Secretion, delivery and function of oomycete effector proteins
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Oomycetes are responsible for multi-billion dollar damages in aquaculture, agriculture and forestry. One common strategy they share with most cellular disease agents is the secretion of effector proteins. Effectors are molecules that change host physiology by initiating and allowing an infection to develop. Oomycetes secrete both extracellular and intracellular effectors. Studying secretion, delivery and function of effectors will hopefully lead to alternative control measures, which is much needed as several chemicals to control plant and animal pathogenic oomycetes cannot be used anymore; due to resistance in the host, or because the control measures have been prohibited as a result of toxicity to the environment and/or consumers. Here the latest findings on oomycete effector secretion, delivery and function are discussed.

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General introduction
Oomycetes contain some of the most devastating pathogens of animals and plants, causing enormous economic and environmental damage in natural and cultured ecosystems [1,2]. In order to infect their hosts these parasites rely on their effector repertoires. Effectors are secreted proteins of the pathogen that can alter the host and which advance the infection process. A large number of effectors are located at the interface between pathogen and its host and fulfil a function on the outside of the host cell. Such effectors are often classified as extracellular effectors, or apoplastic effectors in the case of plant pathogenic oomycetes. At the same time several other effectors of oomycetes are able to translocate into host cells where they can, for example, interfere with defence responses of the host.

These effectors are often referred to as intracellular effectors. Two important groups of translocated effectors are the ‘RxLR-effectors’ and the ‘crinklers’, which are found in abundance in many plant pathogenic oomycetes. The mechanism of translocation or delivery of the intracellular effectors is under intense investigation and several routes of entry have been proposed. Here we will discuss recent advances in understanding oomycete effector secretion, delivery and function.

Extracellular effectors
Extracellular effectors are defined as small molecules and proteins [3] secreted by pathogens into the host extracellular space where they alter the host-cell structure and function [4]. Extracellular (or apoplastic) effectors can be subdivided into two major categories: firstly effectors mediating protection against host defences and secondly effectors mediating invasion (Figure 1).

The first category of extracellular effectors comprises mainly protease-inhibitors and glucanase-inhibitors that either specifically target certain plant proteins or work in a broader fashion. For example, the Phytophthora infestans proteins EPI1 and EPI10 have been found specifically to inhibit the tomato subtilisin-like protease P69B, whereas EPIC1 and EPIC2B can target several different cysteine proteases, namely PIP1, C14 and Rcr3, besides other apoplastic proteases from tomato [4–6]. In addition, Song et al. demonstrated recently that EPIC1 and EPIC2B also inhibit the tomato defence protease Rcr3⁰ further highlighting the importance of these molecules for the infection process [6]. On the other hand glucanase inhibitors prevent the degradation of pathogen cell wall components and subsequent release of oligosaccharide elicitors of host defence mechanisms. Until now these glucanase inhibitors have only been described in vivo for Phytophthora sojae and Phytophthora infestans [7,8]. For a more elaborate description of these hydrolytic protein inhibitors we refer to several recent reviews [4,9].

A second category of apoplastic effectors mediate invasion of host cells in different ways. Like plant cells, oomycetes secrete a number of hydrolytic proteins into the extracellular space. For example glycolysing hydrolyases have been found in Saprolegnia parasitica [10], P. infestans [11] and Aphanomyces euteiches [12] and will aid in breaking down cell wall components and thus allow entry into host tissues.

An alternative way of enhancing the invasion success through extracellular effectors involves toxins. Members
of the PcF toxin family, small cysteine-rich proteins, are thought to be involved in the induction of necrosis [4,9]. The founding member of this gene family, PcF, seemingly has structural homology with a plant pollen protein with allergenic activity (Ole-e6). The authors suggest that PcF could be mimicking the effects of a plant signalling protein [13]. Other toxins secreted by oomycetes belong to the Nop1-like family (NLPs). These proteins appear to be structurally homologous to pore-forming toxins produced by sea anemones (i.e. actinoporins) [14]. On the basis of these similarities and in planta experiments Ottmann et al. suggest that NLPs are involved in the disruption of plasma membranes and subsequent cytolysis [14]. Also proteins/peptides with an Arg-Gly-Asp (RDG) motif are known to disrupt the integrity of plant cells by interfering with the adhesion of the cell wall to the host plasma membrane (CW–PM integrity) [15,16]. The best characterised of these is \( P. \) infestans IPIO that was shown to bind with its RGD motif to the Arabidopsis thaliana lectin receptor kinase LecRK-1.9 and thereby disrupting CW–PM integrity [17,18]. Recently LecRK-1.9 was reported to be involved in the resistance of \( A. \) thaliana to Phytophthora brassicae [19].

**Intracellular effectors**

**RxLR effectors**

In 2005 Rehmany et al. [20] first reported the presence of a highly conserved amino acid motif, Arg-Xaa-Leu-Arg (RxLR), within avirulence proteins from different plant pathogenic oomycetes. This amino acid motif, often followed by an EER (Glu-Glu-Arg), is statistically enriched in the secretome of the Peronosporales, and positioned within the first 40 AA after the predicted signal peptide cleavage sites. This signature has been shown to be present in a number of effector proteins from plant pathogenic oomycetes that interact with proteins that are located within the cytosol of their respective host cells [21,22,23**,24,25]. In addition, the subsequent reports that implicated the RxLR-motif in the host cell translocation process made this group of effectors to a main focus area of research within the community [25,26,27**]. Since 2005 more genome sequences of several oomycete pathogens have become available and computational analysis showed an enrichment of the RxLR-motif within the secreted proteins of species only from the Peronosporales with up to several hundred putative RxLR-effectors [25,28–30] whereas other oomycetes show no enrichment or different conserved motifs [10,31–33,34**]. The problem that arises from this huge number of putative translocated effector proteins is how to identify false positives and on the other hand how to identify RxLR-effectors in genomes of species that do not show an enrichment of this signature within their secretomes. The only way to tackle this problem is to understand the biological role of the RxLR motif.

**How is the RxLR-motif involved in the translocation of oomycete RxLR effectors?**

It is widely accepted that the RxLR-motif and the surrounding sequences are involved in the host cell translocation of the respective proteins but how this occurs is still under debate. However, this assumption is based on only three reports evaluating the function of this conserved motif.
Firstly, in 2007 Whisson et al. used AVR3a to show the involvement of the RxLR-motif in the host cell translocation of this effector [25]. AVR3a is the cognate avirulence protein recognised by the cytosolic potato R3a resistance protein [21]. Two isoforms of AVR3a exist in P. infestans that differ only by two amino acids (position 80 and 103) in the mature proteins lacking the signal peptide. Whilst AVR3aK80,103 is quite efficiently recognised by R3a, AVR3aE80,M103 is weakly recognised. Thus strains that only express the AVR3aEM isoform evade recognition by plants carrying the R3a resistance gene and do not induce a defensive hypersensitive response (HR) reaction, whereas AVR3aKI does [22, 35**]. Whisson et al. used an AVR3aEM homozygote strain transformed with AVR3aKI or RxLR-EER mutants derived from this and probed R3a containing potato with the respective transformants for their ability to induce an HR reaction. They could clearly show that mutations of the RxLR amino acids prevented recognition of the AVR3aKI by R3a. However, they were unable to visualise a WT AVR3a-mRFP reporter fusion construct inside the plant cells. To overcome this obstacle P. infestans transformants carrying a fusion between the signal peptide and the RxLR-leader of AVR3aKI to Escherichia coli ß-glucuronidase (GUS) were utilised. The authors subsequently evaluated GUS activity of infected potato leaves and found signals only in potato cells in contact with haustoria. However, this experiment has been criticised by some researchers because it cannot be reproduced convincingly [36].

Secondly, Dou et al. reported in 2008 that the RxLR-EER motif and surrounding sequences of the P. sojae effector Avr1b are necessary and sufficient to deliver the protein into plant cells [26]. They concluded from protein uptake experiments that this is an intrinsic activity of the Avr1b RxLR-leader. This was based on GFP fluorescence signals found in soy bean root cells after these were soaked with very high concentrations of recombinant produced RxLR-leader-GFP-fusion constructs for 12 hours. Thirdly, the same group in a later publication suggested that the RxLR-leader sequences alone are enough to translocate the respective effectors into eukaryotic cells through binding to surface exposed phosphoinositol-3-phosphate [27**]. However, these two reports have been criticised through several challenging publications [36, 37**, 38*, 39, 40**]. One recent study concluded that binding of AVR3a to phosphoinositol-3-phosphate is most likely an artefact. Using recombinant AVR3a this study showed that actually only denatured protein molecules bind this lipid [40**]. Unfortunately, reports showing alternative models of how RxLR-effectors from plant pathogenic oomycetes enter cells are still missing.

At present the most accepted means of studying effector delivery are experiments based on pathogen assisted delivery assays utilising HR reactions. However, the published data so far using this approach show that mutations of the RxLR amino acids or even truncation of the RxLR leader are, to some extent, giving positive results in these experiments [26].

A detailed description for a host cell translocation process of an animal pathogenic oomycete protein is based on a putative effector from S. parasitica, the host targeting protein 1 (SpHtp1). S. parasitica is a serious fish pathogen of fresh water fish. SpHtp1 contains an RxLR-motif in a position characteristic for RxLR-effectors. However, S. parasitica does not show any enrichment for this signature motif within its secretome. SpHtp1 delivery from the parasite into fish cells was shown by immuno-localisation and recombinant SpHtp1 is also able to enter trout cells [41**]. Utilising recombinant mRFP reporter fusion proteins it was shown that the ability of SpHtp1 to enter fish cells is located within the N-terminus of the protein. Furthermore, the data suggest that the SpHtp1 uptake is a host cell surface protein mediated process. Evidence for this are fish cell specificity, the concentration and tyrosine-O-sulphated dependency as well as the intracellular co-localisation with the lectin wheat germ agglutinin [42*]. However, the function of SpHtp1 is still unknown and thus far others have not tried to independently verify these translocation experiments.

**What are the functions of RxLR effectors?**

One big challenge of oomycete effector research is to identify the function of these proteins. The biggest obstacle here is the great diversity the RxLR-effectors show in their primary amino acid sequences with very little or no similarity to known protein domains. The only exception is the RxLR effector AVR3b from P. sojae, which carries a Nudix hydrolase domain. It is proposed that AVR3b mimics plant Nudix hydrolases to suppress plant immunity [43*].

Since many RxLR-effectors have interaction partners residing in the cytoplasm of their host cells, it is likely that several have evolved to manipulate host immunity. On the basis of this idea a couple of high throughput programmed cell death (PCD) suppression assays have been used during the last few years to identify effector candidates with a function in plant immune response suppression. For instance the screening of 62 P. infestans RxLR effectors for their ability to suppress INF-1 induced PCD in Nicotiana benthamiana after agro-infiltration, revealed two candidates, PexRD8 and PexRD3645 [44]. Using the same agro-infiltration assay, Wang et al. [45] found that the majority of 169 P. sojae RxLR-effectors could suppress PCD triggered by the mouse pro-apoptotic protein BAX, P. sojae effectors, and/or the PAMP INF1. Furthermore, in a Hyaloperonospora arabidopsis screen a high proportion of tested RxLR effectors (35 out of 62) were found to interfere with plant immunity by suppressing callose deposition [46]. The large number of PCD immune response suppressors identified are
remarkable and further studies will reveal how robust and reliable these high-throughput assays are (see also review by Bozkurt [36]).

Similarly, but on a smaller scale, Dou et al. showed that Avr1b and three additional putative RxLR effector candidates from P. sojae and Hyaloperonospora parasitica can suppress PCD in soybean and N. benthamiana cells triggered by the mouse pro-apoptotic protein BAX [47]. The WY motifs [48] of these proteins were found to be essential for this activity. Recently, structural analysis revealed that these amino acids are critical to stabilise a core α-helical fold, which is predicted to be conserved in many oomycete RxLR-effectors despite their primary sequence diversity [49**].

Host immune defence manipulation has also been shown for the H. arabidopsis R protein RxLR effectors ATR1 and ATR13. Both greatly enhanced the virulence of Pseudomonas bacteria when delivered through the type III secretion system into A. thaliana cells by suppressing bacterial PAMP (Pathogen-Associated Molecular Patterns)-triggered callose deposition as well as production of reactive oxygen species (ROS), both hallmarks of plant defence [50].

All these screens have made it apparent that many RxLR-effectors play a role in manipulating host immune response, but only for a few candidates a host interaction partner has been found.

An effective method to identify effector targets is the application of yeast two hybrid screening, which has been used successfully to screen for potential host targets of H. arabidopsis R protein RxLR effectors as well as Pseudomonas syringae type III effectors [51]. Eighteen A. thaliana hosts are likely to be targeted by effectors of both pathogens and for which a function in plant immune system could be proposed. However, experiments to confirm whether these interactions occur in planta need to be followed up and their biological relevance needs to be determined.

Probably the best-studied effector–host target interaction has been described for AVR3aK1 of P. infestans, which suppresses INF1-induced PCD. Because AVR3aK1 is unable to suppress PCD stimulated by the P. infestans effectors PiNPP1 and CRN2, it is likely that this suppression activity is specific [22]. This view is now supported by the observation that AVR3aK1 prevents the degradation of the host ubiquitin-E3-ligase CMPG1, which is an essential component of the INF1-induced cell death response [35**,52].

Suppression of PAMP-triggered PCD of plants helps the infection process. However, plants also produce highly active proteases in response to an evolving infection. Consequently, oomycetes secrete protease inhibitors to suppress the activity of these apoplastic proteases. Interestingly, Bozkurt et al. identified an alternative route to suppress this defence reaction of the plant [23**]. They showed that in planta expression of the P. infestans RxLR-effector AVRblb2 prevented secretion of the immune serine protease C14 into the apoplast, which led to an increased infection susceptibility of the plant.

In contrast to the previously mentioned RxLR-effectors it is suggested that IPI-O from P. infestans fulfills at least part of its function extracellular (see also section ‘Extra-cellular effectors’). This RGD cell-attachment motif containing effector was shown to disrupt plasma membrane (PM)/cell wall (CW) integrity in an RGD motif dependent manner [17,19] and found to interact with LecRK-I.9, a membrane associated legume-like lectin receptor kinase [18]. It is suggested that LecRK-I.9 functions in strengthening CW–PM adhesion upon infection. Furthermore, it is assumed that IPI-O disrupts the CW–PM continuum, possibly by targeting LecRK-I.9 to promote infection [19]. These observations clearly put the function of this RxLR-effector on the host cell surface. At the moment it is unclear whether it is also translocated into the host cells.

IPI-O and the previously mentioned RxLR-effectors demonstrate that oomycetes have developed an arsenal of these effectors to interfere with and manipulate host immune responses by targeting different sites of the immune system.

**Crinklers (CRNs)**

Named because of the leaf-crinkling and cell death phenotype observed during expression in planta [53], crinklers (CRN) are cytoplasmic effectors first discovered in P. infestans but have also been found in other plant pathogenic oomycetes such as P. sojae, Phytophthora ramorum [29], Phytophthora phaeolus [54], H. arabidopsis [30], Bremia lactucae [55] and Pythium ultimum [31].

This group of effectors shares a similar modular structure with the RxLR-effectors exhibiting a highly conserved N-terminal Leu-Xaa-Leu-Phe-Leu-Ala-Lys (LxLFLAK) domain of approximately 50 amino acids. A tri-peptide signature (Asp-Trp-Leu, DWL) marks the beginning of a diverse C-terminal region which ends with a highly conserved His-Val-Leu-Val-Xaa-Xaa-Pro (for details see [29]).

Only a small number of functional studies have been carried out for this class of effectors. Schornack et al. showed that transgenic Phytophthora capsici expressing constructs with the N-terminal portion of CRN2 and CRN16 from P. infestans fused to the C-terminal portion of AVR3a elicited effector-triggered immunity (ETI) in N. benthamiana leaves leading to avirulence of transgenic
P. capsici [34**]. When mutated versions of CRNs N-terminal sequences (LxLFLAK to LxAAAA) were fused to the C-terminus of AVR3a, avirulence was not observed. These experiments lead to the conclusion, that the N-terminal parts of CRNs are responsible for the protein transport into plant cells dependent on the LxLFLAK motif. However, information about which effect these mutations have on the conformation and stability of the respective proteins are still missing.

In addition, in planta expression of CRN-GFP fusion constructs (CRN2, CRN8, CRN15, CRN16 from P. infestans and CRN5 from A. euteiches) through agro-infiltration showed that all constructs were targeted to the plant cell nucleus after translocation. This localisation was found to be dependent on host importin-α, a protein that is part of the nuclear trafficking machinery and responsible for binding the nuclear localisation signal motifs (NLS) [34**].

Timing of CRN expression during the infection is unclear since different results have been reported. In P. infestans, several CRNs are induced during infection [29]. While in P. sojae, CRN transcripts were found more abundantly in germinating cyst and the later infection stages [56]. Therefore, CRNs might fulfil distinct roles during different phases of the infection. Surely, their expression profiles suggest a more evenly distribution of transcripts. Importantly, compared to RxLR effectors a significantly higher number of CRNs are found in oomycetes that do not form haustoria.

Final remarks

The oomycetes contain some of the world’s most serious pathogens that can cause massive mortalities in our natural environments as well as in agriculture, forestry and aquaculture settings. Some oomycetes cause serious issues in highly sensitive natural environments with resulting conservation problems and occasional eradication programmes have to be put in place to safeguard (rare) plants and animals. Fundamental molecular research in how these pathogens infect their hosts is therefore crucial in order to develop sustainable control strategies. Understanding effector functions and trafficking will ultimately help in accomplishing this goal.

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References and recommended reading

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

- of special interest
- of outstanding interest


11. McLeod A, Smart CD, Fry WE: Characterization of 1,3-beta-glucan and 1,3:1,4-beta-glucanase genes from Phytophthora infestans. Fungal Genet Biol 2003, 38:250-263.


24. This paper shows how an effector interferes directly with the secretion of a plant defense protein.


29. This publication puts forward the controversial model that RxLR-effectors can enter eukaryotic cells through binding to surface exposed phosphoinositol-3-phosphate


**functional interactions within the Phytophthora sojae RXLR effector repertoire.** Plant Cell 2011, **23**:2084-2096.


This work shows conservation of core structural elements among several RxLR-effectors.


