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Seeking the interspecies crosswalk for filamentous microbe effectors

Naomi Stuer[1,2], Petra Van Damme[3] , Sofie Goormachtig[1,2,*], and Judith Van Dingenen[1,2,*]

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium

[2]Center for Plant Systems Biology, VIB, 9052 Ghent, Belgium

[3]iRIP Unit, Laboratory of Microbiology, Department of Biochemistry and Microbiology, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium

*** Correspondence: Sofie.Goormachtig@psb.vib-ugent.be, Judith.VanDingenen@psb.ugent.be**

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Highlights

The secretion and translocation pathways trafficking fungal and oomycete effectors to their target host compartments remain largely enigmatic.

Unconventional secretion strongly contributes to effector secretion in diverse fungi and oomycetes of the *Phytophtora* genus.

Distinctive mechanisms for effector translocation were proposed in *Ustilago maydis* and *Magnaporthe oryzae*, relying respectively on a microbial-encoded translocon and clathrin-mediated endocytosis.

A role for structural motifs in effector trafficking is proposed for sequentially distinctive effectors belonging to structurally conserved families within phylogenetically related species.

Together, these studies suggest that diverse effector trafficking pathways might have evolved independently across different fungal and oomycete taxa.

Proteomics-based approaches provide promising perspectives to enable comprehensive effectome characterization, inherently unbiased toward particular secretion and translocation pathways.

Glossary

Appressorium: a flattened, thickened tip of a hyphal branch that acts as an adhesion structure from which a penetration peg emerges for host tissue entry

Arbuscule: intracellular, branched, tree-like hyphal structures formed by arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungus: a broad-host-range, symbiotic plant root-interacting fungus that penetrates and forms arbuscules in cortical cells of land plants

Avirulence protein (Avr): effectors produced by plant-interacting microbes and recognized by plant host cell resistance proteins, triggering effector triggered immunity (ETI)

Biotrophic interfacial complex (BIC): plant membrane-rich structure, focally localized on the tip of the fungal primary invasive hyphae, and on the side of the first bulbous cell, associated with translocation of cytoplasmic effectors of *Magnaporthe oryzae* into plant cells

Clathrin-mediated endocytosis (CME): Vesicular-mediated transport of extracellular matter by a living cell, via invagination of its membrane (endocytosis) initiated by cytoplasmic clathrin scaffold proteins, leading to the formation of intracellular clathrin-coated vesicles

Effectome: the complete set of effector proteins produced by a given microorganism

Extracellular vesicles: particles released from the cell that are delimited by a lipid bilayer

Haustoria: modified fungal and oomycete hyphae, present in host plant cells, facilitating species-species nutrient exchange

Hypha: individual branched structures making up the mycelium of fungi and oomycetes

Lipid-raft-mediated endocytosis: endocytosis mediated by plasma membrane microdomains enriched in cholesterol and sphingolipids, which are involved in the compartmentalization of membrane proteins

Multivesicular bodies (MVBs): specialized endosomes containing multiple intraluminal vesicles

N-terminomics: the isolation and study of protein N-termini

Translocon: a complex of proteins involved in the translocation of proteins across membranes

Abstract

Both pathogenic and symbiotic microorganisms modulate their host's immune response and physiology to establish a suitable niche. Key players in mediating colonization outcome are microbial effector proteins, that act either inside (cytoplasmic) or outside (apoplastic) the plant cells, and modify the abundance or activity of host macromolecules. Here, we compile novel insights into the much-disputed processes of effector secretion and translocation of filamentous organisms, i.e. fungi and oomycetes. We report how recent studies that focus on unconventional secretion and effector structure challenge the long-standing image of effectors as conventionally secreted proteins, translocated with the aid of primary amino acid sequences. Furthermore, we emphasize the potential of diverse, unbiased, state-of-the-art proteomics approaches in the holistic characterization of fungal and oomycete effectomes.

Effector proteins as the microbial strike force

In the molecular cross-talk between a plant host and its microbial colonizer, microbial effector proteins occupy a central position, modulating the host immune response and physiology to facilitate microbial infiltration and proliferation [1–3]. After effectors have been secreted, they either remain in the apoplast or are translocated inside the host cell to alter the fate of host macromolecules such as proteins, DNA, RNA, and metabolites in distinctive subcellular compartments [1–3]. Whilst it is clear that both proteinaceous and non-proteinaceous (RNA and secondary metabolites) effectors play a pivotal role during plant-fungus and -oomycete interactions, this review specifically focusses on proteinaceous effectors, of which the trafficking and function have been best elucidated [4,5]. Well-studied **effectomes** of filamentous plant-interacting organisms include those of the biotrophic fungi *Ustilago maydis* and *Cladosporium fulvum*, the hemibiotrophic fungi *Leptosphaeria maculans* and *Magnaporthe oryzae*, and oomycetes of the *Phytophtora* genus [3]. Despite being taxonomically unrelated, fungi and oomycetes utilize similar mechanisms for host entry and colonization (Figure 1). Consequently, they are often discussed together [6–8], as in this review.

Effectors act at different stages of the infection process [9]. Some are secreted from **appressoria** preceding invasion [10,11], whereas others are secreted from invasive **hyphae** or **haustoria** during infection progression [1]. Moreover, effectors of *M. oryzae* and *U. maydis* move through plasmodesmata to prepare adjacent cells for colonization [12,13] (Figure 1). As such, effectors mediate

host responses in tune with specific requirements at different colonization stages. In resistant plant genotypes, effectors can be detected by the nucleotide-binding oligomerization domain leucine-rich-repeat-containing receptors (NLRs), inducing effector-triggered immunity (ETI) [14]. Consequently, plants and plant-interacting organisms are entangled in a continuous evolutionary arms race with modification, loss and gain of microbial effectors and of plant NLRs, alternating periods of susceptibility and resistance [15].

Although the modes of action have been unraveled for a plethora of effectors in both pathogenic and, to a lesser extent, in symbiotic plant-interacting fungi and oomycetes [1,3], the mechanisms by which they reach their target host cell compartments remain enigmatic, more specifically, the pathways for effector secretion into the extracellular environment, and subsequent translocation into the host cell. The elucidation of these pathways and the supporting effector motifs is especially challenging, because effectors of filamentous organisms often do not share considerable sequence similarities or conserved sequence motifs, equally hampering *in silico* effectome prediction [16,17]. Currently, computational effector prediction is biased toward proteins containing an N-terminal signal peptide (SP), which mediates conventional protein secretion, and toward primary amino acid (AA) motifs with an alleged role in effector translocation (i.e. RXLR and LxLFLAK). However, recent insights into the contribution of unconventional protein secretion and the role of structural, as opposed to primary, effector motifs, curtail these classical criteria, suggesting that they merely uncover a small subset of the true fungal and oomycete effectome.

A way out: effector secretion in fungi and oomycetes

The presence of an N-terminal SP remains a key selection criterion for effector identification. SP-mediated secretion through the endoplasmic reticulum (ER)-Golgi apparatus (GA) secretory pathway has long been considered standard in cell biology, whereas alternative pathways were considered exceptional [18]. Accordingly, the SP-dependent ER/GA secretory pathway is designated conventional protein secretion (CPS) and the other, usually SP-independent secretory pathways, are collectively referred to as unconventional protein secretion (UPS). Such UPS pathways may be vesicular or non-vesicular, in which proteins are released from the cell within vesicles, or freely into the extracellular space, respectively (Box 1) [19]. However, technological advances that allow the holistic characterization of eukaryotic secretomes demonstrated that UPS may not be so unconventional after all.

The comprehensive, proteomics-based characterization of oomycete and fungal secretomes has revealed UPS as key in mediating the plant-microbe crosstalk. For example, leaderless secretory proteins (LSPs), lacking a conventional N-terminal SP, were shown to represent 44% of the *Fusarium graminearum* secretome in wheat (*Triticum* sp.) heads [20], 48% of the *M. oryzae* secretome in rice (*Oryza sativa*) plants [21], 52% of the *Trichoderma virens* secretome in maize (*Zea mays*) [22], up to 90% of *F. oxysporum* f. sp. *cubense* Race 1 and Race 4 (*Foc* R1 and *Foc* R4) secretomes, when cultured with banana (*Musa* sp.) roots or root extracts [23,24], and 78% of the *in vitro Phytophtora infestans* secretome [25]. Theoretically, the abundance of LSPs identified in the fungal and oomycete secretomes could result from both UPS and cellular lysis. However, the authors routinely assayed the samples for the presence of cytoplasmic markers, suggesting that UPS is the main contributing factor [20–22,24]. Similarly, considerable differences were detected between the *in silico* predicted and experimentally identified effectomes of the ectomycorrhizal fungi *Laccaria bicolor* and *Hebeloma cylindrosporum*, with over 50% of the *H. cylindrosporum* secreted exoproteome not being predicted computationally [26,27]. Interestingly, comparative studies of *in vitro* and *in vivo* fungal secretomes reveal that UPS is often favored upon plant colonization [20,24]. Moreover, UPS increases under stress conditions across all eukaryotic kingdoms [28]. While a role for UPS under (a)biotic stress is evident [19,28], the evolutionary purpose underlaying the prioritization of UPS under adverse conditions remains unknown.

Several predicted and experimentally validated effectors were identified among LSPs, e.g. **avirulence proteins (Avrs)** of the powdery mildew fungus *Blumeria graminis f. sp. hordei* [29], isochorismatases (*Ps*ISC1 and *Vd*ISC1) of *P. sojae* and *Verticillium dahliae* [30], and an *U. maydis* peroxisomal sterol carrier protein (*Um*SCP2) [31]. Additionally, two separate pathways for effector secretion were detected in the rice blast fungus *M. oryzae* [32]. Apoplastic effectors (*Mo*BAS4, *Mo*SLP1, and *Mo*BAS113) are secreted through CPS, whereas secretion of cytoplasmic effectors (*Mo*PWL2, *Mo*AVR-PITa, *Mo*BAS1, and *Mo*BAS107) occurs via an UPS pathway (Figure I in Box 1). Similarly, in the oomycete *P. infestans*, the apoplastic effector *Pi*EPIC1 undergoes conventional secretion and the cytoplasmic effector *Pi*04314 utilizes an unconventional route [33]. The substantial contribution of UPS to the secretion of cytoplasmic effectors is further demonstrated by the *in vivo* characterization of 62 *F. graminearum* effectors translocated into the *Arabidopsis thaliana* cytoplasm, of which merely two contained a predicted SP [34]. Whether it is a general rule that apoplastic and cytoplasmic effectors

are preferentially secreted through CPS and UPS, respectively, remains to be explored. Furthermore, although the majority of the currently characterized cytoplasmic effectors contain a conventional SP, as *in silico* prediction approaches often favor such proteins, they can also be unconventionally secreted through a Golgi-bypass route (Box 1)[32,33,35].

Clearly, the UPS contribution to the plant-microbe crosstalk is considerable, but its extent is probably underestimated, because (i) unconventionally secreted effectors are difficult to identify within the proteome, due to a lack of known conserved motifs, and (ii) *in vivo* produced fungal and oomycete secretomes are not isolated straightforwardly [36,37]. Plant colonization induces enormous changes within fungal and oomycete proteomes. For example, many virulence factors and effectors are expressed solely upon recognition of specific plant species and developmental cues [38–40]. Therefore, it is crucial that future efforts focus on the comprehensive understanding of fungal and oomycete secretomes in a biologically relevant infection context.

A way in: effector translocation in fungi and oomycetes

Once secreted, effectors targeting subcellular compartments must cross the plant plasma membrane, a process facilitated by either microbe or host machinery [6]. Well-elucidated cases of pathogen machinery-dependent translocation include the type III, type IV, and type VI secretion systems of Gram-negative bacteria [41,42], allowing direct translocation into the host across 3 membranes (i.e. bacterial inner, outer and host plasma membrane), and the unicellular eukaryotic *Plasmodium* **translocon** of exported proteins (PTEX) [43]. Examples of host cell autonomous translocation comprise bacterial toxins entering human cells through **endocytosis**, following binding to glycosphingolipid receptors [44], and cell-penetrating bacterial effector proteins in *Yersinia enterocolitica* and *Salmonella enterica* serovar Typhimurium [45].

Whether fungal and oomycete effector translocation is host cell autonomous, micro-organismal driven, or both, remains controversial [6,46]. Previously, the oomycete RXLR sequence motif was believed to mediate cell-autonomous effector uptake via endocytosis through binding of phosphatidylinositol-3-phosphate (PI3P) in host membranes (Box 2) [47,48]. Likewise, the oomycete LxLFLAK motif present in CRINKLER (CRN) effectors was hypothesized to facilitate translocation in a similar manner (Box 2) [49,50]. However, the importance of PI3P-mediated translocation and the direct involvement of these motifs in facilitating translocation have been disputed [51–53], especially with the recent

observation that the RXLR motif is cleaved off prior to effector secretion [54]. As such, it is currently hypothesized that co-translational processing of this motif facilitates protein sorting and/or stabilization, similarly to the N-terminal PEXEL motif in *Plasmodium* parasite effector sequences [55–57].

Other effector translocation pathways and motifs include that of the fish pathogenic oomycete *Saprolegnia parasitica Sp*HTP3 effector, which is internalized via **lipid-raft mediated endocytosis** through binding of its C-terminal YKARX region to a gp96-like receptor [58]. The C-terminal RSIDELD motif of the DELD effector family in the fungus *Serendipita indica* [59,60], and the RGD motif of the *Pyrenophora tritici-repentis* ToxA protein were also suggested to mediate translocation [61].

Recently, more detailed translocation mechanisms have been put forward for the fungal pathogens *U. maydis* and *M. oryzae* (Figure 2). Translocation of *U. maydis* effectors into maize leaf cells was suggested to be enabled by a heptameric complex of the *U. maydis* proteins *Um*STP1-6 and *Um*PEP1 [62], providing the first report of an effector **translocon** in filamentous microorganisms. This **translocon,** that seems to be conserved across all known *Ustilaginaceae,* interacts with plant plasma membrane ATPases and PIP2-type aquaporins. Interestingly, the *Xo*HPA1 protein of the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* type III secretion system has also recently been shown to interact with an aquaporin, PIP1;3, to facilitate effector translocation in rice [63]. Nevertheless, further research is needed to assess directly the translocon-dependent translocation of cytoplasmic *U. maydis* effectors. In contrast, effector translocation by *M. oryzae* at the **biotrophic interfacial complex (BIC**) was reported to involve **clathrin-mediated endocytosis** (**CME**) [11], which has also been suggested to assist in effector uptake and in membrane recycling from host exocytosis in the rust fungus *Uromyces vignae* and the *Vigna unguiculata* pathosystem [64]. However, because *U. vignae* is not genetically modifiable, validation of this hypothesis is challenging. Once taken up inside host cells through endocytosis, it remains elusive how effectors escape lysosomal degradation. Possibly, this escape is mediated by a dedicated additional effector, as is the case for the *S. parasitica Sp*HTP3, which is translocated into host cells through lipid raft-dependent endocytosis, and subsequently released from endocytic vesicles with the help of *Sp*Htp1, another host-targeting effector [58].

Despite the enhanced understanding of effector translocation in *M. oryzae* and *U. maydis*, pathways facilitating effector delivery of other host-interacting fungi and oomycetes remain largely unknown. Given the diversity of effector translocation systems, it is conceivable that different infective filamentous organisms may have evolved multiple, unrelated mechanisms for effector translocation to adapt to a host-dependent lifestyle.

A role for structural motifs in mediating effector trafficking?

In addition to the molecular pathways guiding fungal and oomycete effectors to the plant cytoplasm, the effector motifs used as sorting criteria are still largely unknown. Nevertheless, it is clear that only limited information resides in the effector primary AA sequence. More recently, oomycete and fungal effectors have been proposed to belong to restricted sets of structural families conserved within phylogenetically related species, albeit a lack of homology at the sequence level [65–72]. Therefore, the involvement of effector structural motifs has been assumed to mediate effector trafficking.

The largest families of oomycete effectors, i.e., RXLR and CRN, have been shown to adopt conserved tertiary structures. More specifically, many RXLR effectors display a conserved WY domain at the C-terminus [66,71,72], whereas N-termini are generally enriched in disordered residues [73–75]; corresponding with observations in bacterial effectors, in which long disordered regions are assumed to facilitate effector translocation [76]. In contrast, translocation of the *Setosphaeria turcica St*SRE1 effector is reportedly mediated by an N-terminal β-hairpin structure [77], suggesting the existence of distinctive mechanisms for host cell entry. Similarly, long-standing views on CRN effector trafficking have been challenged with a large-scale structure assessment of the N-terminal (header) domain of CRN proteins across the Eukaryote taxon [68]. First, the presence of a functional N-terminal SP in CRN proteins was questioned, as it could not be predicted using sensitive hidden Markov models, and the so-called SP region appeared to fulfill a structural role within the mature effector protein. The most prominently predicted header domain was the Ubiquitin-like Header domain (CR-Ubl) that was observed in practically all oomycete and most fungal CRN proteins. In this domain, the predicted SP and LxLFLAK motif are situated at the conserved strands 1 and 3, implying that the LxLFLAK motif merely contributes indirectly to translocation by maintaining the Ubl domain structure. Moreover, the LxLFLAK motif is absent in several family members. All other header domains consisted of a wide variety of unrelated, structurally distinctive, α-helical domains, with strongly variable phyletic spreads. Consequently, CRN effectors across diverse lineages

contain structurally distinctive N-terminal domains, hinting at effector translocation through diverse pathways. Moreover, only one class of helical header domains contains a hydrophobic region, implying that the vast majority of CRN effectors do not rely on direct interaction with the lipid portion of membranes for translocation.

In contrast to oomycetes, primary sequence analyses have not been able to distinguish large fungal effector families to date. Nevertheless, fungal effectors appear to belong to structurally defined families conserved within phylogenetically related species. Currently, three families of fungal effectors with a conserved protein architecture have been reported: (i) *Magnaporthe* Avrs and ToxB like (MAX) [69], (ii) RNase-Like Proteins associated with Haustoria (RALPH) [67,70], and (iii) *Leptosphaeria* AviRulence-Suppressing (LARS) effectors [65]. Notably, translocation of the LARS effector AvrLm4-7 was shown to require (R/N)(Y/F)(R/S)E(F/W) and RAWG motifs, constituting a positively charged region [78]. It would be interesting to investigate whether this mechanism is conserved within other LARS effectors. Together with the elucidation of RXLR and CRN structural motifs [68,73–75], the study of fungal LARS effectors provides intriguing evidence for the emerging hypothesis that conservation at the structural level, instead of at the sequence level, may be important in effector translocation and putatively also as UPS sorting motifs. Although compelling, the extent to which the hypothesized roles for effector structure hold *in vivo* remains to be empirically substantiated.

It is clear that experimental effector structural and molecular biology may help us uncover the previously hidden motifs and mechanisms facilitating effector trafficking and function, and their conservation across distinctive fungal and oomycete species. Yet, the number of structurally resolved fungal and oomycete effectors remains scarce, and experimental determination of effector structure remains highly time consuming and challenging [79,80]. Therefore, computational tools to predict effector structure pose promising alternatives. Previously, the lack of template structures and rapid effector diversification limited the use of homology-dependent structure modelling. However, recent developments in artificial intelligence (AI)-based, *de novo* protein structure prediction, driven by deep learning, such as Alphafold [81], RoseTTafold [82] and trRosetta [83], offer promising approaches towards resolving effector structure, even at the effectome level, as was previously illustrated in diverse plant-pathogenic fungi, including *M. oryzae* [84] and *F. oxysporum* [79]. Nevertheless, limitations of these deep learning-based tools should be considered, e.g. failure to take into account conformational rearrangements, and the observation that certain protein regions remain challenging to predict [84–86].

Moreover, downstream structure-based functional characterization remains dependent on structurally similar proteins with an experimentally resolved function. This emphasizes the complementarity of computational and experimental research efforts to obtain an integrated understanding of effectome-wide structure conservation, effector function and trafficking.

A role for vesicles in effector trafficking in the host?

How effectors transverse the apoplast poses another enigma, more specifically, whether they do so freely, in the sheltered environment of an **extracellular vesicle** (EV), or both. In bacteria, the concept of effector secretion and transport through EVs, the so-called "secretion system type zero", has gained attention over the last few years [87], and several studies equally suggest the involvement of EVs in fungal and oomycete effector shuttling [88–90]. Firstly, diverse UPS pathways result in the secretion of proteins in vesicles. Secondly, EVs are known to contribute considerably to reciprocal plant-microbe communication, as plants produce secretory vesicles containing antimicrobial agents and defense related proteins [91–94], while fungal and oomycete EVs contribute to virulence [88,95]. Accordingly, ultrastructural characterization of haustoria and **arbuscules** in powdery mildew and the **arbuscular mycorrhizal fungus** *Rhizophagus irregularis*, respectively, revealed abundant **multi vesicular bodies (MVBs)** in the plant and fungal cytoplasm, and EVs in the extrahaustorial matrix (Figure I in box 1) [96–98].

Importantly, validated and predicted effectors were identified within the EV-associated proteomes of plant-colonizing fungi and oomycetes (Figure I in box 1) [88–90]. A total of 18 effectors, of which nine had been reported previously, were detected in *F. graminiarum* EVs [89], three elicitors (*Pc*NLP, *Pc*CBEL and *Pc*GP42) and one confirmed apoplastic effector (*Pc*GIP) in *P. capsici* [88], and several effector candidates in *F. oxysporum f. sp. vasinfectum* [90]. Candidate effectors and other cargos both with and without a conventional SP were present in EVs, a finding also reported in mammalian systems [99,100]. Accordingly, the fluorescently tagged SP-containing *Fusarium* effector Avr2 was observed to accumulate in defined spots alongside cortical plant cell-colonizing fungal hyphae [101], which was hypothesized to be consistent with sequestration of Avr2 inside EVs. The discovery that both LSPs and SP-containing effectors occur in vesicles suggests that multiple UPS pathways contribute to the EV-associated proteome. Alternatively, it has been speculated that conventionally secreted proteins can associate with the EV surface via lipid binding motifs, which have been identified in many fungal and oomycete effectors [102].

Whereas the hypothesized involvement of EVs in effector shuttling is compelling, more research is definitely needed. Improved *in vivo* EV isolation procedures will undoubtably also enhance our understanding of the true contribution of EVs to effector secretion, as effectors are known to be transcribed predominantly during interaction with the host plant [103].

Proteomics-based approaches for holistic effectome characterization

The multitude of UPS and translocation pathways involved in fungal and oomycete effector shuttling complicates *in silico* effector prediction. Not only is the precise knowledge of effector trafficking pathways and the facilitating effector motifs often lacking, but these pathways and motifs may also differ across distinctive taxonomical lineages. To overcome this hurdle, proteomics-based approaches can be applied for the comprehensive, unbiased characterization of effectors in individual host-microbe interactions.

The direct *in planta* identification of effectors offers the best insight into the players at the forefront of the host-microbe interaction (Figure 3). However, *in vivo* secretomics remains challenging, due to the requirement of an adequate sample size for in-depth secretome coverage, specific enrichment of fungal proteins amongst a pool of host proteins, and absence of contamination. Proximity labeling, such as BioID [104,105] and the new improved TurboID [106,107], are especially suitable, because they allow rapid sample collection, and reduce contamination by protein labeling prior to lysis. Generally, BioID is used for the identification of proteins in the proximity of a protein of interest (the bait). To this end, a promiscuous biotin ligase is fused to the bait protein, whereafter interactors can straightforwardly be retrieved by isolation and identification of biotinylated proteins (Figure 3). As such, proteins with a role in effector trafficking and recognition in specific biosystems can serve as BioID baits to capture and identify effectors *in vivo*. Interestingly, this approach offered the rationale behind a translocation assay developed in the *U. maydis*–maize pathosystem, based on enzymatic effector biotinylation in the host cytoplasm as proof of uptake [108]. Alternatively, free biotin ligase can be expressed in a cell type and compartment of interest by means of colonization-inducible host promoters, and organelle targeting sequences, respectively [109], allowing the enrichment of biotinylated proteins in colonized host tissues, and permitting the characterization of fungal effectomes at the subcellular level. Similarly, biotin ligase may be targeted to the apoplast or outer leaflet of the plasma membrane to identify proteins

active at the host-microbe interface [110]. Such untargeted approaches have recently been used for the identification of *F. graminearum* effectors in the *Arabidopsis* cytoplasm and apoplast [34].

Other techniques for the *in vivo* detection of translocated microbial proteins are fluorescence-assisted cell and nuclei sorting (FACS and FANS), in which cells and nuclei of colonized tissue are sorted using fluorescent markers. The probability of effector identification can be increased if colonization-inducible host promoters for the expression of fluorescent proteins are known (Figure 3). Similarly, the isolation of nuclei tagged in specific cell types (INTACT) approach allows the isolation of cell-type specific nuclei, but through a nuclear targeting fusion protein (NTF) instead of fluorescent markers [111] (Figure 3). Although cell and nuclei sorting are occasionally used for proteomics in the biomedical field [112–115], applications in the plant field remain predominantly focused on genomics and transcriptomics [116–118], but with advances in micro-proteomics workflows over the last few years, we expect that single cell-type proteomics approaches will become more prevalent [119–121]. Another useful technique for the enrichment of plant cells contacting microbial structures is laser capture microdissection (LCM) that allows the direct isolation of selected cellular subpopulations under microscopic visualization (Figure 3) with the added value of its applicability in plant species non-amenable to genetic transformation. Moreover, this method has been optimized in plant tissues for low protein inputs [122–124]. However, even with the potential of automated sample collection [125], LCM requires comparatively long hands-on time, mainly due to the preparation of tissue sections [126] and care should be taken to avoid contamination with microorganismal structures. Finally, for the identification of apoplastic effectors, isolation of apoplastic fluid through vacuum-infiltration and centrifugation provides a useful approach (Figure 4) [20–22,127]. Further enrichment of effector candidates from protein samples can be performed through the isolation of small proteins enriched for cysteine residues, as cysteine is often overrepresented in fungal and oomycete effectors [128].

To overcome challenges associated with *in vivo* effector identification, *in vitro* studies pose a compelling alternative. Many fungi and oomycetes can be cultured under infection-mimicking conditions to induce effector secretion, whereafter the secretome can be retrieved from the culture supernatants [20,23] (Figure 3). Moreover, EVs can also be isolated, e.g. through differential ultracentrifugation and immunoaffinity capture, when EV biomarkers and corresponding antibodies are available [37,129]. Importantly, methods for EV production, isolation, and analysis in solid media are being explored, with seemingly higher yields within a shorter processing time, than those in liquid

media [36,130]. In addition, characterization of extracellular proteomes in the presence of chemical inhibitors targeting diverse secretory pathways can help us understand how UPS contributes to fungal and oomycete secretome compositions (Figure 3) [131]. For instance, the inhibitor brefeldin A (BFA), which inhibits ER-to-GA trafficking, has been used to investigate UPS in various fungal and oomycete species, including *M. oryzae* and *P. infestans* [32,33]. Furthermore, **N-terminomics**, a cutting-edge proteomics approach, could help us infer which secretion pathways in diverse interaction systems, are relied upon [132], as a multitude of biological secretory processes, such as protein maturation and translocation are often associated with modification and proteolytic cleavage of protein N-termini [54,133–135]. Therefore, the study of mature N-terminal sequences, potentially combined with inhibitors of selected secretory pathways, could aid the elucidation of the molecular mechanisms, selection criteria, and contributions of the secretion and translocation pathways at play during effector trafficking.

Notably, secretome analysis is challenged by the difficulty of distinguishing legitimate LSPs from intracellular proteins released from lysed cells. However, advances in this area were made in the biomedical field, where approaches such as comparative secretomics and stable isotope dynamic labeling of secretomes (SIDLS) facilitate the detection and removal of contaminating proteins from secretomics data [136].

Concluding remarks and future perspectives

The precise nature of the molecular pathways and sorting criteria that guide effectors of plant-interacting fungi and oomycetes to their target host compartments remain largely puzzling (see outstanding questions). It is however clear that a large variety of pathways have evolved independently across distinctive taxa, underlining the significant contribution of unconventional secretory pathways and diverse, host-autonomous and pathogen-dependent mechanisms for host cell entry [11,32,33,62].

As such, a cautionary note on the utilization of certain model host-fungus/oomycete systems as a proxy to study effector translocation is warranted. Whereas the utilization of model systems poses a compelling alternative for microbial species that are nonamenable to genetic transformation, pathways mediating secretion and translocation within one biosystem possibly differ from another. Therefore, the use of appropriate controls and complementary assays is strongly advised.

The mechanistic diversity in the pathways constituting the interspecies crosswalk is also reflected in the large variety of effector AA and structural motifs that may determine the distinctive effector trajectories. As such, conventional criteria used for *in silico* effector prediction clearly limit the comprehensive characterization of fungal and oomycete effectomes [16,17]. Evidently, structure-guided prediction tools will become indispensable within the field, as recent studies imply that effectors belong to a limited set of structurally conserved families, independently of the primary AA sequence [65,67–71]. Hence, it is crucial that future effector research focusses on the elucidation of effector structure, and how it relates to effector function, secretion and translocation, in distantly related organisms. Such experimental work for resolving effector structure-function relationships remains critical, as even the most state-of-the art structure prediction algorithms require solid references for downstream structure-based functional interference. Moreover, we emphasize the potential of proteomics-based approaches in overcoming our current knowledge-gap, as such approaches enable the holistic and unbiased characterization of fungal and oomycete effectomes in individual biosystems. As outlined in this review, techniques allowing the *in planta* enrichment of fungal and oomycete secretomes are emerging. Especially with the improvement of micro-proteomic workflows [119–121], we expect proteomics to convincingly enhance our future understanding of the fungal and oomycete strike force, and the interspecies crosswalk guiding effectors to their target host compartments.

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Outstanding questions

What is the evolutionary motive underlaying the prioritization of unconventional secretion under stress, and, more specifically, in a biotic interaction context?

The precise nature of unconventional secretion pathways contributing to effector secretion remains largely unknown. Moreover, are these pathways conserved across distinctive phylogenetic taxa? How many distinctive pathways are operational within one species? And do these pathways differ between apoplastic and cytoplasmic effectors?

The existence of host-autonomous and microorganismal driven effector translocation pathways was demonstrated by recent work in the fungi *Magnaporthe oryzae* and *Ustilago maydis*, respectively. However, the precise mechanisms, phylogenetic spread and diversity of effector translocation pathways remain elusive in the vast majority of plant-fungal and -oomycete biosystems.

What is the precise role of extracellular vesicles in effector shuttling? Do effectors merely reside in the vesicular lumen, or equally associate with the outer leaflet of the vesicle through lipid-binding motifs or association with vesicle membrane proteins? How are these vesicles, and/or their content, taken up by the host cells?

How do effectors manage endosomal escape when relying on host endocytic pathways?

Effectors of filamentous microorganisms appear to belong to structurally conserved families with restricted phylogenetic spreads. However, these families and structural motifs remain unknown for the majority of filamentous species. Moreover, how do these effector motifs facilitate effector trafficking?

With the apparent multitude of distinctive effector trafficking pathways and sorting criteria in distinctive host-fungus/oomycete biosystems, how physiologically relevant and truthful is the usage of heterologous systems to study effector trafficking?

How does the diversity in sorting mechanisms and motifs impact the *in silico* prediction of fungal and oomycete effectomes?

Boxes and figures

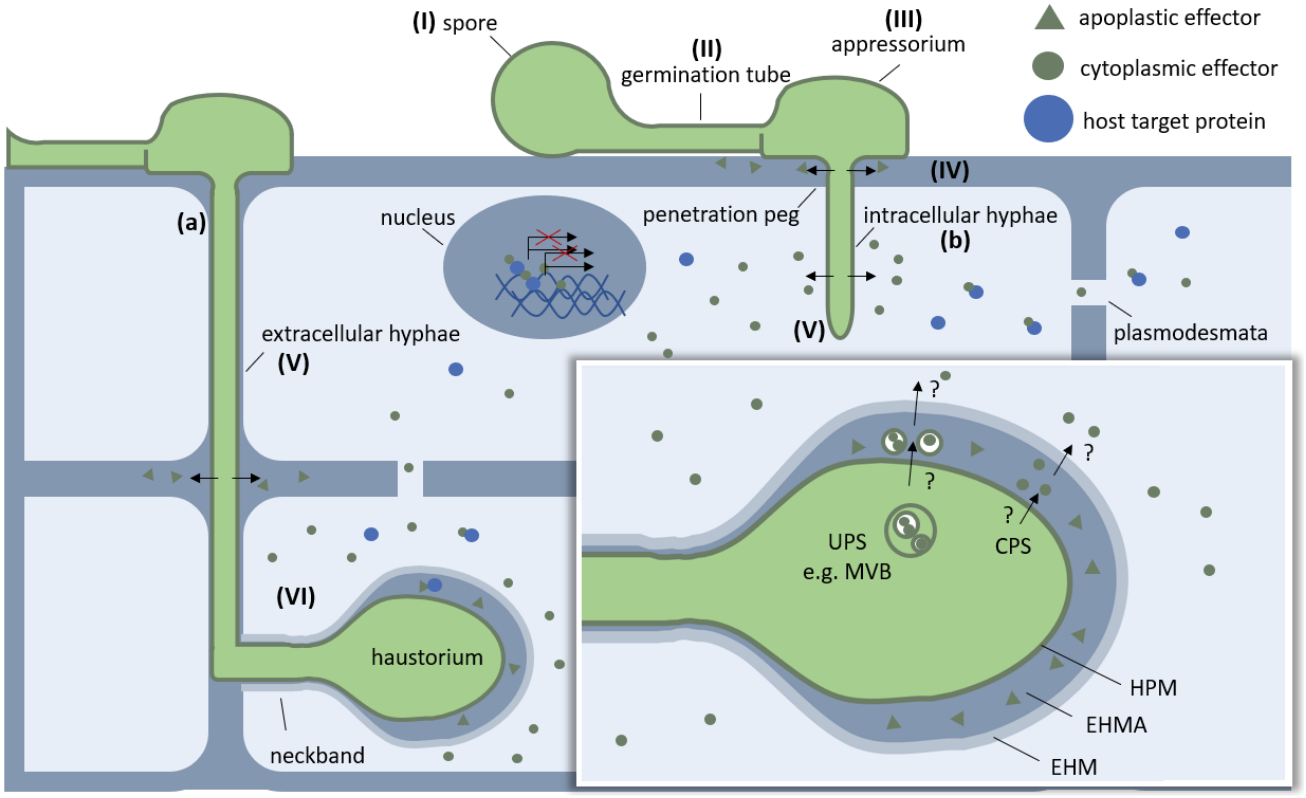

Figure 1. Schematic representation of plant tissue colonization by a filamentous microorganism. The following stages of colonization are represented: (I) germination of the fungal or oomycete spore, (II) formation of the germination tube, (III) formation of the appressorium exerting pressure on the plant cell and allowing extracellular (a) or intracellular (b) invasion, (IV) emergence of the penetration peg, and (V) formation of either invasive intracellular or extracellular hyphae, which subsequently (VI) differentiate into intracellular haustoria. Haustoria are surrounded by a haustorial plasma membrane (HPM), the extrahaustorial matrix (EHMA), and the extrahaustorial membrane (EHM). Apoplastic (green triangles) and cytoplasmic (green circles) effectors are secreted from fungi and oomycetes at diverse developmental stages, including the appressorial pore and penetration peg, intra- and extracellular hyphae, and haustoria. Effectors target host proteins (blue circles) or other host macromolecules, such as DNA and RNA, to directly modulate gene expression. Inset: effector secretion may be facilitated through conventional (CPS) or unconventional (UPS) protein secretion, for instance, via multivesicular bodies (MVBs).

Box 1. Pathways for conventional and unconventional secretion

In eukaryotes, proteins are secreted either through the endoplasmatic reticulum (ER)/Golgi apparatus (GA) secretory pathway, or via various alternative routes collectively referred to as unconventional protein secretion (UPS) [19,137]. Proteins targeted towards the conventional protein secretion (CPS) pathway carry an N-terminal signal peptide (SP), which is recognized by the cytoplasmic signal recognition particle (SRP), enabling co- or post-translational translocation to the ER. Inside the ER, the SP is cleaved off, proteins are folded, and subsequently packed into COAT PROTEIN COMPLEXII (COPII) vesicles for delivery to the cis-GA. During passage through the Golgi cisternae, proteins can be further modified by processes such as glycosylation, sulfation, and phosphorylation. Proteins destined to be secreted are finally stored in secretory vesicles (SVs) that are readily delivered towards the plasma membrane, or in immature secretory granules (ISGs), which accumulate in the cytoplasm and require specific stimuli for maturation and transport to the plasma membrane [137]. As SPs are relatively well conserved, conventionally secreted proteins can easily be predicted by means of bioinformatics tools such as SignalP [138] and Phobius [139]. However, the presence of a predicted SP is not unambiguously indicative of secretion, because SP-containing proteins may be retained in the secretory pathway, or captured in membranes, for instance, when the protein contains transmembrane helices or signals for glycosylphosphatidylinositol (GPI)-anchoring [140].

In addition to the CPS pathway, various UPS pathways have been thoroughly characterized in mammals and fungi [19,141,142]. In contrast to the proteins secreted via CPS, most proteins secreted via UPS lack a typical N-terminal SP for ER entry, although proteins with an SP can also divert from CPS by omitting the Golgi transfer (cfr. Golgi bypass) [35]. For proteins without a SP, the UPS mechanisms are diverse and include: (i) direct passage from the cytosol to the plasma membrane independently of vesicles, such as through pore formation or ATP-binding cassette (ABC) transporters, and (ii) secretion through membrane-bound structures, including endosomes, autophagosomes, amphisomes, compartments for UPS (CUPS), and microvesicles (Figure 1). Although numerous unconventionally secreted proteins have been identified, their underlying molecular mechanisms remain unclear, with only few UPS pathways having been characterized in detail, such as for the yeast a-factor [143], and acyl-coenzyme A binding protein (ACB1) [144]. For an in-depth discussion of the molecular mechanisms facilitating UPS we refer to the recent review of Cohen et al, (2020a).

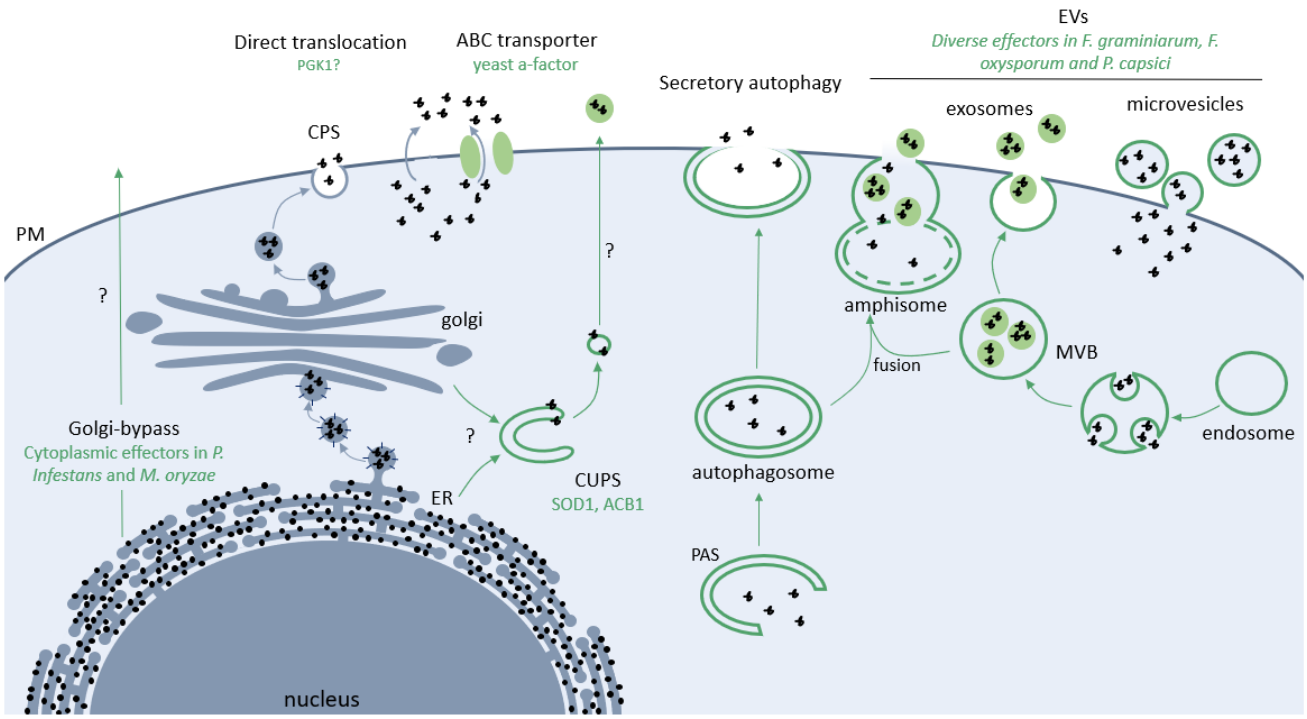

Figure I: Schematic overview of the diverse secretory pathways in eukaryotic organisms. The CPS pathway is shown in blue and examples of UPS pathways are shown in green. Examples of fungal and oomycete proteins secreted through corresponding pathways are in green font. CPS, conventional protein secretion; CUPS, compartments for UPS; EVs, extracellular vesicles; PAS: pre-autophagal structure; PM, plasma membrane; MVB, multi vesicular body.

Box 2. RXLR and CRN effectors

The RXLR and CRN effector families represent the largest groups of reported effector proteins in oomycetes. Both RXLR and CRN effectors display a modular architecture with (i) a conserved N-terminal domain, generally consisting of a SP and a presumed translocation motif, and (ii) a highly variable C-terminal domain, which is hypothesized to mediate effector functioning and to target host macromolecules. In RXLR effectors, the translocation motif consists of an N-terminal Arg-X-Leu-Arg AA motif located within 40 AA of the predicted SP cleavage site, often followed by a EER motif 5 to 25 AA downstream [145]. CRN effectors contain the LxLFLAK translocation motif, followed by one or more DWL domains and ending in a HVLVVVP motif, which presumably acts as a recombination hotspot [146]. Interestingly, mutagenesis experiments suggest that the RXLR motif is highly variable, with various fungal effectors also containing degenerate RXLR-like motifs [48], pointing to a convergent evolution in the taxonomically unrelated fungi and oomycetes.

RXLR and the sequentially similar LxLFLAK motif have been proposed to facilitate translocation into host cells. More specifically, the RXLR motif was suggested to mediate pathogen-independent translocation through specific binding of phosphatidyl-inositol 3-phosphate (PI3P) on the plant cell surface, triggering lipid receptor-mediated endocytosis [47,48]. In addition, fusion of the N-termini of the *Phytophthora* effectors CRN2, CRN8, and CRN16 to the C-terminus of the RXLR effector AVR3a was shown to rescue effector translocation of the latter, hinting at a role similar to that of the LxLFLAK motif in effector translocation [49,50]. The putative role of both motifs in mediating translocation was demonstrated by means of plant transient expression assays (so-called "re-entry" assays) and exposure to purified recombinant protein (so-called "uptake" assays) [47–50]. However, doubts have been raised concerning the reliability of both assays in demonstrating intracellular translocation [51,52]. Moreover, because recently translocation of RXLR and CRN effectors has been demonstrated independently of the RXLR and LxLFLAK motifs (see main text), and since the RXLR motif of the *P. infestans* AVR3a has been shown to be cleaved off prior to secretion [54], a direct role for RXLR and CRN in facilitating effector translocation appears unlikely and, instead, the RXLR motif has been hypothesized to functions as an internal sorting signal, or aids protein stabilization. Similarly, structural analysis has suggested that the LxLFLAK motif mediates translocation only indirectly through maintenance of effector tertiary structure.

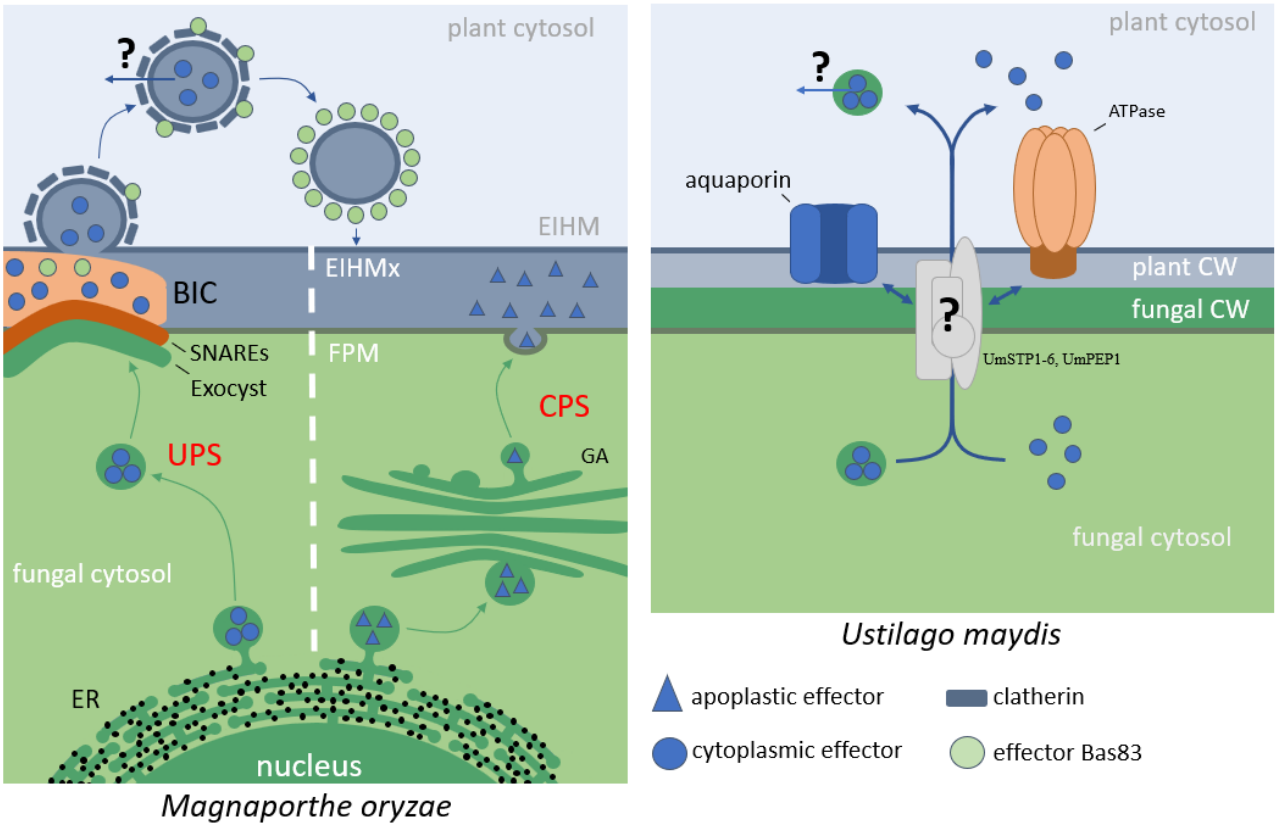

Figure 2. Pathways for effector delivery identified in *Magnaporthe oryzae* and *Ustilago maydis*. In *Magnaporthe oryzae* both pathways for effector secretion and translocation are relatively well determined. Apoplastic effectors are secreted through conventional protein secretion (CPS), while cytoplasmic effectors are secreted through unconventional protein secretion (UPS) in a pathway requiring SNARE proteins and the exocyst complex for delivery into the biotrophic interfacial complex (BIC), a plant-derived membrane-rich structure. From the BIC, cytoplasmic effectors are taken up into host cells through clathrin-mediated endocytosis, whereafter host membranes are suggested to be replenished to the BIC by the fungal Bas83 effector. In *Ustilago maydis*, effector translocation into host cells is mediated by a heptameric translocon, consisting of the fungal proteins UmSTP1-6 and UmPEP1 in an unknown stoichiometry. This complex interacts with plant plasma membrane ATPases and/or PIP2-type aquaporins and that is hypothesized to facilitate effector uptake. In both cases, the mechanisms for endosomal escape remain unknown. CW, cell wall; ER, endoplasmic reticulum; EIHM, extra-invasive hyphal membrane; EIHMx, extra-invasive hyphal matrix; FPM, fungal plasma membrane; GA, Golgi apparatus; SNARE, soluble N-ethylmaleimide-sensitive-factor attachment receptor.

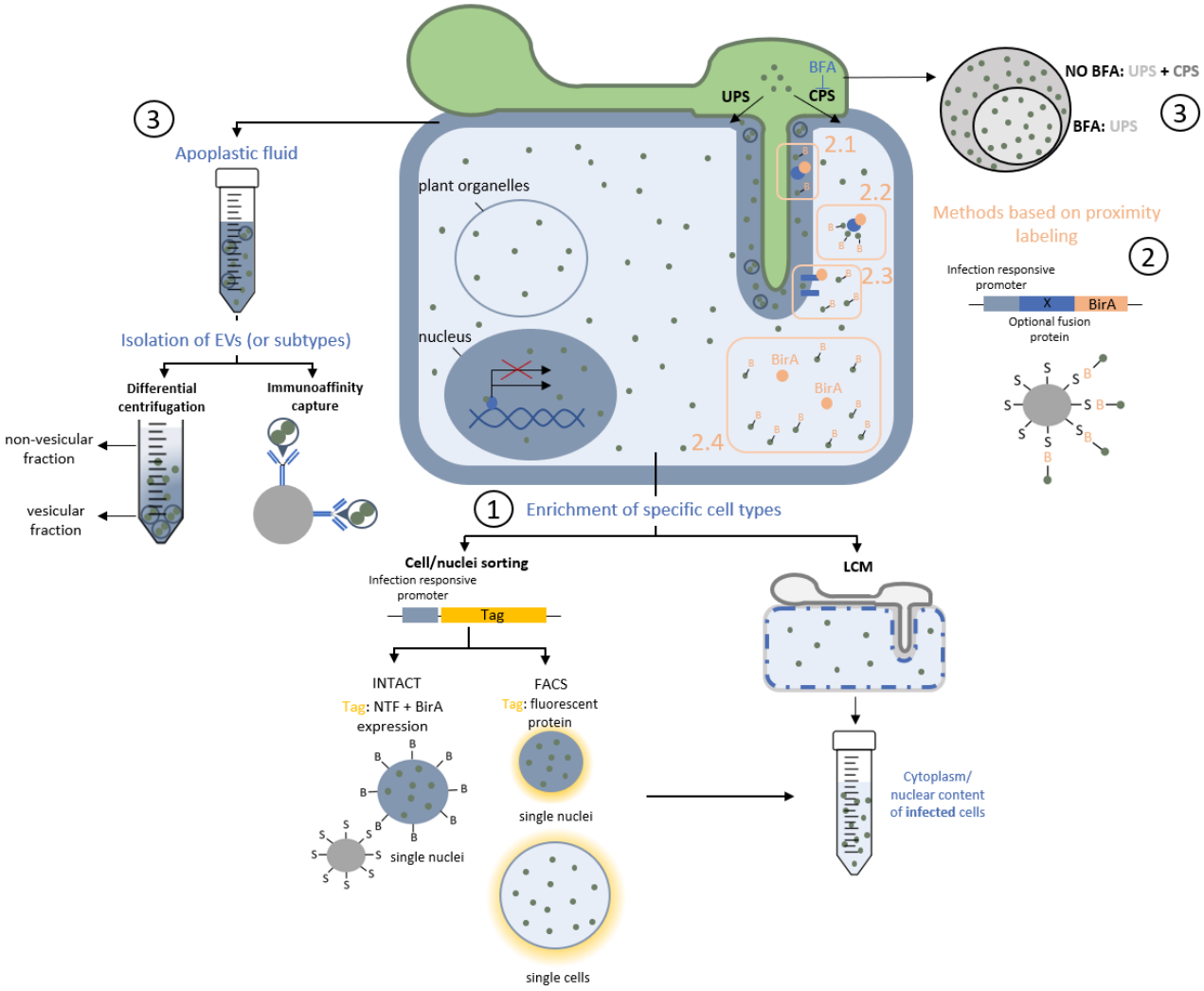

Figure 3. Methods for *in planta* enrichment of fungal and oomycete effectors. (1) Effector-containing host cells and nuclei can be specifically isolated by means of a sorting tag expressed under an infection responsive promoter. In the case of fluorescence-activated cell sorting (FACS) and fluorescence-activated nuclei sorting (FANS) a fluorescent protein is used. Isolation of nuclei in specific cell types (INTACT) uses a nuclear tagging fusion protein (NTF), which tags nuclei and is biotinylated by a co-expressed biotin ligase (BirA) protein, after which nuclei can be purified with streptavidin beads. Alternatively, cell types of interest can also be isolated using laser capture microdissection (LCM). (2) Effector candidates in infected plant cells can be identified and/or enriched by proximity labeling based approaches such as BioID and TurboID, driven by an infection responsive promoter. Different set-ups can be used. BirA can be expressed without a fusion protein in the apoplast (2.1) or plant cytoplasm (2.4) for the identification of apoplastic and cytoplasmic effectors, respectively. To enhance the ratio of fungal-to-host proteins, BirA can also be fused to (2.2) plant

resistance proteins (2.2) or proteins involved in effector translocation (2.3). Biotinylated proteins including translocated effectors are subsequently isolated with streptavidin (S) coated beads. (3) For the identification of apoplastic effectors, apoplastic fluids can be isolated, whereafter extracellular vesicles (EVs) can be separated from non-vesicular fractions by means of, for instance, differential centrifugation and immunoaffinity capture. (4) Chemical inhibitors of conventional protein secretion (CPS), such as brefeldin A (BFA), can be used for the specific identification of proteins secreted through unconventional protein secretion (UPS).

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