Eukaryotic virulence determinants utilize phosphoinositides at the ER and host cell surface

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Abstract

Similar to bacteria, eukaryotic pathogens may also utilize common strategies of pathogenic secretion, as effector proteins from the oomycete *Phytophthora infestans* and virulence determinants from the human malaria parasite *Plasmodium falciparum* share a functionally equivalent host-cell targeting motif (RxLR-dEER in *P. infestans* and RxLxE/D/Q in *P. falciparum*). Here we summarize recent studies that reveal that the malarial motif may function differently than previously envisioned. Rather, binding the lipid phosphatidylinositol 3-phosphate [PI(3)P] is a critical step in accessing the host for both pathogens, but occurs in different locations. Nanomolar affinity for PI(3)P by these short amino acid motifs, suggests a new mechanism of phosphoinositide binding unexpectedly in secretory locations that has been exploited for virulence by diverse eukaryotic pathogens.

Keywords

malaria; oomycetes; host-targeting; phosphoinositides; secretion; pathogenesis

Eukaryotic pathogens target proteins into plant and human cells

Numerous microbes parasitize the intracellular environment of plants and mammals. To invade and survive in a host cell, prokaryotic and eukaryotic pathogens communicate across several membrane barriers and are able to modulate host endomembrane systems. Consequently, intracellular microbes develop strategies to modulate host membrane trafficking. Phosphoinositides (PIs) are key players in trafficking and signaling processes in

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eukaryotes that control membrane–cytoskeleton interactions and vesicle trafficking [1]. In particular, for endocytic trafficking, phosphatidylinositol-3-phosphate [PI(3)P] plays a crucial role in conferring identity to endosomes and regulating vesicle fusion on the cytoplasmic face of the membrane [2] and these properties may be exploited by pathogens to establish intracellular infection.

For some bacterial pathogens, manipulation of cytoplasmic PIs and membrane trafficking begins prior to becoming intracellular. Bacteria such as *Salmonella*, invade epithelial cells that are not phagocytic. They do so by actively injecting effectors into host cells, which induces phagocytosis in non-phagocytic cells [3]. These effectors are delivered through a bacterial secretion apparatus inserted into the host plasma membrane [4]. Once injected, these effectors modulate functions of small GTPases as well as cytoskeleton and membrane lipids in the cytoplasm of the host cell. The *Salmonella* effector protein SopB is a phosphatase whose activity recruits the host Rab5 and PI(3)P kinase Vps34 to the *Salmonella*-containing vacuole (SCV) [5]. This induces remodeling in the cytoplasmic actin network underneath the plasma membrane and facilitates bacterial engulfment by host cells (Figure 1, panel A). Subsequently, SopB-induced enrichment of PI(3)P also contributes to the maturation of the SCV. In contrast, intravacuolar *Mycobacterium tuberculosis* uses a phosphatase SapM (secreted acid phosphatase M) to decrease cytoplasmic PI(3)P levels on the vacuole and thus arrests maturation of the vacuole at the early endosome stage in which *M. tuberculosis* is able to replicate [6].

Viruses are not known to utilize cellular PI(3)P, but utilize other PIs. Lipid enveloped viruses that harbor a lipid membrane bilayer derived from their host cell, such as human immunodeficiency virus-1 (HIV-1) and human T-lymphotropic virus-1 (HTLV-1), utilize $PI(4,5)P_2$ and mono- and polyvalent PIs enriched on the inner leaflet of the plasma membrane (PM) including phosphatidylinositol (4)-phosphate [PI(4)P], phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] and phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$], respectively, to assemble and exit the cell [7, 8]. Further, PI(4)P is required for the replication of RNA viruses from the *Picornaviridae* (i.e., poliovirus, coxsackivirus, Aichi virus, and enterovirus 71) and *Flaviviridae* (i.e., hepatitis C virus) families [9] and inhibition of host PI 4-kinases may serve as a mechanism of panviral therapy [10].

In contrast, eukaryotic pathogens, such as the oomycete *Phytophthorainfestans* and the apicomplexan *Plasmodium falciparum*, appear to utilize PI(3)P at the host plasma membrane and the parasite endoplasmic reticulum (ER) lumen to respectively modulate endocytic and exocytotic trafficking pathways for both secretion and pathogenesis (Figure 1, panel B and C). *P. infestans* causes potato blight. Infection begins when spores germinate and develop into an infection structure called the appresorium to penetrate plant cells. Subsequently, the pathogen develops a structure called haustorium for nutrient acquisition and host immunity modulation. The haustorium is surrounded by an extra haustorial membrane (EHM) that separates the pathogen from the plant cytoplasm (Figure 2A). When blood stage malaria parasites invade human erythrocytes they too become enclosed within a parasitophorous vacuolar membrane (PVM) that forms during invasion and encloses the intracellular parasite during the asexual life cycle (Figure 2B). In *P. falciparum*, invasion is completed in minutes but intracellular development in the erythrocyte takes ~ 48 hours. Parasite progeny proliferate within, then rupture out of the PVM and the infected erythrocyte membrane to re-invade new erythrocytes and thus maintain blood stage infection which is responsible for all the symptoms and pathologies associated with malaria.

Both *P. infestans* and *P. falciparum* employ a host targeting (HT) motif on secretory proteins, to deliver hundreds of effectors that must cross the haustorial/vacuolar membrane

to enter the cytoplasm of host cells (Figure 2 A–B). Interestingly, their major host targeting motifs, RxLR-dEER from *P. infestans* and R/KxLxE/D/Q from *P. falciparum*, not only share protein sequence similarity, but also are functionally interchangeable in both binding PI(3)P [11] and in targeting effectors to the host [12, 13]. These motifs reflect the first well defined pathogenic secretion signals conserved across diverse eukaryotic pathogens. In this review, we will discuss how both oomycetes and *Plasmodium* target PIs but function in unexpected locations, namely the plasma membrane of the plant cell or the lumen of the parasite's ER rather than the cytoplasmic face of cellular membranes.

Eukaryotic pathogens use PI(3)P to target host cells

The utilization of PI(3)P by both *P. infestans* and *P. falciparum* provides remarkable evidence for the presence and function of PIs in secretory locations in eukaryotes. *P. infestans* and additional oomycete pathogens use the RxLR-dEER motif and additional sequences [14] for PI(3)P binding on the plant host plasma membrane for internalization. However, the motif RxLR in absence of dEER sequences recognizes a tyrosine-O-sulfated host membrane protein in the oomycete fish parasite *Saprolegnia parasitica* [15], indicating that in different proteins (as well as possibly in absence of the dEER sequences) the motif RxLR may facilitate binding to different host determinants, distinct from PI(3)P. Moreover in a *P. infestans* effector such as Avh5, both the RxLR-dEER sequence and the C terminal domain bind PI(3)P and the latter shows greater affinity for PI(3)P [16]. Differences in both specificity and amount of binding arise due to use of different methodologies as summarized in Box 1, which discusses best practices for measuring PI-binding and implementing appropriate controls.

P. falciparum secretory proteins with the R/KxLxE/D/Q motif bind PI(3)P in the ER of the parasite to initiate host targeting to the erythrocyte [11]. In the subsequent discussion, we refer to the RxLxE/D/Q motif as the RxL motif, and KxLxE/D/Q as KxL motif. In plasmodial proteins with the RxL motif, the HT signal is subsequently cleaved by an aspartic protease plasmepsin V (pV), which releases the signal anchor [17, 18]. KxL is not a substrate for pV [17]. KxL motifs are present in the major virulence adhesin family *P. falciparum* erythrocyte membrane protein 1 (PfEMP1, or Var), with 59 members that are central to disease in malaria [19]. The N terminal leaders of two *P. infestans* proteins, Avr3a and Nuk10, when expressed transgenically in *P. falciparum*, target reporters like green fluorescent protein (GFP) to the erythrocyte [13] dependent on their RxLR-dEER motifs and with efficiencies comparable to that shown for malarial leaders [20, 21]. Further, cell biological and biochemical analyses confirm that with Nuk10, its export is dependent on its RxLR motif binding to PI(3)P and independent of cleavage by pV [11]. However, it is unknown whether PI(3)P is detected in the *P. infestans* ER and/or whether export of effectors from *Phytophthora* to the haustorium are dependent on interactions of RxLR-dEER with PI(3)P in the pathogen's ER.

The studies of Whisson *et al.* [22] definitively established that during infection export of a *P. infestans* effector like Avr3a from the pathogen to the plant cell was dependent on RxLR-dEER. Subsequent work revealed the presence of PI(3)P on host cells and its ability to bind *P. infestans effectors*, other oomycete RxLR effector proteins, and rust fungus effectors with RxLR-like motifs [23], when recombinant effectors were with cultured host cells in the absence of infection (Table 1). These studies suggest that PI(3)P-RxLR-dEER dependent uptake of effector proteins into endosomes occurs in the absence of additional pathogenic factors and link PI(3)P-dependent uptake to RxLR-dEER as well as possibly other host targeting signals present on *Phytophthora* proteins. However, the consequences of post-translational modification of effectors by the pathogens have not been assessed. Further, uptake of effectors by cell surface PI(3)P results in a concentration of effectors in the

endosome rather than in the cytoplasm where they are normally delivered in infection. Thus, although it is likely that effectors bind to PI(3)P as the first step during uptake into plant cells, how effectors are concentrated and translocated across the endosomal membrane to the host cytosol is poorly understood. Prokaryotic and possibly eukaryotic pathogens actively insert effector translocation machinery into pathogenic vacuoles and it is likely that *Phytophthora* may similarly modify the extra-haustorial membrane (Figure 1).

Detailed comparative analyses of *P. infestans* and *P. falciparum* host targeting signals

Binding to PI(3)P

Functional properties of *P. infestans* and *P. falciparum* host targeting signals have been defined in the context of N-terminal leader sequences of proteins, that when coupled to exogenous reporters such as GFP and RFP, enable their export to the host cell. Using this basic reporter design, Kale *et al.* [14] reported that PI(3)P lipid was present on plant and mammalian cell surfaces and bound by the *P. infestans* HT signal RxLR-dEER to enable effector proteins access to host endosomal compartments. They further showed that a *P. falciparum* host targeting signal (with a consensus motif of RxLxE/D/Q, also known as PEXEL, for plasmodial export element) bound PIs. However, since PI binding was determined by using lipid strips, the mechanism and quantitative affinity for PI(3)P were unknown (as elaborated further in Box 1). Bhattacharjee *et al.* [11] demonstrated that the HT Nuk10 signal (RQLR) of *P. infestans* showed a high affinity interaction for PI(3)P with a K_d of 28 nM. As shown in Table 1, this was comparable to the K_d's (20–35 nM) for PI(3)P with HT motifs of three *P. falciparum* proteins: PfHRPII (PF3D7_0831800; a protein implicated in heme polymerization with an HT motif RLLYE), a RIFIN (member of a large antigenic family, Rif, of unknown function; PF3D7_0401600.1; HT motif RTLSE), and PfEMP1 protein (PF3D7_1200400; HT motif KDVLE). PfEMP1 is a family of variant surface virulent adhesins that enable infected erythrocytes to bind endothelial cells. A second PfEMP1 (PF3D7_1240600) with a motif of KELLD closely related to KDVLE, showed an approximately threefold lower K_d of 90 nM for PI(3)P. Similarly a heat shock protein (Hsp40, PF3D7_0501100.1) with a RSLAE motif and a member of STEVO,R another antigenic family of unknown function (PF3D7_0101800), with a RLLAQ motif display K_d's of 110 nM and 70 nM, respectively. Thus, even a limited set of *P. infestans* and *P. falciparum* HT motifs display a fivefold range for K_d's varying from 20 to 100 nM. As can be inferred from Table 1, this is not easily explained by differences in sequences of the HT motif sequence alone. It is likely that PI(3)P binding is mediated by a domain, a critical component of which is contributed by the R/K residues of the HT signal. As elaborated later in this review, multiple cationic residues are expected to contribute to nanomolar affinity of PI(3)P recognition, hydrophobic residues are likely needed for membrane penetration and hydrogen bonding through consensus D/E and will likely distinguish specificity for PI(3)P relative to other PIs [24]. Requirement of a domain is also consistent with early data indicating that in a vacuolar translocation signal (VTS; of ~40 amino acids) of PfHRPII both the HT motif and sequences upstream are needed for optimal *P. falciparum* protein export to the erythrocyte [13].

Additional host targeting properties

Host targeting is a complex process, with different transport properties ascribed to peptidic regions of effectors known to catalyze export to the erythrocyte. A summary of transport properties for oomycete and *Plasmodium* protein domains is provided in Figure 3. Emerging evidence from *Phytophthora* suggest that in addition to RxLR-dEER, the C-terminal domain of an effector protein can also bind PI(3)P and thus access the extra-haustorial membrane [25]. Indeed for the effector of Avr1b in *Phytophthora sojae*, the C-terminal domain shows a

greater affinity for PI(3)P compared to the N-terminal RxLR-dEER [25]. The four positively charged K residues on the first alpha helix from the N-terminus of the effector domain are important for PI(3)P binding activity. Mutation of any of these residues abolishes the binding activity of PI(3)P. These data are also consistent with the idea that PI(3)P binding is the property of a domain.

C-terminal regions of malarial proteins have not been tested for PI(3)P binding. Gruring *et al.* [26] have recently shown that short leader sequences from PNEPs (or PEXEL negative exported proteins) which lack intrinsic activity to target soluble reporters, are capable of exporting membrane chimeras when used in conjunction with transmembrane domains derived from PNEP proteins [26]. Dependence of this process on PI(3)P was not investigated. It is important to do so because PI(3)P binding is not expected to be restricted to KxL or RxL motif, but rather supported by diverse structural moieties across pathogenic and mammalian proteins (Figures 4 and 5). When the PNEP leaders were substituted by sequences downstream from HT motifs of STEVORs and glycophorin binding protein 130 (GBP130, another parasite protein of unknown function) and erythrocyte membrane protein 3 (EMP3; targets the erythrocyte skeleton), export of the membrane chimeras was preserved. However, the sequences in EMP3 show intrinsic erythrocyte-export activity with soluble reporters [11] even when the PfEMP1 signal anchor is engineered to be cleaved by signal peptidase. Indeed this observation was key to recognizing that the aspartic protease pV does not target export as previously proposed [17] but rather releases the signal anchor from the ER membrane. Sequences downstream of the HT motif in GBP130 and STEVOR have not yet been tested, but those in HRPII also show intrinsic export activity with soluble reporters [27]. This activity is independent of PI(3)P and less efficient than the dominant HT PI(3)P-dependent export when both signals function in an intact VTS [28]. Finally, the distribution of the aspartic protease pV corresponds to that of PI(3)P in the ER lumen (see Figure 3 and [28]). However, PfEMP1 proteins which are the major malarial virulence determinants, contain an HT motif and bind PI(3)P, are not substrates of pV. Thus despite the attraction of a `global' mechanism [29, 26] of export to the erythrocyte, further studies are required to establish whether PNEPs and HT containing proteins are similarly exported at a mechanistic level.

There is as yet no evidence of HT-independent transport associated with *P. infestans* RxLR-dEER leaders. Indeed if the leader of *P. infestans* Nuk10 is expressed in blood stage malaria parasites with a mutated RxLR motif, export to the erythrocyte is abrogated, rather than reduced as seen with the *P. falciparum* VTS and HT motifs [11]. Sequences downstream of Avr3a also appear to have no intrinsic export activity [13, 26]. In *P. infestans*, another group of HT effectors are the crinklers, which possess a FLAK motif in the N-terminal end of the sequences [30]. It remains unknown whether crinklers use PI(3)P dependent or independent mechanisms to target the host.

As elaborated in Figure 3 multiple export properties may be associated with peptidic domains of effectors in both *Phytophthora* and malaria parasites, and additional investigation is required to determine their related hierarchy and/or independence. It should be emphasized that since functional domains involved in protein-lipid interactions are larger than the HT motif, the design of constructs and understanding of the intrinsic export functions of flanking regions requires careful attention. For instance in *P. infestans* effectors the dEER sequences have not been found to have any intrinsic export activity, but they are needed in conjunction with RxLR to detect host targeting [13]. In addition, sequences upstream of the HT motif of HRPII are needed (in conjunction with RLLYE) for targeting the erythrocyte [13]. Finally, HT motifs and their downstream sequences appear inherently unstructured and thus unexpected bends (as well as folding) influences export [26, 31]. Thus

careful biophysical measurements such as SPR or NMR are recommended for measuring lipid binding over more rapid but less discriminating lipid strips (see also Box 1).

Sites of action

A major difference between *P. infestans* and plasmodial HT signals and their binding to PI(3)P are their subcellular sites of action. *P. infestans* secretes proteins that bind at PI(3)P on the host cell surface, which provides the first step for effectors to access the host endosome ([14]; Figure 1). In contrast, the mature erythrocyte has no PI(3)P and thus merozoite proteins secreted during invasion do not bind PI(3)P on host erythrocytes. Further, HT signals with the RxL motif initially bind PI(3)P in the parasite ER but lose this property when they are subsequently cleaved in the ER (Figure 1). Hence RxL motif proteins when exported to the erythrocyte likely do not show affinity for PI(3)P there. Thus, at the end of the intraerythrocytic cycle, when the infected cell lyses and RxL-motif proteins are shed in plasma, they lack the HT motif and PI(3)P binding function.

Common fold for intracellular binding of PI(3)P in oomycete effectors

Emerging structural analysis is starting to provide insights into mechanisms of PI(3)P binding in diverse proteins. Protein structures for a total of five oomycetes effectors have been recently obtained (Figure 4), *i.e.*, Avr3a4 and Avr3a11 (*Phytophthora capsici*), PexRD2 (*P. infestans*), Avh5 (*P. sojae*), and ATR1 (*Hyaloperonospora arabidopsidis*) [32, 33, 16]. *Phytophthora* RxLR-dEER effectors form large rapidly evolving superfamilies, and many effectors contain conserved sequence motifs (W, Y, and L) in their C-terminal domains arranged into tandem repeats [34]. Structural analysis of the four effectors uncovered a common fold conserved across oomycetes species. The conserved unit is termed WY-domain, and comprises a minimum three helices per domain [35].

Structural and functional analyses of the *Phytophthora* effector Avr3a shows a conserved, positively charged surface patch that binds PI(3)Ps on the first alpha helix of the conserved effector structure fold in the host cytoplasm [25]. Once delivered into plant cells, Avr3a is able to suppress host programmed cell death by stabilizing and inhibiting the host E3 ubiquitin ligase CMPG1. Mutations in the PI(3)P-binding patch are able to diminish the Avr3a effect of stabilization of CMPG1 *in vivo* [25]. PI(3)P-binding ability is therefore delineated to the C-terminus of the effector and shown to be involved in host defense suppression. It is possible that this activity is important for cytosolic effector action and also contributes to the PI(3)P recognition at the EHM between the pathogen and host cell (Figure 1). A dual recognition mechanism of PI(3)P has been shown by the RxLR effector Avh5 from *P. sojae* [16]. Structural analysis with NMR and a number of lipid binding assays revealed the C-terminal helical region bound PI(3)P while the RxLR motif also bound PI(3)P but played a more minor role; however, both PI(3)P binding sites mediated entry independently into the host cell. This type of mechanism of lipid recognition by Avh5 is reminiscent of mammalian lipid binding proteins, which are often coincidence detectors for membrane lipids harboring more than one lipid binding site for a lipid headgroup [36].

Structural studies of three mammalian PI(3)P binding proteins and domains (EEA1-FYVE, PX domains and PROPPINS) provide significant insights on how a diverse set of modular domains bind PI(3)P specifically with nanomolar affinity (Figure 5). The X-ray structure of EEA1-FYVE suggests that the FYVE domain *stereo*-specifically recognizes the PI(3)P head group [37], as a homodimer and the PI(3)P-bound FYVE may have an `angled' orientation with respect to the membrane surface, which allows hydrophobic residues in the turret loop to penetrate the membrane (Figure 5a) [38]. Due to the presence of cationic residues, the turret loop region of FYVE domains is surrounded by highly positive electrostatic potential, which drives the initial membrane association [39]. Subsequently, specific PI(3)P binding

induces the interfacial penetration of hydrophobic and aromatic residues in the turret loop [39]. Further, PI(3)P binding is not a consequence of but a prerequisite for the interfacial penetration of turret loop residues. Thus PI(3)P binding serves as an electrostatic switch that greatly reduces the positive potential surrounding the turret loop and thereby promotes the interfacial penetration of hydrophobic and aromatic turret loop residues [39, 40].

The PX domain is a structural module (Figure 5b) composed of 100–140 amino acids [41, 42] shown to interact with different PIs via conserved basic residues [43]. PX domains exhibit broad PI specificity akin to PH domains, but many, including those of Vam7p [44], sorting nexin 3 [45], and p40^{phox} [46], specifically interact with PI(3)P *in vitro* and target proteins to early endosomes in the cell. The crystal structure of the p40^{phox}–PI(3)P complex illustrates how the domain also achieves stereospecific recognition of PI(3)P [46]. Non-specific electrostatic interactions between the cationic molecular surface of the PX domain and the anionic membrane are followed by specific PI binding and subsequent interfacial penetration of hydrophobic and aromatic residues [47, 48]. The CISK PX domain might also be involved in domain homodimerization in addition to PI-induced interfacial penetration [49]. Finally, the first crystal structure of PROPPINs (Figure 5c), which are key autophagic PI(3)P sensors conserved from yeast to humans, delineated a β-propeller structure harboring seven blades and two binding sites for PI(3)P [50]. A hydrophobic loop adjacent to the PI(3)P binding sites acts in tandem with PI(3)P to promote high membrane affinity and full autophagic function in yeast. Pathogenic effectors identified to date are unique in PI(3)P binding specificity. However a well-defined pocket has not yet been determined (Figure 5d) nor is the molecular basis of PI(3)P coordination known, which should undoubtedly provide H-bonding and electrostatic interactions with the 1- and 3-phosphate of PI(3)P if these effectors recognize PI(3)P over other PIs. Instead, Avh5 has been shown to bind PI(3)P using Lys^{62}, Lys^{64}, and Lys^{65} in helix 2 of the WY domain as well as the RxLR motif [16] in contrast to lysine residues present in helix 1 of the WY domain of Avr3a [25]. Thus, these effectors apparently use a cationic patch in different locations in the WY domain or RxLR region to recognize the PI(3)P headgroup. This type of specificity and PI(3)P binding mode is unique among PI(3)P binding proteins identified to date. As summarized earlier, FYVE and PX domains and the PROPPIN family typically insert into the hydrocarbon core of the membrane where aliphatic and aromatic amino acids mediate this step, which is induced by specific PI(3)P binding. The RxLR-dEER and K/RxLxE/D/Q motifs as well as WY domain are rich in aliphatic and aromatic amino acids, which may additionally play a role in increasing PI(3)P dependent membrane affinity and the ability of pathogenic effectors to enter the host cell or exit the ER through vesicular transport.

Concluding remarks and future directions

A major question raised by studies related to PI(3)P and host cell targeting in *Plasmodium* and *Phytophthora* is how an essential signaling molecule appears in unexpected cellular locations. Malaria parasites show PI(3)P in the ER lumen and host cells of oomycetes reveal PI(3)P in the outer leaflet of plasma membrane. Neither cellular location has been previously associated with notable levels of PI(3)P. All known enzymes for PI(3)P production are cytoplasmic and there are no well-defined mechanisms to account for the PIs in the ER or outer leaflet of the plasma membrane. Thus, questions emerge on how PI(3)P is generated and transported to the outer leaflet of the plasma membrane and to the ER lumen? How are their recycling and steady state maintenance performed? Are there other PI(3)P interacting proteins encoded by the parasites that assist with these processes?

A second set of questions arise on why binding of PI(3)P by a pathogenic effector induces endosomal budding at the plasma membrane or is associated with exit from the ER? What additional factors are required at the plasma membrane or in the ER? Are mechanisms

related despite the action in different cellular locations? Expression of EEA1 and p40^{phox} mammalian proteins that bind PI(3)P in *P. falciparum* ER does not result in their exit from the ER [11]. To date, the majority of lipid-binding proteins discovered are coincidence detectors [36] where they bind with high affinity and selectivity to two lipids [such as PI(3)P and PS] or to one lipid and a membrane physical property such as curvature [such as PI(3)P in highly curved membranes]. In addition, two PI(3)P binding sites may be present to increase avidity as observed for PROPPINs, the FYVE homodimer, or Avh5. Thus, PI(3)P binding by pathogenic effectors may be an important determinant of endosomal PM budding or exit from the ER. However, effector interactions with other lipids or membrane physical properties could also exist (Box 1), which may provide spatial and temporal cues in pathogenic export to the host.

Acknowledgments

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Highlights

- Eukaryotic pathogens use common strategies to target virulence proteins into host cells.

- Binding the lipid PI(3)P is a critical step in virulence-related trafficking.

- The high PI(3)P affinity of effectors from pathogens defines them as novel lipid interacting proteins.

Box 1. Methods and conditions of success for monitoring lipid-protein interactions

Cellular membranes are composed of hundreds if not thousands of different lipid species in bilayers that compartmentalize a cell or pathogen with different organelle structure and function. Peripheral proteins, which transiently interact with one side of the bilayer usually, have high affinity and specificity for a key target lipid, which can mediate protein function and elicit communication across membrane bilayers. Lipid binding specificity among peripheral proteins can vary from anionic glycerophospholipids such as phosphatidlyserine and the phosphoinositides to sphingolipids including ceramide and ceramide-1-phosphate. Moreover, many peripheral proteins are now appreciated to be coincidence detectors where they harbor two lipid-binding sites that can provide exquisite regulation of protein localization to discrete membrane sites enriched in both lipids. Thus, to investigate lipid-binding specificity and membrane affinity for newly characterized proteins it is important that lipid-binding studies are carefully and accurately performed.

Methodologies can differ in their sensitivity and reliability

To this end vesicle sedimentation assays and surface plasmon resonance (SPR) with large unilamellar vesicles have robustly served to delineate lipid binding selectivity and membrane binding affinity, respectively. Lipid strips composed of different types of lipids blotted on nitrocellulose have been traditionally useful for probing lipid specificity such as which phosphoinositide a protein selectively binds, however, they are not able to provide quantitative information and their surfaces are not well characterized. This has been a contentious issue for the RxLR leaders where PI(3)P binding has been attributed to the RxLR motif [14], the WY domain [15, 25], or both [16] and also in one case denatured protein binding to PI(3)P [15]. For some of these studies lipid strips were the primary source of data collection. The behaviors and orientations of phosphoinositides spotted on nitrocellulose have not been rigorously investigated and reproducibility from experiment to experiment can be an issue. Assays that incorporate the key lipid into an environment that recapitulates the native environment of the protein binding can play a much more effective quantitative and predictive role of membrane binding mechanisms. For instance, the majority of peripheral proteins identified to date undergo hydrophobic or electrostatic interactions with the membrane surface in addition to phosphoinositide headgroup binding while others have a second binding site for an essential membrane lipid.

Lipid strips cannot provide such an environment for detecting these interactions unlike vesicles, which can be modulated to include a variety of lipid species to provide a surface more akin to a membrane bilayer. To further demonstrate that lipid blots may not accurately assess PI(3)P binding, mutation of the RxLR motif in Avh5 did not show an appreciable change in PI(3)P binding on lipid strips but had ~30% reduction in binding in lipid vesicle binding assays [16]. This may help explain some of the differences in PI(3)P binding analyzed earlier for Avr3a mutants where only lipid strips were used [25]. Lipid strips also may not be the best approach for studying some of these effectors that have two binding sites for PI(3)P. For instance, it is possible the two binding sites may compete for PI(3)P presented on the nitrocellulose making it difficult to quantify the role or affinity for either site for PI(3)P. Mutagenesis of the RxLR or WY domain in this case could just promote more binding of the other motif to PI(3)P or other PIs on the nitrocellulose membrane. Further, because these assays are usually performed at micromolar concentrations in the same vessel it is difficult to discern PI specificity for those effectors that demonstrate binding to two or more PIs without employment of a secondary assay (sedimentation or SPR).

Appropriate controls are important for phosphoinositide binding

It is also wise to use positive and negative controls for lipid surfaces whether it be lipid strips, SPR assays, or vesicle sedimentation assays to demonstrate the viability and reproducibility of the surface employed. For instance, if PI(3)P selectivity is shown for a newly identified pathogen effector one could use the PX domain of p40^{phox} as a positive control for PI(3)P and the PH domain of PLCδ1 as negative control (PI(4,5)P$_2$ specificity). This type of approach allows one to make quantitative and confident conclusions regarding lipid-binding specificity and affinity.

Membrane physical properties such as curvature and charge matter

Recently, membrane physical properties such as charge or curvature have also been shown to play a role in membrane recruitment of peripheral proteins[53]. In addition, some peripheral proteins induce changes to membrane structure (positive or negative curvature changes) upon membrane binding [54]. While testing membrane curvature dependency is not the first set of experiments one pursues it is important to be mindful that some lipid-binding proteins may bind to highly curved membranes more effectively than flat membranes while others themselves may induce changes to membrane structure upon lipid binding. Thus, if binding is not observed to one type of membrane curvature this may not rule out lipid binding affinity or selectivity for a particular protein. The membrane interface, which restricts proteins on the interface to 2-dimensions can promote avidity and protein oligomerization and could be another mechanism of PI(3)P recognition by these motifs. In closing, when performing lipid binding studies of previously uncharacterized proteins it is recommended to utilize at least two different lipid binding assays with appropriate controls to confirm lipid selectivity and to strive for an assay that is sensitive enough to delineate changes in affinity for point mutations if the origin of lipid binding is going to be investigated.
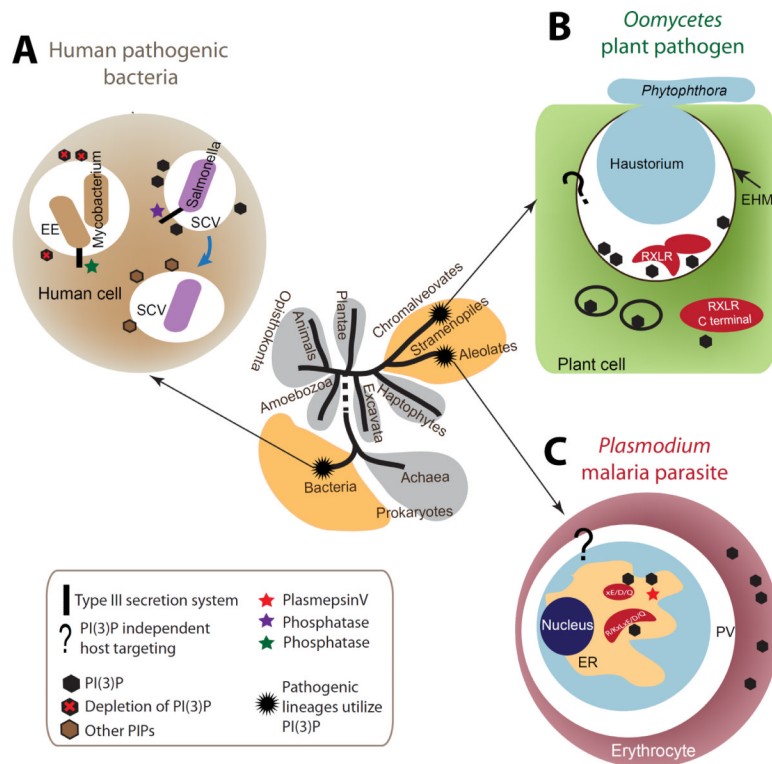
Figure 1. Phylogenetic position, modulation of PI(3)P and host targeting in different intracellular pathogens

The tree of major life domains shown is based on the analysis of [55]. Pathogenic lineages that utilize PI(3)P for host targeting and host modulation are chromalveolates, alveolates and bacteria. The life domains to which they belong are colored yellow. Host targeting refers to the process by which intracellular pathogens deliver pathogen-produced enzymes, toxins and other proteins directly into host cytoplasm and membrane. Bacterial pathogens use a type III secretion system to inject effectors into host cells. Eukaryotic pathogens utilize endocytic pathways to deliver proteins. **(a)** Intracellular bacteria use type III effectors to modulate PI(3)P levels for survival and replication within vacuoles inside the host cell cytoplasm. *Mycobacterium tuberculosis* decreases PI(3)P levels to arrest endosome maturation, avoiding destruction by the host. *Salmonella* transiently increases PI(3)P levels to facilitate invasion and biogenesis of the vacuoles for intracellular survival. **(b)** Plant pathogenic oomycetes partly grow into plant cells with a structure called the haustorium. Host targeting utilizes PI(3)P at the extra haustorial membrane surrounding the haustorium to enable RxLR-dEER effector entry. How effectors are delivered across the extra haustorial membrane into the host cell is not known (and thus shown by a question mark), but once in the cytoplasm, their C-terminal domain(s) can bind cytoplasmic PI(3)P to modulate host physiology. **(c)** The malaria parasite *Plasmodium* survives in a membranous structure in erythrocytes. Host targeting starts at the parasite ER with binding of PI(3)P by a host targeting motif R/KxLxE/D/Q. Plasmepsin V is an aspartic protease that does not cleave KxL motifs. However it is detected in association with PI(3)P (see Figure 3) cleaves after the RxL to yield xE/D/Q, which does not bind PI(3)P but supports export by unknown mechanisms (depicted by question mark). In oomycetes, PI(3)P-independent host targeting pathways also exist. Vaid *et al.* [56] report the presence of PI(3)P in the infected erythrocyte. Abbreviations: EE, early endosome; SCV, *Salmonella*-containing vacuole; EHM, extra haustorial membrane; ER, endoplasmic reticulum; PV, parasitophorous vacuole.
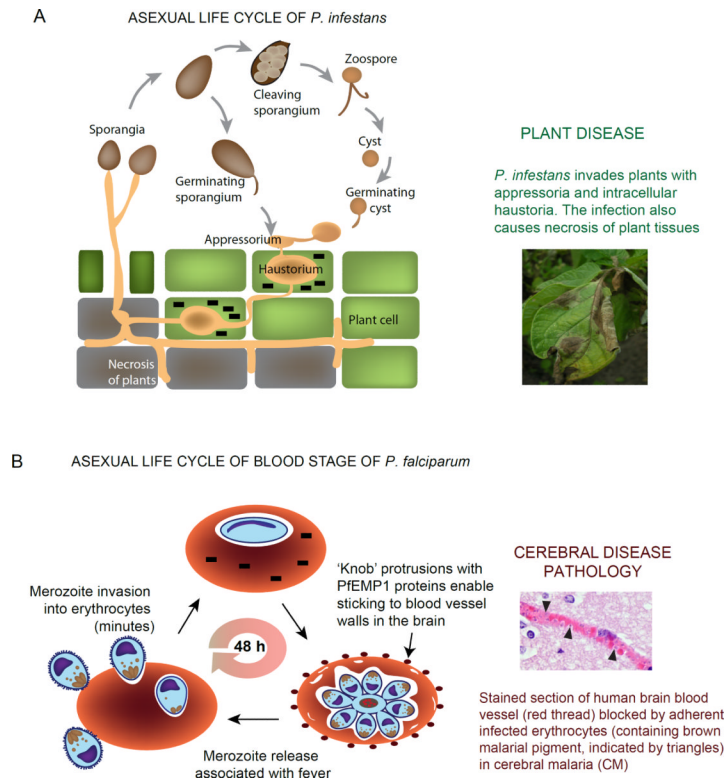
Figure 2. Life cycles and pathologies of *Phytophthora* and *Plasmodium*
(a) *P. infestans* is able to initiate an infection with both sporangia and zoospores. The pathogen forms an appressorium and penetrates plant leaf cells. Subsequently, a haustorium is developed within host cytoplasm for nutrient acquisition and host immunity modulation. Effectors (black bricks) are trafficked into host cells from the haustoria. Later during infection, *P. infestans* causes necrosis in plant tissue and sporulates to form new sporangia. The photo of infected potato leaves with sporulating *P. infestans* is kindly provided by Klaas Bouwmeester and Francine Govers. **(b)** Asexual, *P. falciparum* blood stage infection begins when the extracellular merozoite invades the erythrocyte, a process which although complex, is completed in minutes. Effectors (black bricks) are exported across the vacuolar membrane that surrounds the intracellular parasite. Subsequent parasite development, maturation and emergence of merozoites takes ~48 h. In the second half of the asexual cycle, exported parasite effectors assemble in electron dense `knobs' that display the cell surface parasite protein PfEMP1. PfEMP1s are endothelial adhesins that enable infected erythrocytes to bind to the walls of blood vessels. In the brain this can lead to inflammation as well as occlusion of blood vessels (as seen in the adjacent tissue section) and the fatal, neurological pathology of cerebral malaria. The photo is kindly provided by Dan Millner.
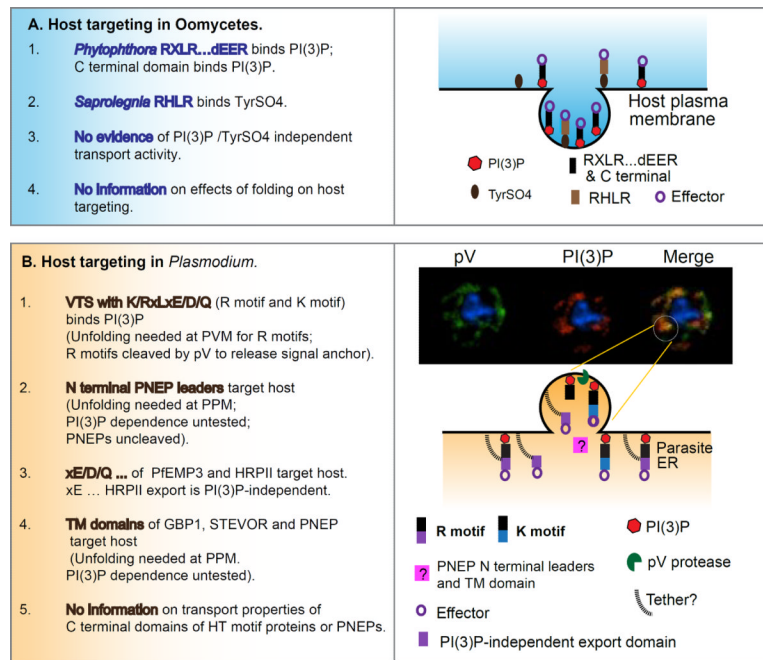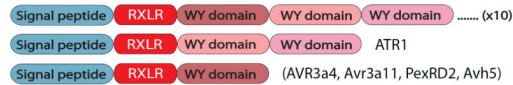
A. Host targeting in Oomycetes.

1. *Phytophthora* RXLR...dEER binds PI(3)P; C terminal domain binds PI(3)P.

2. *Saprolegnia* RHLR binds TyrSO4.

3. **No evidence** of PI(3)P /TyrSO4 independent transport activity.

4. **No Information** on effects of folding on host targeting.

B. Host targeting in *Plasmodium*.

1. **VTS with K/RxLxE/D/Q** (R motif and K motif) binds PI(3)P (Unfolding needed at PVM for R motifs; R motifs cleaved by pV to release signal anchor).

2. **N terminal PNEP leaders** target host (Unfolding needed at PPM; PI(3)P dependence untested; PNEPs uncleaved).

3. **xE/D/Q ...** of PfEMP3 and HRPII target host. xE ... HRPII export is PI(3)P-independent.

4. **TM domains** of GBP1, STEVOR and PNEP target host (Unfolding needed at PPM. PI(3)P dependence untested).

5. **No Information** on transport properties of C terminal domains of HT motif proteins or PNEPs.

Figure 3. Summary of transport properties of (a) oomycete and (b) *Plasmodium* protein domains **(a)** In *Phytophthora*, leaders containing RxLR- dEER and/or C terminal domains (black bars) of effector proteins (purple circles) binds the lipid PI(3)P (red dots) at the host plasma membrane resulting in their endosomal internalization. In *Saprolegnia*, leaders containing RHLR (brown bars) binds tyrosine sulfate (TyrSO$_4$; grey dot, a post translational modification of host proteins) to also undergo endosomal internalization. Presently, there is no evidence of host targeting properties independent of binding PI(3)P or TyrSO$_4$ or protein folding, such as seen in *Plasmodium*. **(b)** In *Plasmodium*, leaders known as vacuolar translocation sequences (VTS) containing a host targeting motif KxLxE/D/Q (black/blue bar) or RxLxE/D/Q (black/purple bar) bind PI(3)P (red dots) in the ER lumen. Micrographs in the right hand side top panel show distribution of the lipid PI(3)P in the ER lumen (red) and a parasite protease plasmepsin V (pV; green) colocalized (yellow/orange) in membrane domains in the ER. This suggests a model (shown immediately below) for a PI(3)P enriched vesicle (containing pV) emerging from the ER. In this regard, PI(3)P-dependent exit from the ER is topologically equivalent to PI(3)P-dependent endosomal uptake at the plasma membrane. Proteins with RxLxE/D/Q may be cleaved by the protease pV in the emerging PI(3)P vesicle, (which is reinforced by the finding that the specificity of PI(3)P binding mimics that of pV mediated cleavage). The xE/D/Q fragments of PfEMP3 and PfHRPII (purple rectangle) also have intrinsic export activity suggesting a second mechanism of inclusion and retention of soluble proteins (indicated by black tethers). N terminal leaders lacking K/RxL domains called PNEPs (pink rectangles) appear to share a common export mechanism whose dependence on PI(3)P has not been tested (and thus indicated by a question mark). Unlike *Phytophthora*, C-terminal domains of plasmodial effectors have not yet been shown to have intrinsic PI(3)P binding or export activity and parasite host targeting is sensitive to protein folding.
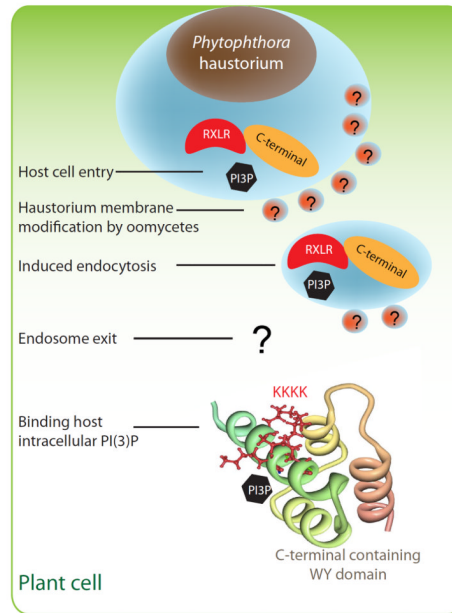
Figure 4. Conserved RxLR-dEER effector structure fold and different sites for PI(3)P binding
(a) RxLR effector domain architecture. RxLR effectors have an N-terminal secretory leader, a host targeting RxLR motif and conserved C-terminal domains that are often arranged into tandem repeats. **(b)** Host targeting model and involvement of PI(3)P at different cellular sites. The N-terminal RxLR motifs play an important role in host cell entry at host plasma membrane; and *in vitro* recombinant proteins of Avr1b bind PI(3)P. The C-terminal region has a conserved effector fold and is able to suppress host defense responses. The positive patches in the C-terminal domain of Avr1b and Avr3a are important for PI(3)P and PI(4)P binding. The Avr1b C-terminal structure is used in the illustration. Several important conceptual steps such as modification of membrane by pathogen are postulated and indicated with question marks.
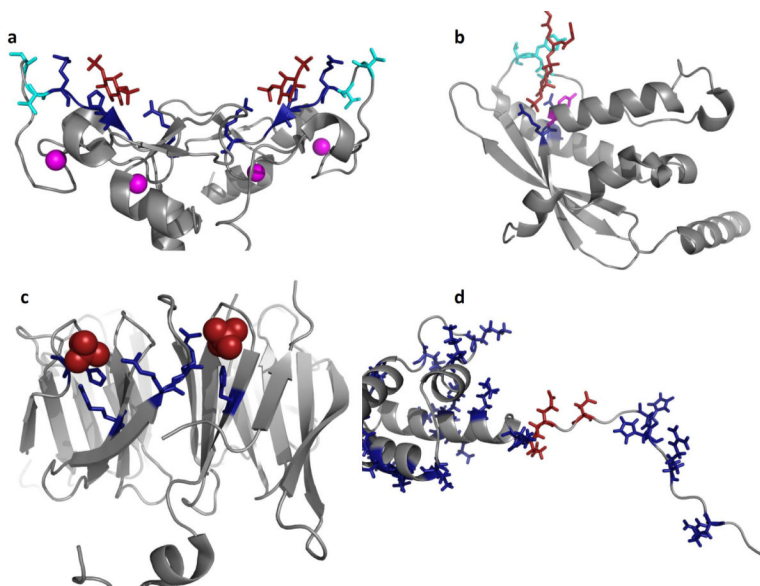
Figure 5. Mechanisms and models of PI(3)P binding by FYVE, PX, PROPPIN, RxLR and WY domains

PI(3)P binding has been attributed to at least three distinct eukaryotic domains including FYVE, PX, and PROPPINS while pathogens utilize the RxLR or RxLxE/D/Q motif and/or WY domains. **(a)** The homodimeric structure of the EEA1 FYVE domain is shown (PDB ID: 1JOC), which binds PI(3)P (shown in red) in a sideways fashion where both electrostatic and H-bonding with PI(3)P can be optimized while achieving hydrophobic interactions through the turret loops [39]. Cationic residues that coordinate the PI(3)P headgroup are shown in blue, hydrophobic residues that penetrate into the hydrocarbon portion of the membrane are highlighted in cyan, and the zinc ions coordinated by the FYVE domains for structural stability are shown in magenta. **(b)** The p40^{phox} PX domain (PDB ID: 1H6H) X-ray structure was solved bound to PI(3)P. Here, two critical arginine residues (shown in blue) mediate the recognition of PI(3)P, while a tyrosine residue (magenta) forms the bottom of the PI(3)P binding pocket. The PI(3)P binding pocket is deeper than that observed for the FYVE domain and harbors two loop regions that have been shown to facilitate nonspecific electrostatic interactions and membrane penetration (hydrophobic residues in cyan) [57]. **(c)** The yeast PROPPIN structure from Hsv2 (Homologous with SVP1), which is a key autophagic sensor protein, was solved bound to two sulfate ions (PDB ID: 4EXV). PROPPINs are PI(3)P sensors necessary for autophagy and are conserved from yeast to humans. The PROPPIN structure is a β-propeller composed of seven blades where two PI(3)P binding sites (sulfate ions in red and coordinating residues in blue) were discovered on blades five and six. In a similar manner to the FYVE and PX domains, a hydrophobic loop between blades five and six promotes membrane penetration of Hsv2, which is essential to act in concert with PI(3)P binding for membrane recognition and full autophagic function in yeast. **(d)** Shown is the N-terminal unstructured region and WY domain of Avr1b from *P. sojae*, which was modeled and generated using the available *P. infestans* Avr3a structure (PDB ID: 2LC2) [25]. The N-terminus harbors the RxLR motif (Arg residues in blue on the left) as well as number of other cationic (blue) and anionic (red) residues that may be critical tophosphoinositide binding. The WY domain of Avr1b is shown to highlight the cationic nature of this module (Arg, Lys and His shown in blue). In contrast to FYVE, PX, and PROPPINs a distinct phosphoinositide binding pocket or region is not obvious and nonspecific electrostatic association may occur based upon lipid charge.

Table 1

Host targeting characteristics for PI(3)P binding and protease sensitivity of effectors in *Plasmodium*, oomycetes and fungi

Species	Protein	Protein function [a]	HT motif	Cellular localization for motif activity [b]	PI(3)P binding	K_d for PI(3)P binding	Cleavage by protease	Experimental methods for host targeting	Experimental methods for PI(3)P binding [c]	Refs
Plasmodium parasites										
P. falciparum	PfHRPII	Unknown	RLLHE/RLLYE	Parasite ER	Yes	36 nM	Yes	*in vivo*	RP/SPR/In cells	[11, 51]
P. falciparum	RIFIN	Large antigenic family	RTLSE	Parasite ER	Yes	20 nM	Yes	*in vivo*	RP/SPR	[11]
P. falciparum	PfEMP1	Cell surface adhesin	KDVLE	Parasite ER	Yes	30 nM	nd	*in vivo*	RP/SPR	[11]
P. falciparum	STEVOR	Antigen family	RLLAQ	Parasite ER	Yes	70 nM	Yes	*in vivo*	RP/SPR	[11, 20, 21]
P. falciparum	PfEMP1	Cell surface adhesin	KELLD	Parasite ER	Yes	90 nM	No	*in vivo*	RP/SPR	[11, 21]
P. falciparum	Hsp40	Heat shock protein	RSLAE	Parasite ER	Yes	110 nM	Yes	*in vivo*	RP/SPR	[11, 20]
Oomycetes										
P. infestans	Nuk 10	nd	RQLR..dEER	h	Yes	28 nM	No	*in vitro*	RP/SPR	[11]
P. infestans	Avr3a	Suppress host immunity	RLLR..dEER	h	No	nd	No	*in planta*	RP/LS	[22, 25]
P. sojae	Avr1b	Suppress host immunity	RFLR..dEER	h	Yes	nd	nd	*in planta*	RP/LS	[12, 14, 25]
P. sojae	Avh5	nd	RFLR..dEER	h	Yes	nd	nd	*in vitro*	RP/LS	[14]
P. sojae	Avh331	nd	RSLR..dEER	h	Yes	nd	nd	*in vitro*	RP/LS	[14]
S. parasitica	SpHTP1	nd	RHLR	Host plasma membrane	No	nd	nd	*in vitro*	RP/LS	[15]
Fungi										
Melampsora lini	AvrL567	nd	RFYR	h	Yes	nd	nd	*in vitro*	RP/LS	[14]
Fusarium oxysporum	Avr2	nd	RIYER	h	Yes	nd	nd	*in vitro*	RP/LS	[14]
Puccinia striiformis	Ps87	nd	KRLTG	h	nd	nd	nd	*in vitro*	RP	[23]
Leptosphaer ia maculans	AvrLm6	nd	RYWT	h	Yes	nd	nd	*in vitro*	RP	[14]

[a] nd, no data available.

[b] h, host membrane surrounding the haustorium.

[c] RP, *in vitro* recombinant protein; SPR, surface plasmon resonance; LS, lipid strips.