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Review

Towards an understanding on how RxLR-effector proteins are translocated from oomycetes into host cells

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ABSTRACT

The most notorious oomycetes, such as *Phytophthora infestans*, are pathogens of higher plants, although numerous other species of these fungal-like microorganisms infect algae, crustacea, nematodes, fish and mammals. While there is now ample evidence that oomycetes and fungi deliver effector proteins inside the host cell during infection, like bacteria using the well characterised type III secretion system, the mechanism employed by eukaryotic pathogens remains unclear. In oomycetes this process depends on an N-terminal motif defined by a short conserved amino acid sequence (RxLR) located near the signal peptide of many secreted proteins. This motif resembles the host-cell targeting signal found in virulence proteins from the malaria parasite *Plasmodium falciparum* (RxLxE/D/Q).

This review will focus on the recent findings contributing to the understanding of the delivery of oomycete effector molecules into the host cells, with emphasis on how they compare with various models proposed for filamentous fungi and the malaria parasite.

1. Introduction

Oomycetes belong to the Stramenopila, along with golden-brown algae and diatoms. The Stramenopiles form with the Alveolates, including the apicomplexans such as malaria parasites, a group named the Chromalveolates. This group of organisms is considered to have derived from a common photosynthetic ancestor (Baldauf *et al.*, 2000, 2003; Tyler *et al.*, 2006; Burki *et al.*, 2007). Despite their morphological resemblance, oomycetes are thus phylogenetically distant from fungi, which are closely branched with the animal kingdom. Unlike fungi, oomycetes are mainly diploid during their life cycle, they have coenocytic hyphae, and the main components of their cell walls are cellulose and β1-3-glucans, with little chitin present (Erwin and Ribeiro, 1996). Convergent evolution has possibly led oomycetes to share similar growth habits, lifestyle and infection strategies with filamentous fungi. Comparison of the expressed gene content from *Phytophthora infestans* with fungal gene and protein sequence databases revealed shared components of pathogenicity

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such as pectate lyases. Surprisingly, genes encoding proteins similar to chitin synthases were also detected (Randall *et al.*, 2005). Moreover, there is now some evidence that horizontal gene transfer has occurred between eukaryotic species (Richards *et al.*, 2006; Whitaker *et al.*, 2009; Morris *et al.*, 2009).

Oomycetes include not only several of the most destructive plant and animal pathogens, but also some mycoparasites (*Pythium oligandrum*) as well as many saprophytes. At least one species, *Pythium insidiosum*, infects mammals (including humans), causing pythiosis (Vanittanakom *et al.*, 2004). The most economically important oomycetes come from two orders: the Saprolegniales and the Peronosporales. *Phytophthora* species belong to the Peronosporales, along with the downy mildew pathogens, such as *Hyaloperonospora arabidopsidis* (formerly *Peronospora parasitica*, infecting model plant *Arabidopsis thaliana*), and also the genera *Pythium* and *Albugo*. The *Saprolegniales* include the fish pathogen *Saprolegnia parasitica* and *Aphanomyces* species.

Plant-pathogenic species, especially from the genus *Phytophthora*, which include more than 80 species (Blair *et al.*, 2008), certainly have devastating effects on crops. The most notorious and also the best-studied of all oomycetes is the potato blight pathogen, *P. infestans*. This disease led to the great Irish famine in the 1840s, and today it remains the most important potato disease, costing US$3 billion annually worldwide in control measures and loss of production (Duncan, 1999), and more than €1 billion in the European Union alone (Haverkort *et al.*, 2008). Some species also affect native plant communities, such as the recently emerged *Phytophthora ramorum* causing sudden oak death, a disease that is decimating Californian forests and other ecosystems (Rizzo *et al.*, 2005).

2. Oomycete-plant interactions: the importance of haustoria

As an example for many oomycete species, the life cycle of *P. infestans* initiates with the direct germination of sporangia or with the release of motile zoospores, which encyst on the plant surface. A germ tube is then produced and differentiates into an appressorium, which allows the pathogen to penetrate a leaf cell. Not all oomycetes have been shown to produce appressoria, and some species may invade host tissues through stomata instead (Dale and Irwin, 1991). Intercellular hyphae grow into the mesophyll, and specialised intracellular structures called haustoria are formed (van West and Vleeshouwers, 2004). The haustorial stage of *P. infestans* has been shown to be essential for infection (Avrova *et al.*, 2008). In the plant, hyphae secrete various proteins from apical vesicles, such as degradative enzymes, as well as molecules involved in adhesion, cell wall synthesis and also in counter-defence (Hardham, 2007). After 3–4 d, hyphae spread saprophytically in the necrotised tissue of the growing lesion, and finally emerge through the stomata. The pathogenic life cycle is completed with the formation of sporangiophores producing numerous sporangia on the leaf surface (Grenville-Briggs and Van West, 2005; Hardham, 2007).

Many fungal pathogens establish a biotrophic relationship with their hosts through the formation of haustoria. The haustorium is encased in an extra-haustorial matrix, which is separated from the host cytoplasm by a specialised membrane thought to be derived mainly from the host and called the extra-haustorial membrane (EHM) (Woods *et al.*, 1988; Soylu *et al.*, 2004). Hexose transporters have been identified in the haustoria of the rust fungus *Uromyces fabae*, and in this species, carbohydrate uptake would take place exclusively in the haustorium (Voegele *et al.*, 2001). It has been suggested by studies with rust fungi (Voegele and Mendgen, 2003) that haustoria are also sites of biosynthesis, can influence host metabolism and play a role in the suppression of host defences. In addition to uptake of nutrients at the haustorial membrane, proteins are also secreted by fungal haustoria (Dodds *et al.*, 2004; Kemen *et al.*, 2005; Catanzariti *et al.*, 2006). Some of these have been localised to the host cell cytoplasm or the nucleus, where they may interact with host proteins to influence the fungus/host interaction (Dodds *et al.*, 2004; Kemen *et al.*, 2005; Catanzariti *et al.*, 2006). Similarly, transcript analysis of the flax rust fungus revealed haustoria to be enriched for transcripts encoding secreted proteins (Catanzariti *et al.*, 2006).

Many oomycetes also form haustoria. The cell biology of oomycete haustoria is less well characterised, although recently confocal microscopy and monomeric Red Fluorescent Protein (mRFP) were used to show the secretion of the *P. infestans* avirulence protein AVR3a from haustoria (Whisson *et al.*, 2007). Using a similar approach, a predicted membrane protein, Pihmp1 (*P. infestans* haustorial membrane protein 1), was shown to be localised to the *P. infestans* haustorial membrane, giving the first demonstration that the haustorial membrane in oomycetes differs from the plasma membrane of the intercellular hyphae (Avrova *et al.*, 2008). The function of Pihmp1 remains to be determined, but it appears likely to be a structural protein essential for pathogenicity. Indeed, *Pihmp1*-silenced lines were unable to produce haustoria, and thus could not establish a successful infection (Avrova *et al.*, 2008). Unlike for *U. fabae*, it is yet to be demonstrated that oomycete haustoria are the site of nutrient uptake for these pathogens.

Mims *et al.* (2004) used transmission electron microscopy (TEM) and cryofixation to examine the interface between haustoria from *H. arabidopsidis* and *A. thaliana* leaf cells at the ultrastructural level. They observed many rounded and finger-like protrusions on the outer surface of the extra-haustorial matrix. They also observed numerous small vesicles in the host cell cytoplasm around the EHM, with which some of these vesicles appeared to be fusing with or blebbing off. Interestingly, a study of the ultrastructure of *Albugo candida* and host interaction revealed some striking tubular elements continuous with the EM and extending into the host cell cytoplasm (Baka, 2008).

Subcellular reorganization and cytoplasmic aggregation have been observed in many plant-pathogen interactions, specific or not. Takemoto *et al.* (2003) have visualized the response to oomycete pathogen attack in epidermal cells from transgenic *A. thaliana*, in which microtubules, actin filaments, endoplasmic reticulum (ER) and the Golgi apparatus were tagged with the Green Fluorescent Protein (GFP). Three different types of interactions were investigated, although no obvious differences between non-pathogenic, incompatible

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and compatible interactions were observed at the penetration site. In all cases, the actin microfilaments were re-arranged, and the ER and the Golgi were reorganised around the penetration site, possibly to favour site-specific secretion (Takemoto *et al.*, 2003). The existence of a plant secretory pathway specifically targeted to the haustorium was strongly supported by the recent finding that a powdery mildew resistance protein was targeted to the EHM (Wang *et al.*, 2009). These studies demonstrated that the interface between host and pathogen is highly specialised, on both the host and pathogen sides of the interaction.

3. Secretion of effector proteins from oomycetes

Effector proteins are secreted by pathogens during a biotrophic interaction with their host not only to facilitate successful infection, but also to suppress host defences. Secreted effectors from oomycetes may be broadly grouped as those secreted into intercellular spaces including the EHM, such as protease and glucanase inhibitors, and those that are secreted and subsequently translocated into the host cell (Kamoun, 2006). While a small number of oomycete cytoplasmic effectors have been shown to influence phenomena such as programmed cell death (Bos *et al.*, 2006; Bos *et al.*, 2009; Dou *et al.*, 2008a), and can facilitate colonisation by some incompatible pathogens (Sohn *et al.*, 2007; Rentel *et al.*, 2008), their virulence functions are not completely understood.

3.1. *The RxLR sequence in effector proteins*

The assumption that some oomycete effectors are delivered inside the host cell was made following the discovery of the RxLR protein family by cloning of race-specific avirulence genes (Rehmany *et al.*, 2005). Indeed to date, each cloned *Avr* gene belongs to the RxLR family (Allen *et al.*, 2004; Armstrong *et al.*, 2005; Dong *et al.*, 2009; Qutob *et al.*, 2009; Rehmany *et al.*, 2005; Shan *et al.*, 2004; Vleeshouwers *et al.*, 2008; van Poppel *et al.*, 2008; Lokossou *et al.*, 2009) and the recognition of the proteins they encode have been shown to involve the products of resistance genes belonging to the NBS-LRR class of proteins (Botella *et al.*, 1998; Bittner-Eddy *et al.*, 2000; Huang *et al.*, 2005; Song *et al.*, 2003; Lokossou *et al.*, 2009), which have an intracellular location. In case of Avr1b from *Phytophthora sojae* and Avr4 from *P. infestans*, the nature and location of the corresponding resistance proteins RPS1b from soybean and R4 from potato are at present unknown (Shan *et al.*, 2004; van Poppel *et al.*, 2008). The *AVR* and *R* genes in plant-oomycete interactions are described in detail in Hein *et al.* (2009).

The term RxLR is generally used to define a larger N-terminal region with a core conserved motif, RxLR (Arginine-any amino acid-Leucine-Arginine) frequently followed by a less well conserved EER (Glutamic acid-Glutamic acid-Arginine) sequence within 30 amino acids towards the C terminus. Various search algorithms have now been used to identify hundreds of potential RxLR-EER effectors amongst predicted secreted proteins in several oomycetes (Bhattacharjee *et al.*, 2006; Whisson *et al.*, 2007; Win *et al.*, 2007; Tyler *et al.*, 2006; Haas *et al.*, 2009) and this sequence, with its specific positional characteristics, is over-represented in oomycetes relative to other eukaryotes (Win *et al.*, 2007).

One avirulence gene, *ATR13* from *H. arabidopsidis*, differs from other cloned avirulence genes in that it encodes a protein possessing only the RxLR sequence, but not the EER sequence, and interestingly, ATR13 contains several conserved heptad repeats and direct repeats downstream of its RxLR (Allen *et al.*, 2004).

The sequence including the RxLR-EER motifs has now been shown to mediate the translocation of effector proteins from haustoria into host cells, and importantly, mutation of the motifs prevented transport beyond the extra-haustorial matrix (Whisson *et al.*, 2007). This sequence has also been shown to mediate import of effector Avr1b into soybean cells in the absence of the pathogen (Dou *et al.*, 2008b). Furthermore, the C-termini (minus RxLR-EER) of the AVR proteins have been shown to be sufficient to carry the virulence function in addition to the avirulence function, implying a modular organisation of RxLR-EER class effectors (Bos *et al.*, 2006, 2009; Dou *et al.*, 2008a; Allen *et al.*, 2008; van Poppel *et al.*, 2008; van Poppel *et al.*, 2009). The C-termini of RxLR-EER effectors are highly diverse in amino acid sequence, although many exist as gene families (Win *et al.*, 2007). Surprisingly, for ATR13 some level of recognition has been shown to involve the heptad repeats, which are a conserved region adjacent to the RxLR motif (Allen *et al.*, 2008). This is consistent with the demonstration that sequences downstream of the RxLR sequence in ATR13 are not required for translocation, despite the lack of an EER sequence (Grouffaud *et al.*, 2008). Consequently, the number of potentially translocated effectors from *Phytophthora* is much higher than anticipated, as most predictions are based on the presence of the dual motif RxLR-EER. Micro array analysis and expression analysis have shown that a large number of RxLR-only genes selected from *P. infestans* ESTs are up-regulated during the biotrophic interaction with potato, with similar expression profiles to the RxLR-EER genes (Whisson *et al.*, 2007; Haas *et al.*, 2009).

One RxLR-EER effector, Nuk10 from *P. infestans*, carries a functional nuclear localisation signal (Kamoun, 2006), suggesting that it may have a role in modulation of host transcription. Other secreted proteins from *P. infestans*, Nuk6/Nuk7/Nuk12, also contain functional NLS sequences, although these proteins do not contain an RxLR motif (Kanneganti *et al.*, 2007); if they do function inside host cells, these proteins must somehow follow an alternative route into host cells.

3.2. *CRN families*

The existence of another class of oomycete cytoplasmic effectors, the Crinkler (CRN) family, has been proposed (Torto *et al.*, 2003; Kamoun, 2006), after leaf-crinkling and necrosis were observed following expression of *crn1* and *crn2* of *P. infestans* from a potato virus X vector in *Nicotiana* and in host plant tomato. CRNs appear to be modular proteins, with a highly conserved N-terminal domain including a signal peptide followed by the consensus sequence LXLFLAK, and very diverse C-termini (Haas *et al.*, 2009). Moreover, this family seems to be also well represented in *H. arabidopsidis*, where, surprisingly, it overlaps the RxLR motif in several proteins (Win *et al.*, 2006 and Win *et al.*, 2007). The functions of CRN proteins

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are currently under investigation, and current proposed models suggest the ability of their N-termini to mediate their translocation and that the C-terminal domains trigger cell death (Haas et al., 2009).

4. Mechanism of translocation of effector proteins

4.1. *Translocation of effectors: evidence from fungi*

In the broad bean rust *U. fabae*, Kemen et al. (2005) identified a glycoprotein, named RTP1p, which is secreted from haustoria and carries a nuclear localisation signal. This protein was detected by immunofluorescence in the cytoplasm and also in the nucleus of a host cell. They also showed that only selected proteins are translocated from extra-haustorial matrix to host cell. This study provided the first direct evidence that some fungal proteins are transferred from haustoria to the host cell.

Avirulence genes have been cloned from numerous fungal plant pathogens, and many of the R proteins recognising fungal AVR proteins are predicted to be cytoplasmic (Ellis et al., 2006; Catanzariti et al., 2007). AVR-Pita secreted from *Magnaporthe oryzae* has been shown to interact directly with the rice Pi-ta resistance protein, an NBS-LRR protein, demonstrating that a fungal protein can enter host cells (Jia et al., 2000).

The products of the three *AvrL567* genes from flax rust *Melampsora lini* were the first avirulence proteins to be identified from haustoria-forming fungi. It has been shown that they were secreted from haustoria, and *in planta* expression of *AvrL567* genes lacking the signal peptide sequence triggered *R* gene-dependant necrosis, suggesting that recognition occurred inside the host cytoplasm (Dodds et al., 2004). *AvrP4* and *AvrM* were identified by screening a haustorium-specific cDNA library and the proteins they encode behave in a similar manner to AvrL567 suggesting that they are also recognised inside host cells (Catanzariti et al., 2006).

Interestingly, AVRk1 and AVRa10 from barley powdery mildew *Blumeria graminis* f. sp. *hordei* (*Bgh*) do not have a secretion signal peptide, but are recognised by intracellular resistant proteins MLK1 and MLA10, implying that they are probably secreted from haustoria by an alternative pathway (Ridout et al., 2006). Since then, it has also been shown that AVRa10 interacts with resistance protein MLA10 and WRKY transcription factors in the host nucleus (Shen et al., 2007).

Although the rice blast fungus *M. oryzae* does not produce haustoria, its invasive hyphae are sealed in a plant-derived extra invasive hyphal membrane (EIHM), invading successive live cells by crossing through plasmodesmata (Kankanala et al., 2007). How effectors such as AVR-Pita are secreted across the EIHM into the host cytoplasm remains unclear. Most recently, a novel pathogen induced structure was identified within the EIHM compartment matrix and named the biotrophic interfacial complex (BIC) (Kankanala et al., 2007, Mosquera et al., 2009, Khang et al., 2009). Effectors fused to fluorescent proteins have been shown to be secreted from the intracellular hyphae and to accumulate in the BIC, suggesting that it could mediate effector translocation into the host cytoplasm. One new BIC is generated each time an intracellular hypha invades a new cell, and these caps consist of plant lamellar membranes and vesicles. In a remarkable experiment (Khang et al., 2009) the signal peptides from known effectors were sufficient to direct BIC localisation while the signal peptide from cutinase was not, supporting the hypothesis that the signal peptide sequences of fungal effectors may contain the information required for directing secretion to the site of translocation, despite the lack of discernable amino acid motifs.

4.2. *Effector translocation in* Phytophthora *and* Plasmodium

Similar to the intimate association with the host cell plasma membrane created by many haustoria-forming oomycete and fungal pathogens during infection, the malaria parasite *Plasmodium falciparum* produces a parasitophorous vacuole within infected red blood cells. Both malaria parasites and oomycetes export proteins into the lumen of the parasitophorous vacuole (PV)/EHM, respectively, by means of a conventional hydrophobic signal sequence. Transport of effector/virulence proteins through the host-derived membrane requires an additional host-targeting signal, located within the N-terminal region of these proteins (Marti et al., 2004; Hiller et al., 2004; Haldar et al., 2006). Interestingly, the malaria host targeting signal (also called PEXEL) and the oomycete translocation motif share similar sequences, RxLxE/D/Q and RxLR-EER respectively. Moreover, these sequences have been shown in independent studies to be functionally interchangeable (Bhattacharjee et al., 2006; Grouffaud et al., 2008; Dou et al., 2008b).

Evidence strongly suggests that the malaria host-targeting signal sorts proteins into the lumen of novel pathogen structures called Maurer's clefts prior to their export beyond the parasitophorous vacuole membrane (PVM) (Bhattacharjee et al., 2008). However, it is not clear if sorting occurs within the parasite or in the PV. Using minimal reporters, it was found that the RxLxE/D/Q motif alone was not able to translocate proteins directly across the PVM (Bhattacharjee et al., 2008). However, the question of how parasite proteins enter and exit these intermediate compartments remains unanswered. Electron tomography has been used to analyse the three dimensional structure of Maurer's clefts, revealing that these complex organelles are connected to both the host cell plasma membrane and the parasitophorous vacuole membrane *via* tether-like structures (Hanssen et al., 2008).

4.3. *Effector transport by a translocon?*

The mechanism employed by malaria parasites to export virulence proteins beyond the parasitophorous vacuole membrane remains poorly understood, although several models have been proposed to involve a protein-conducting channel (Marti et al., 2004; van Ooij and Haldar, 2007; Charpian and Przyborski, 2008). Two independent studies supporting this model have been published recently (Gehde et al., 2009 and de Koning-Ward et al., 2009). In the first study, it was found that parasite proteins were most likely translocated directly from the lumen of the parasitophorous vacuole across the PVM, and that protein transport was dependant on their unfolded state (Gehde et al., 2009), consistent with the abundance of chaperones identified in

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the PV of infected erythrocytes (Nyalwidhe and Lingelbach, 2006). In the second study, it was found that a novel PEXEL-protein translocon associated with the PVM (de Koning-Ward *et al.*, 2009). Proteomic analysis of parasite membranes combined with specific criteria were used to predict potential translocon proteins, including a secreted heat shock chaperone protein (HSP101/ClpA/B) containing AAA+ ATPase domains, a novel protein called PTEX150, and the previously characterised vacuolar membrane protein EXP2 (Fischer *et al.*, 1998), as well as two other potential accessory proteins thioredoxin (TRX2) and PTEX88. Despite the lack of a predicted transmembrane domain, there is evidence that EXP2 is associated with the PVM (Fischer *et al.*, 1998), and evidence suggests EXP2 to be the channel forming element of the translocon complex (de Koning-Ward *et al.*, 2009). Other components of this machinery are likely to be involved in the recognition of PEXEL-protein cargo. de Koning-Ward *et al.* (2009) were able to demonstrate a specific interaction between the translocon proteins and four different exported proteins with a PEXEL motif. Yet, how these proteins destined for export are distinguished and recognised by the translocon remains an issue. The PEXEL sequence defines a cleavage site recognised by a specific protease in the parasite endoplasmic reticulum (Chang *et al.*, 2008; Boddey *et al.*, 2009). Both of these reports demonstrate that the motif RxLxE/D/Q is cleaved after the conserved leucine, that the resulting N-terminus is also acetylated, and that this processing is required prior to export beyond the PVM. However, PEXEL processing and N-acetylation are not sufficient for export, as acetylation of N-termini following signal peptidase cleavage appears to be common in *P. falciparum* (Boddey *et al.*, 2009). Several studies using fusions of exported proteins to GFP reported that fluorescence typically appeared like a ''necklace of beads'' in the parasitophorous vacuole (de Koning-Ward *et al.*, 2009; Adisa *et al.*, 2003), suggesting the presence of subcompartments within the PV housing the translocons. Therefore, Boddey *et al.* (2009), proposed that processed proteins destined for export could be sorted by specific PEXEL cargo receptors at the ER or Golgi into functionally distinct vesicles targeted to these translocon containing compartments.

Previously, Morgan and Kamoun (2007) have also proposed a model for the translocation of effectors from oomycetes involving RxLR binding proteins and chaperones, which would recruit the effectors secreted in the extra-haustorial matrix, and a translocon embedded in the host-derived extra-haustorial membrane. The effectors would be transferred through the translocon in a folded state maintained by the chaperones (Morgan and Kamoun, 2007). However, this model was mainly based on observations that the RxLR domain shares structural similarities with other proteins using known translocation systems (Wickner and Schekman, 2005). Inevitably, since both RxLR and PEXEL motifs appear to be functionally similar (Bhattacharjee *et al.*, 2006, Dou *et al.*, 2008b; Grouffaud *et al.*, 2008), it is tempting to assume that the putative malaria export machinery would be conserved in oomycetes. However, not even distant homologues were identified when the sequences of the five protein components of the putative *Plasmodium* translocon were compared by BLAST to the *P. infestans* genome sequence and predicted proteome databases (Leighton Pritchard, personal communication).

4.4. *Uptake of effectors in absence of a pathogen*

Various proteins have previously been reported to have the ability to translocate through the cell plasma membrane. These cationic cell-penetrating peptides (CPPs) include Tat, derived from the HIV-1 Tat protein (Frankel and Pabo, 1988; Mann and Frankel, 1991), penetratin, derived from the Antennapedia homeodomain (Derossi *et al.*, 1994 and Derossi *et al.*, 1996), and even oligoarginines (Futaki *et al.*, 2001 and Mitchell *et al.*, 2000). Intracellular delivery of arginine-rich peptides has also been demonstrated in plant cells (Mizuno *et al.*, 2009). Nevertheless, after 20 y of controversy and conflicting findings, underlying molecular mechanisms for the internalization of these CPPs remain elusive. Recent models involve several endocytic pathways occurring simultaneously, as well as an endocytosis-independent process in some cases (Duchardt *et al.*, 2007 and Ter-Avetisyan *et al.*, 2009 respectively). Another study showed that poly-lysines and poly-arginines induced pore formation upon electrostatic binding to negatively charged phospholipid vesicles (Reuter *et al.*, 2009).

Inconsistent results have been obtained regarding the requirement of the signal peptide (SP) for constructs to elicit the HR in experiments where the protein is being synthesised in the host *via* particle bombardment or Agro-infiltration. The SP, which is removed during secretion, leads the protein outside the host cell in principle. Therefore, the protein has to re-enter the host cell so the interaction with the intracellular resistance protein can take place. On the other hand, when a construct lacking a SP is expressed in a cell, the protein stays inside the cytoplasm. For instance, in particle bombardment experiments involving other known avirulence genes, similar HR phenotypes were observed when *H. arabidopsis ATR13* and flax rust fungus *M. lini AvrM* and *AvrP4*, and also *PsAvr3c* were expressed with or without the signal peptide (Allen *et al.*, 2004, Catanzariti *et al.*, 2006 and Dong *et al.*, 2009, respectively). In contrast, when ATR13 was secreted by bacteria *via* the type III system, the presence of the signal peptide prevented intracellular recognition (Rentel *et al.*, 2008). In the case of *Avr3a* from *P. infestans*, only expression constructs lacking a signal peptide for secretion elicited the HR in particle bombardment experiments (Armstrong *et al.*, 2005). Like *Avr3a*, *H. arabidopsidis ATR1* also exhibited a more pronounced HR in similar experiments when the signal peptide for secretion was removed (Rehmany *et al.*, 2005). However, during *Agrobacterium* co-infiltration of *Avr3a* and its cognate resistance gene, *R3a*, in *N. benthamiana*, presence or absence of the SP made little difference to the ability of *Avr3a* to trigger the HR (Bos *et al.*, 2006). Perhaps some of the results can be explained by varying expression levels and sensitivity in the assays. However at present this remains unclear.

Recently, Dou *et al.* (2008b) have reported that RxLR-mediated delivery of *P. sojae* effector Avr1b into host cells did not require the presence of the pathogen, and thus would depend only on the intrinsic properties of the RxLR domain and host molecules. Co-bombardment of soybean cells with DNA encoding native Avr1b and β-glucuronidase (GUS) showed that secreted proteins were probably able to re-enter

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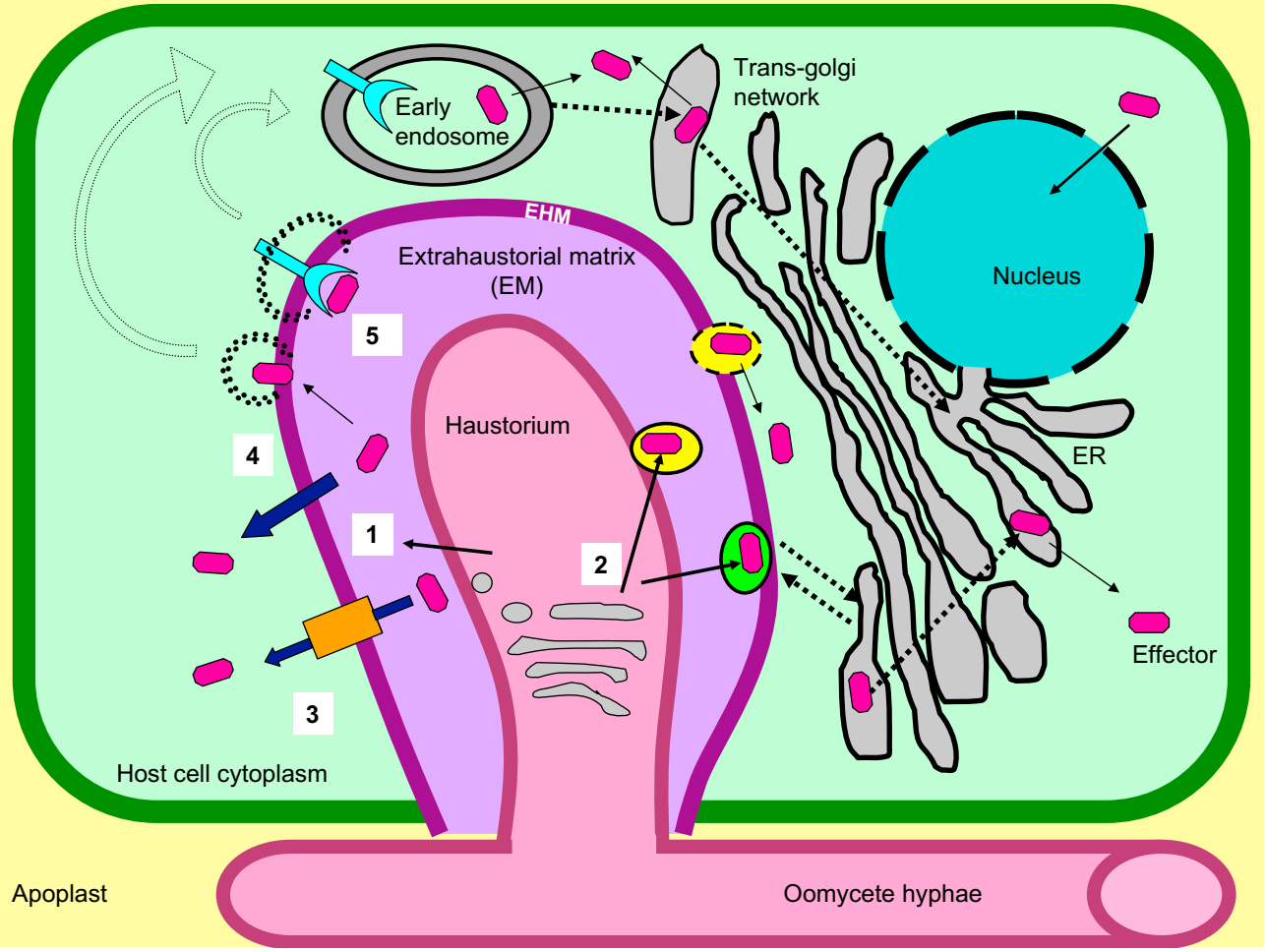

Fig 1 – Hypothetic models of translocation of oomycete effectors into the plant cell. After synthesis at the oomycete endoplasmic reticulum (ER), proteins to be exported are sorted for transport through the Golgi to the extra-haustorial matrix (EM) *via* the default secretory pathway (1), or may be targeted to an alternative route involving novel vesicles derived from the pathogen (in yellow) or the plant (in bright green) endomembrane system (2). Therefore, effectors would traffic through the EM inside membrane-bound compartments, and subsequently fuse with the extra-haustorial membrane (EHM), and be released into the host cytoplasm. There is also a possibility that plant-derived structures present in the EM are connected to the host ER, allowing trafficking of effectors through the plant system. Exported proteins may also translocate across the EHM through a transporter (translocon) (3), more likely to be from pathogen origin. Another potential export mechanism could depend on intrinsic properties of RxLR effectors, enabling them to directly cross the EHM by endocytotic or non-endocytotic processes (4). A further model involves recognition of effectors at the EHM by host or pathogen-encoded receptors (5) inducing clathrin-mediated endocytosis. Vesicles containing internalized ligand-bound receptors fuse to the early endosome, inside which effectors and receptors dissociate. From there, effectors may be sorted to the ER *via* the TGN by the retrograde pathway, or may escape directly into the cytosol. Alternatively, it is possible that the mechanisms illustrated by (3), (4) and (5) may be compartmentalized within the EM as described for (2) (not shown). Note that effectors depicted by purple rounded rectangles represent oomycete proteins that are translocated inside the host cell. Apoplastic effectors are not shown on this figure.

the cell to trigger the HR, while mutation of the motif prevented recognition (Dou *et al.*, 2008b). Additionally, visualisation of onion bulb epidermal cells bombarded with Avr1b fused to GFP supported these results. Moreover, they also show that the N-terminal domain including the RxLR-EER motif was sufficient for translocation by incubating root tips of soybeans with a partially purified protein construct consisting of the N-terminal domain of Avr1b (without the signal peptide) fused to GFP. After overnight incubation, fluorescence appeared in the plant cells, including in the nucleus, while no fluorescence was observed with mutated versions of the construct (Dou *et al.*, 2008b).

5. Future perspectives

In a recent review, Birch *et al.* (2008) revealed the interesting finding that the RXLR-EER motif was represented in the

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proteome of the model plant *A. thaliana*, and some of those predicted proteins may be involved in the endocytosis pathway and/or associated with membranes. This may explain why this highly conserved oomycete feature is not recognised as a pathogen associated molecular pattern (PAMP) by the host.

Remarkably, the *in planta* induced *ipiO* gene product (van West *et al.*, 1998) also contains the cell adhesion motif RGD (arginine glycine aspartate) overlapping an RxLR motif. Now renamed AVR-blb1, its avirulence property has recently been confirmed, and it is presumably translocated inside the host cell (Vleeshouwers *et al.*, 2008). Previously, the RGD motif from AVR-blb1 was shown to bind specifically to the lectin domain of a transmembrane protein with an intracellular kinase activity in *A. thaliana* (Gouget *et al.*, 2006). These findings, together with the recent demonstration in *Arabidopsis* that receptor FLS2 undergoes internalization upon activation with bacterial flagellin (Robatzek *et al.*, 2006), raises the hypothesis that AVR-blb1 binding to its cognate receptor could induce its internalization. It is now becoming clear that some plant pathogens could have evolved strategies to exploit host-cell vesicle trafficking to manipulate immune responses (Frei dit Frey and Robatzek, 2009 and Robatzek, 2007).

Other oomycetes that seem to have only very few or no putative RxLR-effector proteins, such as *Aphanomyces euteiches* (Gaulin *et al.*, 2008), are likely able to deliver effectors to their host cells by means of different mechanisms. It is interesting to note that several pathways are used by the malaria parasite (Sam-Yellowe, 2009). Various hypothetic routes for the delivery of oomycete effectors into the plant cell are illustrated in Fig. 1.

In the last few years, the genomes of six oomycetes have been sequenced (Lamour *et al.*, 2007 and Tyler *et al.*, 2006; Haas *et al.*, 2009): *P. sojae*, *P. ramorum*, *P. infestans*, *P. capsici*, *Pythium ultimum* and *Hyaloperonospora arabidopsis*. If the mechanism of translocation is broadly conserved across pathogens from taxa as divergent as *Plasmodium* and *Phytophthora*, it is expected that translocation motifs will be found in the secreted proteins of oomycete pathogens from more distantly related genera, such as *Saprolegnia* (van West, 2006).

Although there is now good evidence that the RxLR effectors are translocated into the host cell (Dou *et al.*, 2008b and Whisson *et al.*, 2007), visualisation of translocated effectors has proven difficult to achieve so far. Despite the prediction of a nuclear localisation for some effectors, the subcellular targeting of most RxLR proteins is still unclear.

Current research in this field is intensive and, undoubtedly, the coming years will shed light on the mechanisms employed by these fascinating organisms to manipulate their host. Ultimately, understanding how oomycetes are able to deliver their weaponry inside plant cells may facilitate the limitation of the devastating impact they inflict on food security.

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