plasmodium*

Mutations in the RXLR consensus abolished export. In addition, the RXLR region of PH001D5, another candidate effector identified computationally from *P. infestans* sequences, was also functional in *Plasmodium*. Interestingly, regions upstream and downstream of the RXLR motif were required for host targeting, suggesting that

01 the RXLR sequence defines a ca. 30 amino acid leader sequence. Thus the targeting  
02 domain extends beyond the RXLR sequence. Consistent with this view, sequence  
03 biases were observed in the regions flanking RXLR, particularly a high rate of E/D  
04 residues following RXLR. In summary, these findings suggest that plant and animal  
05 eukaryotic pathogens share similar secretory signals for effector delivery into host  
06 cells (Bhattacharjee et al., 2006). At this stage, it is unclear whether this functional  
07 conservation reflects conserved export machinery between these divergent eukary-  
08 otes (see below for further discussion on this topic). However, the functional analogy  
09 with bacterial type III secretion system is remarkable. In both cases, export signals  
10 are highly conserved across unrelated pathogen species but the effector secretome  
11 that is delivered to the host is highly divergent (Bhattacharjee et al., 2006).

#### 14 **4 The RXLR Domain Is Not Required for Effector Activities**

16 The view that the RXLR region functions in translocation into host cells sug-  
17 gests that RXLR effectors are organized into two main functional domains  
18 (Kamoun, 2006) (Fig. 1). The first domain encompassing the signal peptide and  
19 RXLR leader functions in secretion and targeting, while the remaining C-terminal  
20 domain carries the effector activity. This model predicts that the RXLR region  
21 should not be required for activity when the effector is expressed inside host cells.  
22 Indeed, Bos et al. (2006) recently showed that mutation of *P. infestans* AVR3a<sup>KI</sup>  
23 RXLR sequence into AXAA did not interfere with induction of R3a hypersensitivity  
24 when the protein is directly expressed in *N. benthamiana* leaves. In fact, deletion  
25 analyses of AVR3a<sup>KI</sup> showed that the C-terminal 75-amino acid, which excludes  
26 the RXLR region but includes the two polymorphic amino acids K<sup>80</sup> and I<sup>103</sup> that  
27 are mutated in the nonfunctional allele, was sufficient for avirulence function when  
28 expressed directly inside plant cells (Bos et al., 2006). These findings are consistent  
29 with the view that the N-terminal region of AVR3a<sup>KI</sup> and other RXLR effectors is  
30 involved in secretion and targeting but is not required for effector activity.

#### 32 **5 The C-Terminal Region of RXLR Effectors Is Typically More 33 Polymorphic than the Signal Peptide and RXLR Domains**

36 Higher levels of polymorphisms, particularly non-synonymous substitutions, have  
37 been detected in the C-terminal regions of RXLR effectors than in the signal pep-  
38 tide and RXLR leader region. For example, the C-terminal regions of *H. parasit-*  
39 *ica* ATR1 and ATR13 exhibit higher levels of non-synonymous polymorphisms  
40 than the N-terminal regions, suggesting that the effector activity is localized to  
41 the C-terminal domain (Allen et al., 2004; Rehmany et al., 2005). Also, two out  
42 of the three polymorphic residues between the two *Avr3a* alleles of *P. infestans*,  
43 amino acids 80 and 103, are located in the C-terminal effector domain (Armstrong  
44 et al., 2005). The observation that these effectors are under diversifying selection  
45 is consistent with the view that pathogen effectors with avirulence functions are

01 caught in a coevolutionary arms race with host factors, particularly their cognate R  
02 genes (Allen et al., 2004). Signatures of selection are expected in regions involved in  
03 effector activity rather than targeting. Thus, the observation that the RXLR domain  
04 is less polymorphic than the C-terminal region of RXLR proteins is consistent with  
05 the view that it is not exposed to selection pressure by host defenses and that it  
06 functions in targeting.

## 08 **6 Can RXLR Effectors Enter Host Plants in the Absence** 09 **of the Pathogen?**

11  
12 Whether RXLR effectors require pathogen machinery or structures (e.g., haustoria)  
13 to enter plant cells is currently unclear. In our laboratory, we have failed so far  
14 to trigger R3a hypersensitivity using recombinant AVR3a<sup>KI</sup> proteins (unpublished  
15 data). Shan et al. (2004) reported that infiltration of *P. sojae* RXLR effector Avr1b-  
16 1, produced in *Pichia pastoris*, into Rps1b soybean leaves resulted in cell death.  
17 However, it is unknown (although highly likely) whether Rps1b is a cytoplasmic  
18 protein and confirmation of *Avr1b-1* activity by *in planta* expression inside soybean  
19 cells has not been reported yet. Clearly, it would be highly informative to test Avr1b-  
20 1 recombinant proteins mutated in the RXLR sequence using the infiltration assay  
21 of Shan et al. (2004).

22 The issue of host translocation is also relevant to cytoplasmic effectors of fungal  
23 pathogens. In their work with Avr proteins of the flax rust fungus *Melampsora lini*,  
24 Catanzariti et al. (2006) concluded that the avirulence protein AvrM enters plant  
25 cells in the absence of the pathogen. AvrM is a secreted protein with a canonical  
26 signal peptide that is recognized inside flax cells in an *M* gene dependent man-  
27 ner. In addition, *Agrobacterium tumefaciens*-mediated expression of a full-length  
28 AvrM construct in flax plants carrying the *M* gene resulted in hypersensitive cell  
29 death. Interestingly, similar constructs carrying the ER retention sequence HDEL  
30 were unable to trigger cell death. The authors concluded from this experiment  
31 that AvrM first exits plant cells into the apoplast and then reenters through an  
32 unknown process (Catanzariti et al., 2006). We are not in absolute agreement with  
33 this interpretation and are of the opinion that this experiment is inconclusive in  
34 evaluating whether AvrM can enter plant cells in the absence of the pathogen. An  
35 equally plausible explanation is that the signal peptide carrying AvrM is translo-  
36 cated back from the ER into the cytosol through the well-established retrograde  
37 transport pathway (Brandizzi et al., 2003; Di Cola et al., 2001). In such case, the  
38 HDEL motif would retain the protein into the ER and prevent retrograde translo-  
39 cation. In summary, although the experiments of Catanzariti et al. (2006) suggest  
40 that AvrM needs to shuttle through the ER, perhaps to achieve maturation, infil-  
41 tration experiments with purified AvrM proteins are necessary to obtain conclusive  
42 evidence as to the ability of this protein to enter host cells in the absence of the  
43 pathogen. Such data has been obtained conclusively with ToxA, a host-selective  
44 toxin produced by the plant pathogenic fungus *Pyrenophora tritici-repentis*. Man-  
45 ning and Ciuffetti (2005) demonstrated elegantly that a recombinant ToxA protein

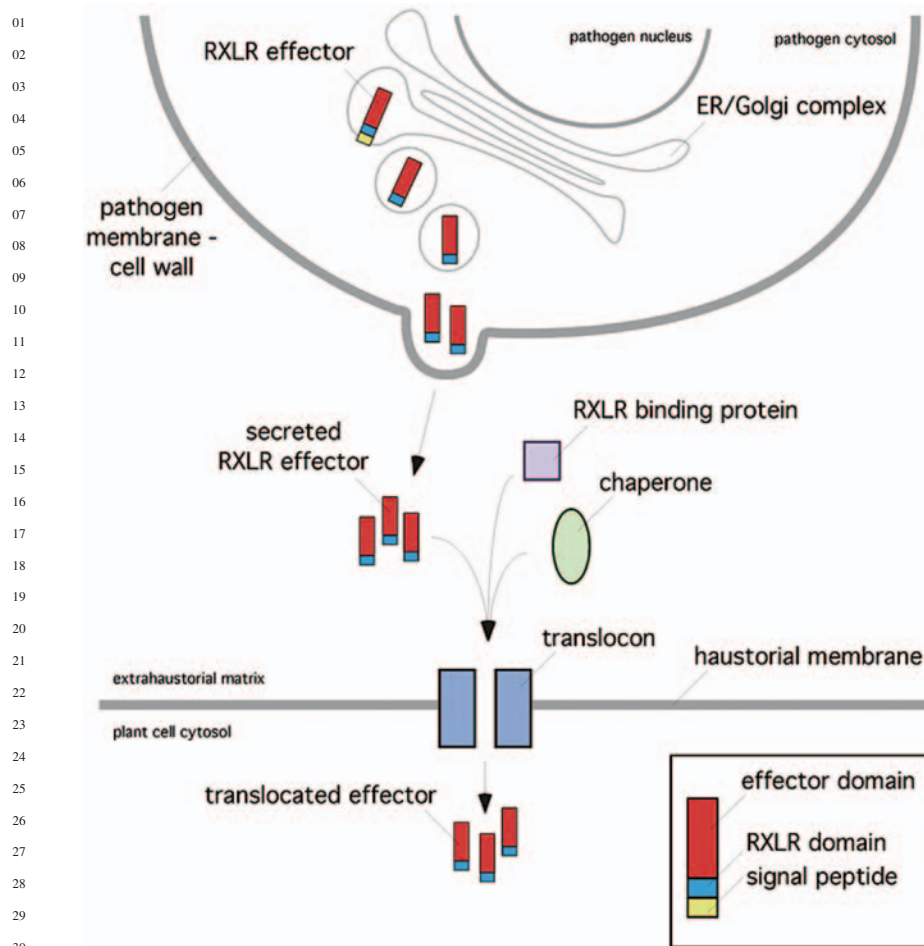
01 tagged with GFP internalizes inside sensitive wheat cells, where it localizes to  
02 cytoplasmic compartments and chloroplasts. This implies that effector proteins can  
03 translocate inside host cells in the absence of pathogen machinery, probably using  
04 host-derived machinery. Host-translocation of ToxA is likely to occur via receptor-  
05 mediated endocytosis, and might implicate the RGD sequence that is identical to  
06 a cell adhesion motif of mammalian extracellular matrix proteins (Manning and  
07 Ciuffetti, 2005).

08 In our own work with AVR3a<sup>KI</sup>, we noted that high levels of expression of a full-  
09 length construct resulted in R3a-dependent hypersensitivity (Bos et al., 2006). These  
10 experiments are not particularly informative since they could be equally explained  
11 by (1) mis-targeting of the protein to the cytoplasm or (2) secretion of AVR3a<sup>KI</sup>  
12 followed by re-entry of the protein inside the plant cell resulting in R3a activation.  
13 Also, in other experiments, attempts to exploit this full-length AVR3a<sup>KI</sup> to evaluate  
14 the role of the RXLR motif in translocation inside host cells were inconclusive  
15 since a RXLR to AXAA mutant retained the ability to elicit R3a response (Bos  
16 et al., 2006).

## 17 18 **7 A Model for RXLR Effector Delivery into the Host**

19  
20 The process through which the RXLR leader sequence might function in host tar-  
21 geting of effectors remains unknown. Many key questions still need to be addressed.  
22 What is the export machinery of effectors in eukaryotic pathogens? Is it derived from  
23 the pathogen or are the effectors exploiting host transport systems? Is the export  
24 machinery conserved between oomycetes and *Plasmodium*? Despite these persist-  
25 ing questions, some reasonable assumptions about the translocation process can be  
26 made. For instance, it seems sensible to break down the export process into two steps  
27 (Bhattacharjee et al., 2006). First, the effectors are secreted outside the pathogen  
28 cell through the general secretory pathway and endoplasmic reticulum (ER) type  
29 signal peptides. Then, the secreted effectors are transported across a host-derived  
30 membrane, most likely the haustorial membrane, via the RXLR leader. In the GFP  
31 export experiments of Bhattacharjee et al. (2006), constructs with a mutated RXLR  
32 sequence accumulated GFP outside the parasite but within the parasitophorous vac-  
33 uole suggesting that the main function of the RXLR leader consists of transport  
34 across this host-derived membrane.

35 Here, we propose a model for effector delivery (illustrated in Fig. 3). The model  
36 is based on the fact that mechanisms of protein transport across membranes follow  
37 canonical processes involving recurrent themes (Wickner and Schekman, 2005). We  
38 propose that host translocation of the effectors via the RXLR leader involves at least  
39 a RXLR leader binding protein, one or more chaperones, and a translocon, which  
40 could be of either pathogen or plant origin. Translocation into host cells initiates  
41 with the RXLR binding protein recruiting secreted mature effectors in coordination  
42 with the chaperones. The effector cargo is then transferred to a translocon embedded  
43 in the haustorial membrane, and is then released across the membrane into the plant  
44 cytosol. The chaperones are important for maintaining the folding state of the trans-  
45 ported effectors both prior and after transit through the translocon.



**Fig. 3** A hypothetical model for RXLR effector secretion and delivery into host cells (see text for details)

At this point, this model is highly speculative. But our purpose is to outline a useful working model to serve as a hypothesis generator and 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.

## 8 Virulence Functions of RXLR Effectors

The virulence function of RXLR effectors, i.e. their activity in plants that do not carry cognate *R* genes, remains in great part unknown. Bos et al. (2006), in an effort to assign virulence-related functions to AVR3a, discovered that AVR3a<sup>K1</sup> suppresses



01 the hypersensitive cell death induced by the major *P. infestans* elicitor INF1 in *Nico-*  
02 *tiana benthamiana*. The cell death suppression activity of AVR3a<sup>KI</sup> exhibited some  
03 level of specificity. AVR3a<sup>KI</sup> did not suppress the cell death induced by other *P.*  
04 *infestans* effectors, like PiNPP1 and CRN2, which elicit distinct and antagonistic  
05 cell death signaling pathways compared to INF1 (Kanneganti et al., 2006). The  
06 biological relevance of this activity of AVR3a<sup>KI</sup> is unknown but could be signifi-  
07 cant considering that suppression of innate immunity is a widespread function of  
08 plant pathogen effectors, particularly the type III secretion system (TTSS) effectors  
09 of bacterial phytopathogens (Espinosa and Alfano 2004). AVR3a<sup>KI</sup> could interfere  
10 with the avirulence activity of INF1 or other unidentified effectors that trigger hyper-  
11 sensitivity using similar pathways as INF1 (Bos et al., 2006). Future work is needed  
12 to clarify these issues and determine whether cell death suppression is a common  
13 function among RXLR effectors.

## 16 9 Outlook: Too Many Effectors, Too Little Time

18 Considering that five oomycete species, *H. parasitica*, *P. capsici*, *P. infestans*, *P.*  
19 *ramorum*, and *P. sojae*, are undergoing genome sequencing and annotation, we are  
20 moving rapidly toward genome-wide catalogues of RXLR effectors. Already it is  
21 evident that the RXLR effector secretome of plant pathogenic oomycetes is much  
22 more complex than expected, with perhaps several hundred proteins dedicated to  
23 manipulating host cells (Kamoun, 2006; Tyler et al., 2006). Recent estimates of  
24 RXLR effectors based on the draft genome sequences of *P. sojae* and *P. ramorum*  
25 range from ca. 150 to 350 per genome (Bhattacharjee et al., 2006; Tyler et al., 2006).  
26 The task of tackling the study of so many effectors is daunting. One of the challenges  
27 is to establish “effectoromics” approaches, or global studies of effector function  
28 and activity. Ultimately, comprehensive understanding of RXLR effector activities  
29 and the perturbations they cause in plants is a precondition for understanding the  
30 molecular basis of oomycete pathogenesis and disease.

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