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Trends in Plant Science

Forum

Filamentous pathogen effectors enter plant cells via endocytosis

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Recent findings demonstrate that cytoplasmic effectors from fungal and oomycete pathogens enter plant cells via clathrin-mediated endocytosis (CME). This raises several questions: Does effector secretion pathway facilitate host uptake? How is CME triggered in host cells? How are the effectors released from endosomal compartments to reach diverse subcellular destinations?

Effector delivery

Plant pathogens secrete effector proteins that are critical for infection of their hosts. **Effectors** (see Glossary) can act in the **apoplast**, outside host cells [1], or can be delivered to the inside of living plant cells (cytoplasmic effectors) to manipulate host immunity, often by directly targeting plant proteins [2]. Bacterial pathogens of plants and animals can inject effectors directly into host cells with specialized machinery, such as the type III secretion system [3]. Filamentous (fungal and oomycete) pathogens cause some of the major diseases of crops and are therefore threats to global food security. However, the precise means by which they deliver cytoplasmic effectors into plant cells largely remains unknown [4].

Oomycete cytoplasmic effectors often feature an Arg-any amino acid-Leu-Arg (RxLR) motif, that is required for **translocation** [2,5]. The means by which RxLR effectors are delivered into host cells is controversial; claims of lipid binding of the RxLR motif to the host plasma membrane and cell-autonomous uptake have been challenged [4]. Evidence suggests that the RxLR motif is a site of proteolytic processing and it is cleaved and removed during secretion [5]. By comparison to oomycete effectors, fungal cytoplasmic effectors lack obvious amino acid motifs associated with translocation. However, conserved structural folds in both oomycete and fungal effectors have been postulated to contribute to effector delivery [4]. Interestingly, cytoplasmic effectors from both the fungal pathogen *Magnaporthe oryzae* [6] and oomycete *Phytophthora infestans* [7] are exported from these pathogens by **unconventional protein secretion (UPS)** pathways, in that, although they possess secretion signal peptides, their export is insensitive to the inhibitor, brefeldin A, which blocks intracellular vesicle movement and thereby prevents conventional secretion via the endoplasmic reticulum (ER) and Golgi apparatus. The secretion route may be a key step in determining host delivery from these pathogens. Indeed, evidence suggests that cytoplasmic effector export from filamentous pathogens by UPS pathways is widespread [4]. In addition to understanding the secretion of cytoplasmic effectors a critical question is: how do they enter plant cells?

Clathrin-mediated endocytosis of filamentous pathogen effectors

Recent companion publications have provided evidence that uptake of cytoplasmic effectors from *M. oryzae* [8] or *P. infestans* [9] into host rice or *Nicotiana benthamiana* cells, respectively, can occur via clathrin-mediated endocytosis (CME). These studies complemented each other because they involved a mixture of similar and different experimental approaches. Each study used confocal microscopy to visualise fluorescent protein-tagged clathrin light chain components and saw clathrin-labeled bodies accumulating around pathogen infection structures; the **biotrophic interfacial complex (BIC)** in the case of *Magnaporthe*, and the **haustoria** of *Phytophthora*. Wang *et al.* [9] observed similar localization patterns with a tagged endosome-associated Rab GTPase, the plant-specific Rab5 homolog Ara6. Transformed *M. oryzae* lines expressing tagged cytoplasmic effectors delivered sufficient quantities of them from the BIC to detect their colocalization in membranous effector compartments (MECs) with GFP-tagged clathrin light chain [8]; whereas Wang *et al.* [9] were limited by the low levels of translocated effector fluorescence from *Phytophthora*.

Silencing expression of clathrin heavy chain components in either rice or *N. benthamiana* reduced infection by the respective pathogens. This was also observed following silencing of CME-associated *ADAPTOR PROTEIN COMPLEX 2 subunit 2a* in rice and the endocytic Rab GTPase, *Ara6*, in *N. benthamiana* [8,9]. Silencing to prevent CME in rice also resulted in abnormally enlarged and misshapen BICs lacking punctate MECs in infected

Glossary

Apoplast: the intercellular area between plant cells.
Biotrophic interfacial complex (BIC): a plant membrane-rich structure associated with *Magnaporthe oryzae* invasive hyphae that is the site for cytoplasmic effector translocation.
Effectors: proteins secreted by pathogens that act inside or outside plant cells to promote pathogenesis.
Haustoria: fungal and oomycete hyphal projections that are intimate with host cell membranes and are sites for cytoplasmic effector translocation and potential nutrient uptake.
Pattern-triggered immunity (PTI): the first layer of plant defence triggered by the perception of pathogen-associated molecular patterns by pattern recognition receptors.
Translocation: a process by which molecules, such as proteins, can be actively transferred from one membranous compartment to another.
Unconventional protein secretion (UPS): vesicular or nonvesicular protein secretion pathways that do not utilize the conventional ER–Golgi apparatus route.

cells. In contrast, silencing the clathrin-independent endocytosis (CIE)-associated gene *FLOTILLIN 1* had a negligible impact on *M. oryzae* pathogenicity or BIC morphology. The swollen BIC morphology was also observed with chemical inhibitors of CME and not with CIE inhibitors [9].

In the *P. infestans–N. benthamiana* pathosystem, *in planta* expression of GFP-tagged clathrin or Ara6 was used to specifically enrich for endosomes in vesicle preparations from infected plant cells using immunoprecipitation. Endosome-enriched preparations contained RFP-tagged cytoplasmic effectors secreted from transgenic *P. infestans* lines, observed by confocal microscopy and immunoblotting [9]. Proteomic analysis of immunopurified endosomes from infections with wild-type *P. infestans* revealed enrichment of host proteins associated with vesicle trafficking, along with various cytoplasmic RxLR effectors, but not apoplastic effectors [10]. These observations support host cell entry via CME.

Questions and next steps

The observation that cytoplasmic effectors from both fungi and oomycetes can enter plant cells via CME suggests that this might be a common mechanism across filamentous pathogens. However, there may be more than one route to gain host cell entry. Indeed, although apparently not playing a major role in *M. oryzae* effector delivery [8], CIE has been implicated in cytoplasmic effector uptake by fish cells following secretion by the oomycete pathogen *Saprolegnia parasitica* [10]. In addition, the fungal pathogen of maize, *Ustilago maydis*, forms a complex of seven proteins that spans the host membrane and associates with plant channel-forming plasma membrane proteins [11]. The protein complex potentially forms a translocon to facilitate cytoplasmic effector delivery. The *Plasmodium* translocon of exported proteins delivers into human blood cells cytoplasmic effectors that possess a motif, RxLxE/D/Q, which is similar to the RxLR in oomycete pathogen cytoplasmic effectors and is also cleaved during secretion [12]. It is thus important to investigate whether all cytoplasmic effectors from pathogens such as *M. oryzae* and *P. infestans* follow the same route of host cell entry.

Cytoplasmic effectors from *M. oryzae* and *P. infestans* that are taken up by CME are secreted by UPS [6,7]. What is the nature of this UPS pathway, and is this secretion route important to how the effectors are presented to the host membrane to facilitate CME? Several studies indicate that large proportions of fungal and oomycete secretomes comprise proteins that may be externalized by UPS, many of which lack signal peptides. Such UPS pathways may be favored during plant colonization [4]. Moreover, whether possessing or lacking SPs, secreted fungal and oomycete proteins, including effectors, can be associated with extracellular vesicles (EVs) [4]. EVs represent an attractive means to package a range of effectors that are destined to function in the host cytosol and protect them from the potentially adverse conditions in the apoplastic environment. How such EVs are formed, what the composition of their cargo is and what proteins decorate their outer surface, are important questions to address.

CME can be triggered by cell surface receptor activation, including pathogen-derived ligand binding to pattern recognition receptors leading to **pattern-triggered immunity (PTI)** [13]. Does receptor internalization during PTI facilitate cytoplasmic effector uptake? If so, whether effectors are secreted as individual proteins, as components of protein complexes, or in association with EVs (Figure 1), how are

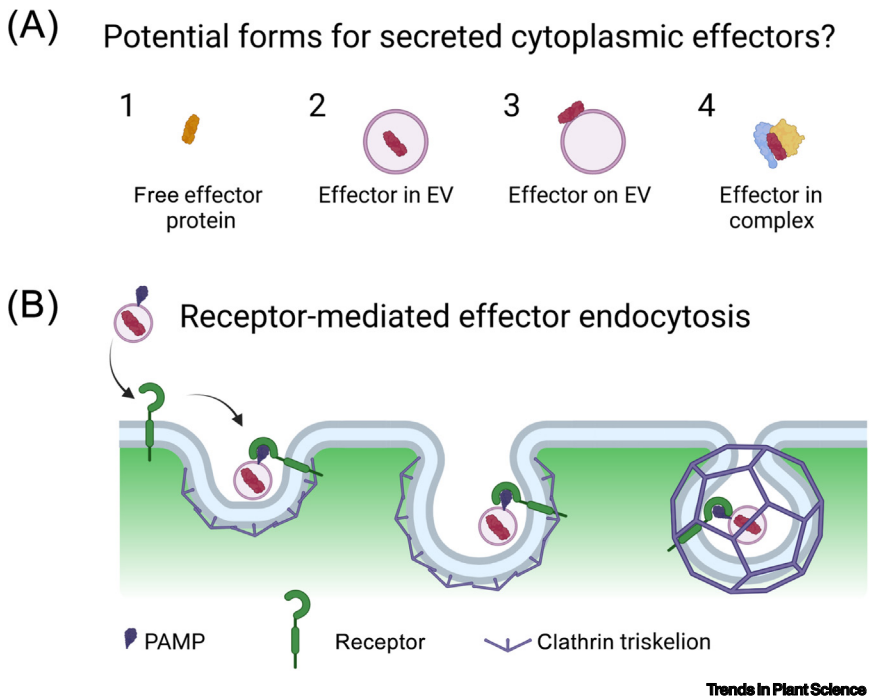

Figure 1. Presentation of cytoplasmic effectors for endocytosis. (A) Cytoplasmic effectors (orange or red rod shape) could potentially be secreted as: 1, free proteins; 2, within extracellular vesicles (EVs; pink circle); 3, associated with the exterior of EV; or 4, in complex with other pathogen molecules (blue and yellow shapes). (B) The clathrin-mediated endocytosis of effectors, which could be in EVs for example, could involve receptor-mediated recognition of a pathogen-associated molecular pattern that may be associated with the surface of EVs.

they and pathogen-associated molecular patterns (PAMPs) simultaneously presented to the host membrane for CME to be triggered? If cytoplasmic effectors are within or associated with EVs, are PAMPs co-associated and, if so, what are they (Figure 1)? The nature of the host interface is also likely to be critical for effector uptake. For example, genome editing of a rice CDP–diacylglycerol synthase, implicated in phospholipid biosynthesis at the BIC, was recently shown to enhance blast disease resistance [14].

A potential conundrum in cytoplasmic effector uptake via CME is that the effectors would then be compartmentalized in endosomes (Figure 2). How are they subsequently released to reach their host targets in diverse subcellular localizations? EVs exchanged between plants and microbes, whether pathogens or symbionts, have been reported to be taken up by recipient cells via endocytic processes [4]. One route to protein release could simply be fusion between EV and endosome membranes (Figure 2) to directly release cargo into the cytosol. Are EVs decorated with proteins that could facilitate such membrane fusions? A further route for release from endosomes could be by rupturing the endosome membrane, perhaps triggered by membrane disrupting proteins that are co-endocytosed (Figure 2). Potential rupturing of MECs was observed following CME in the rice–*M. oryzae* pathosystem [8]. A potentially cytolytic *P. infestans* NLP protein was evident in the host endosome proteome during infection [9]. Could it lead to endosome rupture? Finally, could endocytosed cytoplasmic effectors follow a retrograde trafficking route to the ER where protein export functions such as ER-associated degradation (ERAD) occur (Figure 2)? ERAD is a mechanism by which misfolded or misassembled proteins are dislocated into the cytosol from the ER for proteasome-mediated degradation. This is a well-characterized route by which viral proteins and bacterial toxins enter host cells [15]. If this is a route also for filamentous pathogen effectors to be released into the host cytosol following CME, presumably degradation of them by the 26S proteasome would need to be prevented? Future research will need to explore a number of options to fully understand the pathogen exit and host cell entry routes of cytoplasmic effectors during infection.

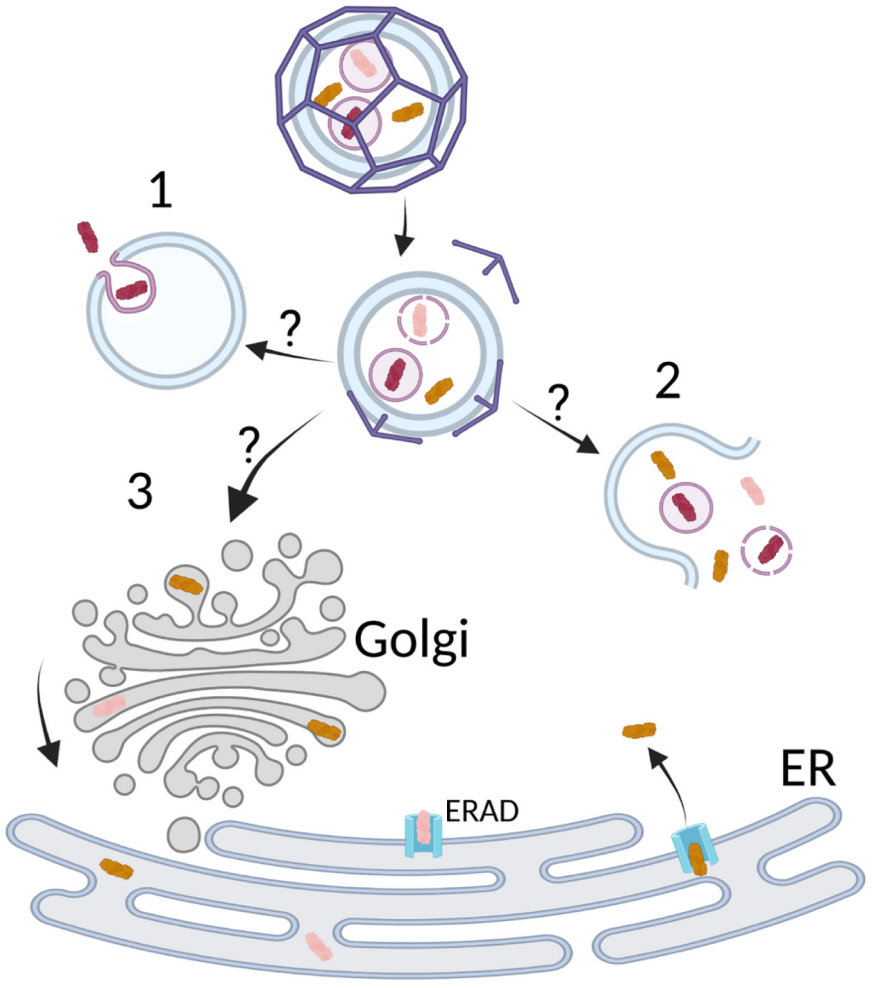

Figure 2. Effector escape from the endomembrane system. Effectors taken up by clathrin-mediated endocytosis are compartmentalized within endosomes. How do they escape to reach their targets? Effectors initially in extracellular vesicles (EVs) become soluble in endosomes if the EVs degrade in the endosome (broken pink circle, pink rod shape). If instead, they are located within intact EVs (red rod shape in pink circle), then those EVs could perhaps fuse with the endosome membrane to release their cargo (1). If the endosomes were induced to rupture, soluble effectors (orange and pink rod shapes) would be released immediately (2), whereas effectors in EVs released intact would need the EVs to degrade in the cytoplasm. Effectors that are soluble in the endosomal lumen could undergo retrograde transport (3) through the Golgi apparatus to the endoplasmic reticulum (ER) and then exploit the ER-associated degradation (ERAD) apparatus to translocate to the cytoplasm.

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Declaration of interests

No interests are declared.

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