Filamentous plant pathogen effectors in action

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Abstract | Live-cell imaging assisted by fluorescent markers has been fundamental to understanding the focused secretory 'warfare' that occurs between plants and biotrophic pathogens that feed on living plant cells. Pathogens succeed through the spatiotemporal deployment of a remarkably diverse range of effector proteins to control plant defences and cellular processes. Some effectors can be secreted by appressoria even before host penetration, many enter living plant cells where they target diverse subcellular compartments and others move into neighbouring cells to prepare them before invasion. This Review summarizes the latest advances in our understanding of the cell biology of biotrophic interactions between plants and their eukaryotic filamentous pathogens based on *in planta* analyses of effectors.

Biotrophic
A biotrophic organism feeds and completes its life cycle on living plant tissue and lacks a necrotrophic phase of killing host cells before feeding.

Hemibiotrophic
A hemibiotropic organism feeds on living tissues for a period of time and then switches to necrotrophic colonization of dead tissues.

Apoplast
Plant extracellular space; a tissue-level compartment outside the plasma membrane that includes the cell walls and xylem vessels, through which water and solutes freely diffuse.

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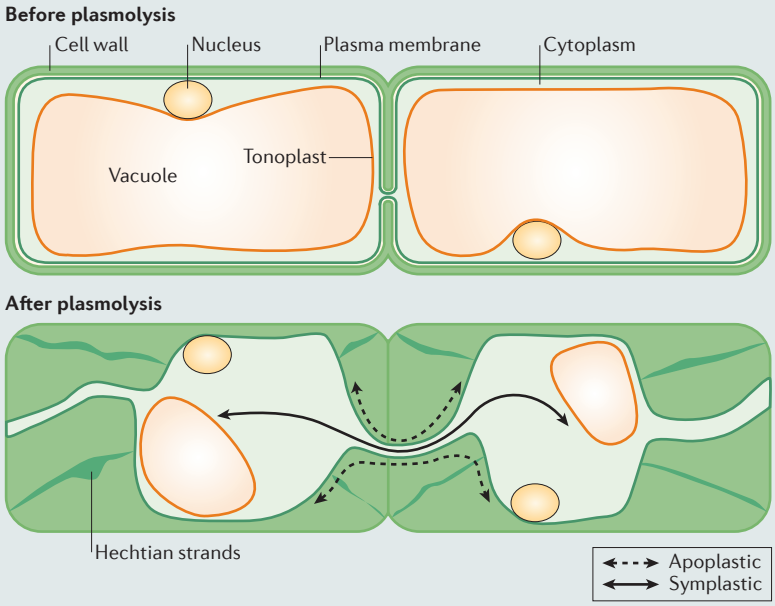
In a world facing increasing demands for food, fibre and biofuels, our main challenge is to meet these demands in spite of limited water and land resources[1–3]. Among the biotic factors that limit our ability to overcome this challenge are the eukaryotic filamentous plant pathogens that cause extensive annual yield losses of staple crops worldwide[4]. These include fungi such as the ascomycete rice blast fungus *Magnaporthe oryzae*[5], the basidiomycete rust fungi (order Pucciniales) that plague diverse crop species[6], and oomycetes such as the potato late blight pathogen, *Phytophthora infestans*[7]. These pathogens rapidly evolve to infect previously resistant plants, which necessitates constant renewal of disease control strategies; for example, the new Ug99 lineage of the wheat stem rust pathogen *Puccinia graminis* f. sp. *tritici*, which was identified in Uganda in 1999 (REF. 8), and the distinct wheat-infecting *M. oryzae* population (which causes wheat blast), which was identified in South America in 1985 (REFS 9,10), both currently threaten global wheat production.

Filamentous biotrophic or hemibiotrophic pathogens that feed on living plant cells must block host defences and sustain the host processes that they require for feeding and growth[11–14]. The first line of the plant immune system involves basal defence responses that are triggered by the detection of broadly conserved molecular features of pathogenic microorganisms called pathogen-associated molecular patterns (PAMPs) or microorganism-associated molecular patterns (MAMPs)[15–17]. PAMP-triggered immunity (PTI) involves pattern recognition receptors (PRRs), which are transmembrane receptor kinases and transmembrane receptor-like proteins. PAMPs include pathogen cell wall components (chitin for fungi and glucans for fungi and oomycetes) and detection occurs in the apoplast, which is the plant extracellular compartment (BOX 1). Plant defences include the production of reactive oxygen species (ROS), as well as the secretion of antimicrobial compounds, hydrolytic enzymes (proteinases, chitinases and glucanases) that are damaging to the pathogen, and inhibitors of pathogen hydrolytic enzymes, which are damaging to plants. Pathogens secrete effectors, which are generally small unique proteins, many of which function to defeat PTI[12,14,18]. In turn, plants have developed a second line of defence, effector-triggered immunity (ETI), which involves the detection of specific avirulence effectors (AVR effectors), either through direct ligand–receptor interactions or through indirect detection of effector action on host targets[16,17,19,20]. Apoplastic effectors of the extracellular fungus *Cladosporium fulvum* are detected by transmembrane receptor-like CF resistance proteins[21]. More commonly, pathogen effectors called cytoplasmic effectors are delivered to the host cytoplasm, and they are recognized by intracellular resistance (R) proteins of the cytoplasmic nucleotide binding site-leucine-rich repeat (NBS-LRR) class[16,19]. This recognition triggers the hypersensitive response (HR) and blocks pathogen growth. Mutation or complete loss of effector genes enables the pathogen to avoid R protein recognition, which leads to the boom-and-bust cycle that has often defeated efforts to control important plant diseases[8,22].

Box 1 | **Apoplastic and symplastic compartments in plants**

Plant tissue is divided into the symplastic (also known as cytoplasmic) compartment on the inner side of the plasma membrane and the apoplastic (also known as extracellular or cell wall) compartment on the outer side. Plasmodesmata, which are microscopic channels that enable the transport of small molecules and some proteins between plant cells, connect the cytoplasms of most cells in plant tissue to form a symplastic continuum throughout the plant (see the figure; not drawn to scale)[121,122]. The size exclusion limit of proteins that can pass through plasmodesmata varies depending on plant cell type. Guard cells lack plasmodesmata altogether. Plant cells typically contain a large vacuole separated from the cytoplasm by a vacuolar membrane, known as the tonoplast. Incubation of plant tissue in hypertonic solutions (such as molar sucrose solution) results in plasmolysis[90,123], during which water is drawn by osmosis from the cytoplasm and vacuole into the apoplast, and the plasma membrane and enclosed protoplasm shrink away from the cell wall. The accumulation of extracellular defence responses seems to require plasma membrane–cell wall connections[90]. In animal cells, the extracellular matrix–plasma membrane–cytoskeleton continuum is maintained by receptors that recognize RGD-containing matrix proteins, and RGD-proteins also seem to be important in plants. The plant plasma membrane–cell wall continuum is visible as Hechtian strands, which are thin connections between the protoplast and cell wall, after plasmolysis[90,123]. Gentle, stepwise plasmolysis, which maintains Hechtian strands and is reversible[90,123,124], can be achieved by the stepwise addition of hypertonic solutions to tissue on a microscope slide[54,90,123]. Plasmolysis shows that the plasma membrane remains intact as the pathogen grows inside host cells and defines the relationship of hyphae to the apoplast. To assay cytoplasmic translocation, plasmolysis separates putative cytoplasmic fluorescence from autofluorescence in cell walls and enlarges the apoplastic space for better differentiation of apoplastic from cytoplasmic localization[53,54,88,115].



Recent reviews have focused on the functional and structural biology of effectors from plant pathogenic and symbiotic fungi and oomycetes[12,14], on the nature of the biotrophic interface for diverse pathogens and symbionts[23], on effectors from necrotrophic fungi[24], and on oomycete effector translocation mechanisms[12,14,25,26]. In this Review we focus on the molecular and cellular biology of effectors during biotrophic invasion of plant cells by eukaryotic filamentous pathogens, with an emphasis on live-cell imaging of effector dynamics during natural plant invasions. Recent results illustrate the great progress that such techniques have facilitated in our understanding of the cellular biology of the complex sets of effectors that fungi and oomycetes use to cause plant diseases.

Effectors
Pathogen molecules that modify host cell structure, metabolism and function. They often interfere with signal pathways, either those required for host invasion or those that trigger host resistance.

Setting the scene

Biotrophic and hemibiotrophic filamentous pathogens enter plant tissue by direct penetration through the cuticle and cell wall using either melanized or non-melanized appressoria, or through stomata, often using non-melanized appressoria (FIG. 1). Once inside plant tissue, cell invasion is accomplished by diverse types of biotrophic hyphae. Some pathogens grow from one plant cell to the next as intracellular invasive hyphae (IH)[23,27,28] (FIG. 1a–c), some grow only as extracellular hyphae[21,29] (FIG. 1d) and others grow as extracellular hyphae that insert haustoria, which are terminal feeding structures, inside host cells[6,30,31] (FIG. 1e,f). Some pathogens are obligate biotrophs that only grow on living host tissue, whereas others are culturable outside the plant.

Powdery mildew–epidermal plant cell encounter sites provide a useful illustration of the general components of the biotrophic interface (FIG. 2), although the structural details can vary in other pathosystems[23]. Powdery mildew fungi are obligate biotrophs that grow as epiphytic hyphae on the leaf surface and that feed by inserting haustoria into host epidermal cells[32] (FIG. 2a). Intracellular hyphae remain separated from the host cytoplasm by the extrahaustorial membrane (EHM), which is continuous with the plant plasma membrane. The extrahaustorial matrix (EHMx) between the fungal wall and the EHM is separated from the host apoplast by a molecularly uncharacterized neckband. The haustorium complex, including the EHM, can be purified from plant tissue for gene expression and structural analyses[33,34]. Fluorescence microscopy has shown that the powdery mildew EHM is highly differentiated from the host plasma membrane: it lacks multiple plasma membrane components and contains at least one specialized protein, the transmembrane R protein Rpw8 (REFS 33,34).

The cell biology of host resistance — our understanding of which is most advanced in the powdery mildew–barley or powdery mildew–*Arabidopsis* systems[35,36] — highlights the challenges faced by potential plant pathogens (FIG. 2b). Live-cell confocal imaging, performed on fungus invading fluorescently labelled *Arabidopsis thaliana* epidermal cells, has documented marked host membrane polarization and focal accumulation of proteins at sites of attempted penetration[33,35–37]. Host responses occur even before appressorium penetration[33], in the form of cytoskeletal reorganization, cytoplasmic aggregation and deposition of a papilla at the site of attempted pathogen entry[35]. Fluorescently labelled membrane proteins show marked reorganization into concentric rings surrounding the site of attempted penetration. Proteins that are involved in vesicle secretion (Penetration 1; PEN1, a t-SNARE protein), and in the biosynthesis (PEN2, a myrosinase) and export (PEN3, an ABC transporter) of toxic compounds focally accumulate beneath the penetrating fungus (FIG. 2b).

New ultrastructural detail at the powdery mildew–host interface suggests that both plants and fungi use multivesicular bodies (MVBs) and exosomes (which are

Avirulence effectors
(AVR effectors). Effectors that are recognized by a corresponding plant resistance (R) protein, triggering the hypersensitive response and rendering pathogen strains expressing these effectors unable to infect (known as avirulent toward) host genotypes expressing the R protein.

Apoplastic effectors
Effectors that are secreted into and function in the plant extracellular space.

Cytoplasmic effectors
Effectors that are secreted and translocated across the plant membrane into the host cytoplasm, where they target different subcellular compartments.

Necrotrophic
An organism that kills host cells before invasion and gains nutrition from the dead cells.

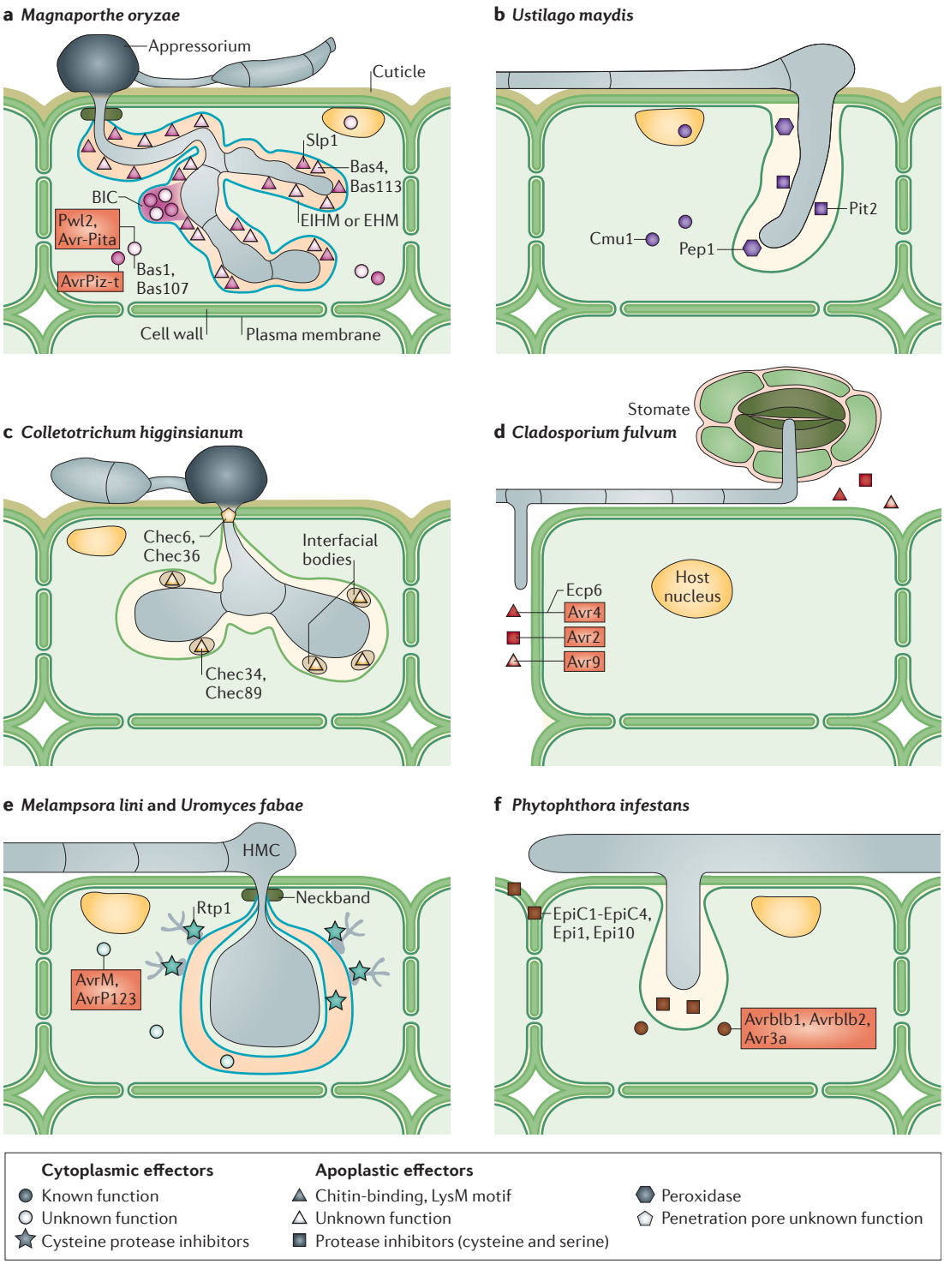
Extrahaustorial matrix
(EHMx). A substance that resides between the pathogen cell wall and the surrounding extrahaustorial membrane. Called the extrainvasive hyphal matrix when it surrounds invasive hyphae.

Neckband
An undefined structure that seals the interface between host and pathogen plasma membranes; sometimes observed as an electron-dense ring around haustorial necks by electron microscopy.

Papilla
Cell wall apposition at a site of attempted penetration; contains callose, phenolic compounds, lignin, reactive oxygen species, proteins and even membranes and exosomes; it is thought to function as a physical barrier to penetration.

Multivesicular bodies
(MVBs). Membrane-bound vesicles associated with late endosomes.

Exosomes
Intact vesicles that are secreted when multivesicular bodies fuse with the plasma membrane; suggested as an alternative route for secretion of virulence and pathogenicity factors into the host.



intact vesicles in the apoplast) for secretion at the biotrophic interface[34,35,38] (FIG. 2a). Membranes and exosome-like vesicles were observed in papillae and in the EHMx using transmission electron microscopy (TEM) of infected tissues prepared by high-pressure freezing and freeze substitution (HPF-FS), which provides superior membrane preservation[34,39]. MVBs were observed apparently fusing with fungal and host plasma membranes. This suggests that plants secrete defence components using MVBs and exosomes, and that filamentous plant pathogens, like filamentous animal pathogens[40], secrete virulence factors in exosomes. Exosome-mediated secretion might be an explanation for secretion of the barley powdery mildew AVR effectors Avrk1 and Avra10, which lack signal peptides for classical secretion[41–43].

Genome sequencing of diverse plant pathogens has led to the identification of hundreds of candidate effectors that each pathogen potentially secretes at the

◀ Figure 1 | **Biotrophic strategies and identified secreted effectors for diverse pathogens.** Intracellular biotrophic hyphae remain separated from the plant cytoplasm by a membrane (the extrahaustorial membrane (EHM) or the extrainvasive hyphal membrane (EIHM)) that is continuous with the plant plasma membrane. The matrix inside the EHM or the EIHM can be continuous with the plant apoplast or separated into a distinct compartment by a neckband-like structure. The effectors shown in red boxes have avirulence activity. **a** | *Magnaporthe oryzae*, which is a hemibiotrophic ascomycete fungus, enters host tissue using melanized (pressurized) appressoria. A neckband seals off the extrainvasive hyphal matrix (EIHMx) surrounding intracellular invasive hyphae in living host cells. **b** | *Ustilago maydis*, which is a biotrophic basidiomycete fungus, enters host tissue using non-melanized appressoria. The matrix surrounding intracellular hyphae is continuous with the host apoplast. **c** | *Colletotrichum higginsianum*, which is a hemibiotrophic ascomycete fungus, enters host tissue using melanized appressoria. The matrix surrounding intracellular hyphae is continuous with the host apoplast. **d** | *Cladosporium fulvum*, which is the biotrophic ascomycete tomato leaf mould fungus, enters leaf tissue through stomata and grows as extracellular hyphae. **e** | *Melampsora lini* and *Uromyces fabae*, which are biotrophic basidiomycete rust fungi, enter through stomata using non-melanized appressoria (not shown). Intercellular hyphae form haustorial mother cells (HMC) that in turn form haustoria in host mesophyll cells. Neckbands separate the extrahaustorial matrix (EHMx) from the host apoplast. **f** | *Phytophthora infestans*, which is a hemibiotrophic oomycete, enters host tissue using non-melanized appressoria and produces finger-like haustoria, surrounded by EHMx that is continuous with the host apoplast.

biotrophic interface[28–32,44]. Fungal effector proteins are generally characterized by the presence of a signal peptide for secretion and by specific expression during biotrophic invasion of plant cells[10,14,18,27]. Oomycete effectors have amino acid motifs associated with host translocation (RXLR, where X represents any amino acid, and CRN) closely following the signal peptide[12,45,46]. Most effectors are small proteins that lack motifs to predict their function[12,14,18]. Even when gene knockout strategies are feasible for functional analysis, effectors rarely show virulence phenotypes, presumably because of functional redundancy; for example, targeted gene replacement of 78 *M. oryzae* candidate effector genes found that only one, *MC69*, was associated with a pathogenicity defect[47]. Although cytoplasmic effectors generally show restricted phylogenetic distribution, this is not always the case. The *MC69* orthologue in the cucumber pathogen *Colletotrichum orbiculare* is also required for pathogenicity. Therefore MC69 has been identified as an effector that is important in both monocotyledonous and dicotyledonous hosts. Thus, effectors seem to range from core effectors that are broadly present, even across taxa, to variable effectors that are restricted to particular pathogen species (TABLE 1).

Focused secretion of effectors by appressoria

As host plants are known to respond before the pathogen enters the host tissue, it is reasonable to hypothesize that pathogens secrete effectors before penetration. Recent results with the crucifer pathogen *Colletotrichum higginsianum* invading *A. thaliana* cells have supported this prediction[48]. Specifically, *C. higginsianum* effector candidate (ChEC) proteins were expressed in waves, which suggests that distinct effectors were needed at different invasion stages. Wave 1 ChECs were only expressed in appressoria before penetration and wave 2 ChECs were expressed before and during penetration[48]. Interestingly, the fungus focally secreted fluorescently labelled wave 2 effectors Chec6 and Chec36 from the appressorium pore (FIG. 3). This general feature of melanized appressoria provides an opening to the host surface where the melanin diffusion barrier is not present[48,49]. ChECs predominate among highly expressed appressorial genes, which indicates that many effectors might be focally secreted through this pore[28,48]. This suggests that appressoria function in effector delivery, in addition to their well-established function in penetration of the host surface[48].

Fungal secondary metabolites can also be effectors secreted from appressoria during penetration. The unique *M. oryzae AVR* gene *ACE1* encodes Avirulence conferring enzyme 1, which is a hybrid protein with both polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) domains[50–52]. Fluorescent Ace1 protein specifically localizes to the cytoplasm of appressoria. It is probable that an uncharacterized secondary metabolite synthesized by the Ace1 enzyme is the secreted AVR effector that triggers *Pi33*-mediated resistance in rice[17,52]. *ACE1* resides in a secondary metabolism-associated gene cluster that is specifically expressed for a short period of time at the onset of penetration. The genomes of *M. oryzae* and *Colletotrichum* species are enriched for secondary metabolism-associated genes[28,51], and many of these genes are upregulated during biotrophic invasion. However, secondary metabolites are not general features of biotrophy, because powdery mildew fungi lack a marked capacity for PKS and NRPS secondary metabolism[32].

Live-cell imaging of *in planta* effector dynamics

Pathogens secrete effectors into the apoplast or the EHMx, which is known as the extrainvasive hyphal matrix (EIHMx) when it surrounds invasive hyphae. Cytoplasmic effectors must be translocated across the EHM or the extrainvasive hyphal membrane (EIHM) to reach their intracellular targets. New details on biotrophic invasion strategies are now available from live-cell imaging of pathogens secreting fluorescently labelled effectors *in planta*[48,53–55], or of pathogens growing in plant cells that are directly expressing fluorescently labelled effectors[56,57].

Targeted secretion of rice blast effectors. Live-cell imaging of fluorescently labelled pathogen and/or plant cellular components during fungal invasion is facilitated by the use of optically clear rice leaf sheath epidermal cells[54,58–62]. IH undergo successive colonization of living rice cells (FIG. 4a–c), although host cells eventually die as they fill with fungus[61]. IH switch from filamentous tubular hyphae to bulbous pseudohyphae in each new cell and they grow in a closed EIHM compartment[61,63]. Chimeric fluorescent effectors secreted by IH show two localization patterns in the EIHMx. Apoplastic effectors (such as the biotrophy-associated secreted protein 4 (Bas4)[64], the LysM protein 1 (Slp1)[62], and Bas113 (REF. 60)) accumulate throughout the EIHMx, outlining the entire IH[54,62,64]. Known AVR effectors (such as Pwl2 (REF.65), Avr-Pita[66] and AvrPiz-t[67]), as well as many Bas proteins, are first secreted into the biotrophic interfacial complex (BIC) at primary hyphal tips[47,54,60,64,68] (FIG. 4a).

Appressorium pore
A cell wall-less region of the appressorium adjacent to the plant cuticle that also lacks the melanin layer, which results in fungal plasma membrane in direct contact with the cuticle; it is sealed against the cuticle by a 'pore ring' that surrounds the perimeter of the pore.

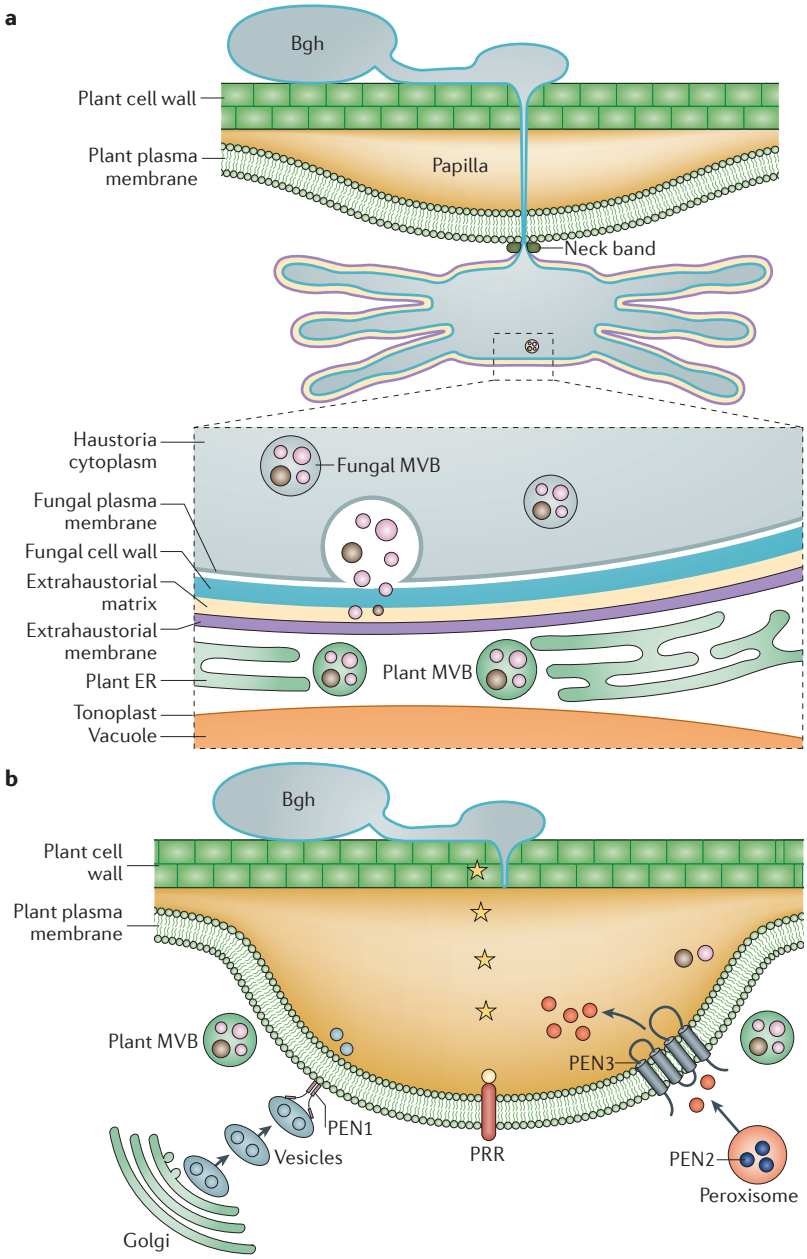


Figure 2 | **Structure and secretion dynamics at the biotrophic interface.** **a** | Powdery mildew haustoria in epidermal cells are sealed in extrahaustorial membrane (EHM) by one or more neckbands. The EHM is differentiated in protein content from the plant plasma membrane (PM) and occurs in close proximity to host endoplasmic reticulum (ER) and vacuoles. The abundance of multivesicular bodies (MVBs) in the plant and haustorial cytoplasms and of vesicles in the fungal paramural space and extrahaustorial matrix (EHMx) suggest a role for exosome secretion by the fungus and the host. **b** | Focused host secretion is involved in the formation of papillae and penetration resistance. *Arabidopsis thaliana* penetration-defective mutants have been used to identify the proteins that accumulate at sites of attempted penetration. Penetration 1 (*PEN1*) encodes a t-SNARE (a target SNAP receptor), which works with a vesicle-associated v-SNARE and a SNAP25 homologue to mediate fusion of vesicles and secretion at the penetration site. *PEN2* encodes a peroxisome-localized enzyme involved in biosynthesis of toxic indole glucosinolates (peroxisomes also accumulate at penetration sites). A *PEN3*-encoded ATP-binding cassette (ABC) transporter may be involved in extruding the toxic metabolites to the apoplast. In addition, plant MVBs are thought to be potential sources of membranes and vesicles observed in papillae, in haustorial encasements and in the EHMx. A pathogen associated molecular pattern (PAMP) recognition receptor (PRR) is also shown. Bgh, *Blumeria graminis* f. sp. *hordei*.

BIC localization requires expression of the secreted effector from its native promoter[54,69]. BICs are left behind beside the first bulbous IH cell after differentiation and further growth by the IH[54] (FIG. 4b). Fluorescence recovery after photobleaching (FRAP) experiments showed that effectors are still secreted into BICs while IH grow elsewhere in the rice cell[54]. Hyphal differentiation and BIC relocalization recur for each new hypha that enters a living host cell (FIG. 4c). Fluorescent effectors accumulating in BICs are a fundamental feature of successful infection and are not observed in interactions with resistant plants[64] (FIG. 4d).

Recent results suggest that *M. oryzae* effectors are secreted into BICs using a specialized secretion system[60] (FIG. 4b). AVR effectors require a signal peptide as well as function of the *M. oryzae* ER chaperone gene *LHS1* (REF. 70), which indicates that these effectors enter into the fungal ER for secretion; however, secretion pathways for cytoplasmic and apoplastic effectors seem to diverge from there. Brefeldin A (BFA), which is a chemical known to inhibit conventional Golgi-mediated secretion in fungi, totally blocked secretion of EIHMx-localized apoplastic effectors; however, as clearly confirmed by FRAP analysis, BFA treatment had no effect on the secretion of AVR effectors and other host-translocated Bas proteins into BICs[60]. In the opposite direction, targeted gene replacement mutants showed that the *M. oryzae* exocyst complex components Exo70 and Sec5 (also known as Exoc2) that have a role in tethering vesicles to target membranes before fusion, and the Sso1 t-SNARE were required for efficient secretion of effectors into BICs[60]. In the *Δexo70* and *Δsec5* mutants, BIC-localized effectors were partially retained inside the BIC-associated IH cells. These mutants secreted apoplastic effectors normally. Subapical BIC-associated IH cells retain abundant secretion machinery components (FIG. 4b), even though protein secretion is generally expected only at hyphal growth points[71]. Taken together, these results suggest that BIC-associated cells focally secrete effectors into BICs using a specialized, Golgi-independent secretion system[60].

Imaging of biotrophic invasion by a fungal strain expressing a fluorescently labelled BIC-localized effector and a fluorescently labelled fungal plasma membrane protein clearly showed that BICs lie in the interfacial zone outside the fungal plasma membrane[60]. BICs are surrounded by an accumulation of host cytoplasm, and the BIC region contains intense fluorescence in hosts expressing fluorescently labelled plant plasma membrane and ER markers[54,60]. Further definition of BIC structure requires higher resolution microscopy and immunolocalization studies using TEM techniques that retain membrane structures. BICs clearly concentrate secreted effectors that move into host cells, but their exact role — if any — in effector translocation remains to be determined.

Focal accumulation of* C. higginsianum *effectors. The crucifer pathogen *C. higginsianum* carries out localized biotrophy, in which biotrophic primary hyphae establish in the first *A. thaliana* cells that are invaded and then differentiate into necrotrophic hyphae that kill

and colonize neighbouring cells[28,72] (FIG. 4e). Following on from appressorial penetration (FIG. 3), a third wave of effectors is associated with the growth of biotrophic hyphae[48]. Live-cell imaging of monomeric red fluorescent protein (mRFP) fusions of the wave 3 effectors Chec34 and Chec89, expressed under the control of their native promoters, identified multiple fluorescent foci, called interfacial bodies (FIGS 1c,4e), that were randomly distributed on the biotrophic hyphal surface[48]. Using TEM immunolocalizations, these fluorescent foci were identified as discrete pads of electron opaque material within the EIHMx[48]. ChEC proteins diffused into the plant cell wall, but they were not observed in the cytoplasm of the invaded host cells, which is another example of effectors localizing to stage-specific compartments at the biotrophic interface during natural infection.

Imaging of effector-labelled plant cells after infection. For some pathogens, imaging of transformed strains is not feasible. An alternative strategy is heterologous expression of effectors in host cells followed by imaging of these cells after pathogen invasion[12]. Such assays are typically performed using *Agrobacterium tumefaciens*-mediated transient expression in tobacco. Using this strategy for *P. infestans* effectors, Avrblb2 was shown to localize to the cell periphery in uninvaded host cells and to focally accumulate around haustoria in invaded cells[56]; Crn8 was shown to accumulate in the host nucleus[73]. For the *A. thaliana* oomycete downy mildew pathogen, *Hyaloperonospora arabidopsidis*, screening for subcellular locations of 48 candidate effectors showed that one-third localized to host nuclei[57], which suggests that they have a role in host transcriptional reprogramming. Other candidates targeted diverse host compartments in the vicinity of haustoria, including the plasma membrane, tonoplast and ER; however, some caution is required in these cases as the effectors are overexpressed in host cells using constitutive plant promoters, and the corresponding localization patterns might vary from those that are characteristic of natural pathogen delivery.

Apoplastic effectors target host defences

In some cases, apoplastic effectors from diverse pathogens can be used to identify conserved host defence targets[13,74] (FIG. 1; TABLE 1).

Protease inhibitors. Papain-like cysteine proteases (PLCPs) are integral components of the plant immune response in the apoplast[13,75], and diverse pathogens secrete PLCP inhibitors. The apoplastic AVR effector Avr2 of *C. fulvum* inhibits the tomato plant-derived cysteine protease RCR3. Indeed, it is the Avr2–RCR3 complex that is recognized by the CF-2 resistance protein to activate HR[76]. *P. infestans* secretes EpiC1 and EpiC2B (which are members of a family of secreted proteins with cystatin-like protease inhibitor domains) that also inhibit the RCR3 protease[77]. EpiC2B interacts with and inhibits PIP1, another PLCP that is closely related to RCR3 (REFS 77–79). The *P. infestans* apoplastic effectors EpiC1 and EpiC2 inhibit activity of the tomato protease C14, and the cytoplasmic RXLR effector Avrblb2 focally accumulates near the haustorium and blocks secretion of C14 into the apoplast[56]. The *Ustilago maydis* apoplastic effector Pit2 inhibits cysteine proteases in maize[80,81]. Serine proteases are also targeted. In *P. infestans*, the Kazal-like extracellular protease inhibitors Epi1 and Epi10 are multidomain secreted serine protease inhibitors that inhibit the pathogenesis-related subtilisin-like serine protease P69B of tomato[82].

Chitin-binding effectors. Chitin is an unbranched β-1,4-linked homopolymer of *N*-acetyl-glucosamine (GlcNAc), which is a component of fungal cell walls. Chitin fragments function as a PAMP for triggering the plant immune response, and fungi secrete effectors to block chitin-induced immunity. Diverse pathogens secrete effectors that contain LysM amino acid domains (carbohydrate-binding modules that generally bind GlcNAc) that either prevent the release of chitin oligosaccharides from fungal cell walls or that sequester these oligosaccharides to prevent recognition. *C. fulvum* secretes the LysM effector Ecp6, which sequesters chitin oligosaccharides released from the fungal cell wall[83]. The *C. fulvum* effector Avr4 contains a different chitin-binding domain and functions to protect the fungal cell wall from degradation by plant chitinases[84]. One of the three LysM effectors in the intercellular wheat pathogen *Mycosphaerella graminicola* has both wall protection and sequestering functions[85]. In *M. oryzae*, the LysM effector Slp1 binds chitin oligosaccharides and suppresses chitin-induced immunity mediated by the rice PRR, chitin elicitor binding protein (CEBiP)[62]. Chitin-binding effectors are required for virulence in all three fungi.

Peroxidase inhibitor. The apoplastic effector Pep1 of *U. maydis* functions to protect hyphae from ROS, which are a major component of the plant immune response[53,55]. Fluorescently labelled Pep1 protein surrounds hyphae in the apoplast and concentrates as rings around hyphae at cell-to-cell passage sites (FIG. 1b). In *U. maydis* and the related smut *Ustilago hordeii*, *Δpep1* deletion mutants are defective in penetration of the initial epidermal cell and in cell-to-cell spread. They encounter extensive host resistance, including H_2O_2 accumulation, papilla formation and host cell death[53]. Bimolecular fluorescence complementation (BiFC) assays showed a direct interaction between the Pep1 protein and the maize defence peroxidase POX12 *in vivo*. Pep1 seems to enable penetration by scavenging ROS[55].

Cytoplasmic effectors

High-throughput assays for effector virulence function are based on screens for suppressors of plant cell death induced by the *P. infestans* infestin 1 (Inf1) protein, necrosis- and ethylene-inducing peptide 1-like proteins (NLPs) from various pathogens, or the mouse BAX protein[12,48]. Sometimes >70% of effectors tested suppress host cell death in these assays[7,12]. Cytoplasmic effectors characterized in detail defeat host defences using a range of mechanisms (TABLE 1).

Tonoplast
The vacuolar membrane in a plant cell.

Table 1 | **Examples of filamentous plant pathogen effectors**

Organism	Gene name	Gene accession ID and Uniprot code	Associated functions*	Distribution (such as species- or taxon-specific)	Refs
Magnaporthe oryzae	*SLP1*	MGG_10097.6 and G4N906	LysM domain protein; binds chitin oligosaccharides; suppresses chitin-induced immunity in rice; BFA-sensitive secretion	Unspecific; also in *Cladosporium* and other fungi	62
M. oryzae	*AVRPiz-t*	HE578813 and C6ZEZ6	Targets rice ubiquitin ligase APIP6; AVR protein for rice Piz-t; translocated into rice cells	Specific to *Magnaporthe*	68
M. oryzae	*PWL2*	U26313 and Q01144	AVR protein blocking infection of *Eragrostis curvula*; translocated and moves from cell to cell; BFA-insensitive secretion	Specific to *Magnaporthe*	54,60,65
M. oryzae	*AVR-Pita1*	AF207841 and Q9C478	Homology to metalloproteinases; AVR protein for rice Pita; BFA-insensitive secretion	Specific to *Magnaporthe*	60,66, 98,108
M. oryzae	*AVR1-CO39*	AF463528 and Q8J183	AVR protein corresponding to *R* gene pair functioning as *Pi-CO39*	Specific to *Magnaporthe*	69,100
M. oryzae	*BAS1*	MGG_04795 and G5EHI7	Candidate cytoplasmic effector; translocated and moves from cell to cell; BFA-insensitive secretion	Specific to *Magnaporthe*	54,60,64
M. oryzae	*BAS2*	MGG_09693 and G5EI08	Accumulates at cell wall crossing points	Specific to *Magnaporthe*	10,64
M. oryzae	*BAS3*	MGG_11610 and G5EHH0	Accumulates at cell wall crossing points	*Magnaporthe* and *Colletotrichum*	48,64
M. oryzae	*BAS4*	MGG_10914.6 and G5EI20	Apoplastic effector; marker for intact EIHM; BFA-sensitive secretion	Specific to *Magnaporthe*	64
M. oryzae	*BAS107*	MGG_10020.6 and G4N9C5	Translocated and localized to rice nuclei; moves from cell to cell; BFA-insensitive secretion	Specific to *Magnaporthe*	60
M. oryzae	*BAS113*	MGG_05785.6 and G4N0X0	Candidate apoplastic effector; homology to glycosyl hydrolase; BFA-sensitive secretion	Specific to *Magnaporthe*	60
M. oryzae	*MC69*	MGG_02848.6 and G5EI17	Required for full virulence in both monocot and dicot hosts	Also in *Colletotrichum*, CoMC69 (AB669186 and H7CE70)	47
Ustilago maydis	*pit2*	XP_757522 and Um01375	Apoplastic effector; cysteine protease inhibitor	Unspecific; also in *Cladosporium* and *Phytophthora*	80,81
U. maydis	*pep1*	UM01987 and G0X7E8	Apoplastic effector; peroxidase inhibitor	Specific to *Ustilago*	53,55
U. maydis	*cmu1*	AB116236 and Q6L8Q0	Cytoplasmic effector; secreted chorismate mutase; converts chorismate to prephenate; translocated; moves from cell to cell	In many biotrophic and hemibiotrophic fungi, and *Phytophthora*	88
Colletotrichum higginsianum	*ChEC6*	HE651161 and K7N7G2	Candidate effector secreted at the appressorial pore	Specific to *Colletotrichum*	48
C. higginsianum	*ChEC34*	HE651193 and K7N7F7	Candidate effector secreted into interfacial bodies	Specific to *Colletotrichum*	48
C. higginsianum	*ChEC36*	HE651195 and K7N7G5	Candidate effector secreted at the appressorial pore	Specific to *Colletotrichum*	48

Targeting the host ubiquitylation system. Recent results from studies of filamentous pathogen effectors support the importance of the plant ubiquitylation system to both positive and negative regulation of plant immunity[68,86,87]. The *P. infestans* AVR effector Avr3a was shown to bind to and to stabilize a potato U-box E3 ubiquitin ligase CMPG1 and to block Inf1-induced death of potato cells[86]. CMPG1 did not have an effect on HR-induction through recognition of Avr3a by the R3a resistance protein. Inf1-induced cell death is suggested to have a role during necrotrophic growth of the pathogen, and an effector that blocks this death would promote biotrophic growth. The *M. oryzae* AVR effector AvrPiz-t binds to and destabilizes the rice RING E3 ubiquitin ligase APIP6 to suppress chitin-induced PAMP immunity[68]. In support of this effector function, silencing of APIP6 reduced resistance to *M. oryzae*. AvrPiz-t is a BIC-localized protein that is translocated into rice cells[68].

Targeting defence signalling. The *U. maydis* effector Cmu1 is a secreted chorismate mutase that is highly expressed during biotrophic invasion and that is required for full virulence on maize[88]. Cmu1 protein was detected in the maize cytoplasm by TEM immunolocalizations, and the protein spread into neighbouring

Table 1 (cont.) | **Examples of filamentous plant pathogen effectors**

Organism	Gene name	Gene accession ID and Uniprot code	Associated functions*	Distribution (such as species- or taxon-specific)	Refs
C. higginsianum	*ChEC89*	HE651252 and I2G7G9	Candidate effector secreted into interfacial bodies	Specific to *Colletotrichum*	48
Cladosporium fulvum	*Ecp6*	4B9H_A and 4B9H	LysM domain protein; binds chitin; suppresses host immunity	Unspecific; also in *Magnaporthe* and other fungi	21,83
C. fulvum	*Avr4*	Y08356 and CAA69643	Binds chitin oligomers; inhibits plant chitinases from degrading fungal cell walls; AVR protein for tomato CF-4	Specific to *Cladosporium*	21,84
C. fulvum	*Avr2*	AJ421628 and CAD16675	Inhibits tomato proteases RCR3 and PIP1; AVR protein for tomato CF-2; RCR3 required for Avr2 recognition	Unspecific; also in *Ustilago* and *Phytophthora*	21,76
C. fulvum	*Avr9*	A19194 and CAA01455	Structural homology to carboxypeptidase inhibitor; AVR protein for tomato CF-9	Specific to *Cladosporium*	21,29
Uromyces fabae	*RTP1*	AJ971426 and Q334H6	Rust transferred protein 1; cysteine protease inhibitor; fibril formation; and translocated into host cells	Specific to *Uromyces*	92,94,95
Melampsora larici-populina	*AvrM*	DQ279870 and Q2MV46	AVR protein for flax M; and translocated into host cells	Specific to *Melampsora*	104,105, 115
Melampsora lini	*AvrP123*	EU642499 and Q2MV43	Homology to serine protease inhibitor; AVR protein for flax P, P1, P2 and/or P3	Specific to *Melampsora*	101,105
Phytophthora infestans	*EPIC1*	DS028131 and EEY55256	Cystatin-like protease inhibitor, inhibits PLCPs RCR3 and C14	Unspecific; also in *Ustilago* and *Cladosporium*	77–79
P. infestans	*EPIC2*	AY935251 and A1L016	Cystatin-like protease inhibitor; and inhibits PLCP C14	Unspecific; also in *Ustilago* and *Cladosporium*	77–79
P. infestans	*EPIC2B*	EEY55258 and D0NBV3	Cystatin-like protease inhibitor; inhibits PLCP RCR3; interacts with and inhibits PLCP PR protein PIP1	Unspecific; also in *Ustilago* and *Cladosporium*	77–79
P. infestans	*EPI1*	JN021528 and G8FQ60	Extracellular serine protease inhibitor; and Kazal domain motifs	Specific to *Phytophthora*	82
P. infestans	*EPI10*	AY586282 and Q6PQG3	Extracellular serine protease inhibitor; Kazal domain motifs	Specific to *Phytophthora*	82
P. infestans	*AVRBLB1*	DS028419 and D0P3S7	RXLR cytoplasmic effector; contains RGD motif; AVR protein to Rpi-blb1	Specific to *Phytophthora*	89,97
P. infestans	*AVRBLB2*	DS028242 and D0P1B2	RXLR cytoplasmic effector; accumulates around haustoria; prevents secretion of tomato PLPC C14; AVR protein to Rpi-blb2	Specific to *Phytophthora*	56
P. infestans	*AVR2*	EEY61966 and D0NN59	RXLR cytoplasmic effector; binds putative plant phosphatase BSL1; AVR protein to *S. demissum* R2; interaction with R2 requires BSL1	Unspecific; also in *Ustilago* and *Cladosporium*	96
P. infestans	*AVR3a*	EF587759 and A5YTY8	RXLR cytoplasmic effector; stabilizes host E3 ligase CMPG1; AVR protein to *S. demissum* R3a	Specific to *Phytophthora*	86
P. infestans	*CRN8*	AY961456 and Q2M405	CRN cytoplasmic effector; and nuclear-localized kinase that induces host cell death	Specific to *Phytophthora*	56

AVR, avirulence; BFA, brefeldin A; EIHM, extrainvasive hyphal membrane; PLCP, papain-like cysteine protease; *R*, resistance. *Translocation to the host cytoplasm and cell-to-cell movement are listed when shown microscopically.

cells when transiently expressed in maize cells[88]. This fungal enzyme catalyses the conversion of chorismate to prephenate in the shikimate pathway, which shifts production towards aromatic amino acids and away from production of the defence signalling hormone salicylic acid. Plants infected by *Δcmu1* mutants have increased levels of salicylic acid and increased resistance to the fungus[88].

Targeting plant cell wall–plasma membrane attachment. The *P. infestans* cytoplasmic RXLR-effector Avrblb1 (also known as IpiO)[7,89], contains an RGD motif, which suggests that it might have an effect on the plant cell wall–plasma membrane attachment that is required for extracellular defences (BOX 1). Avrblb1 binds *in vitro* to the *A. thaliana* lectin receptor kinase LECRK-1.9, which is reported to play a role in maintaining this attachment. Therefore, Avrblb1 might decrease basal resistance by interrupting the connection between the host plasma membrane and the cell wall[89–91].

An effector with a proposed structural role. The *Uromyces fabae* rust transferred protein 1 (Rtp1) was the first fungal effector to be visualized in the host cytoplasm and nucleus after *in planta* secretion by the rust fungus[92]. Rtp1

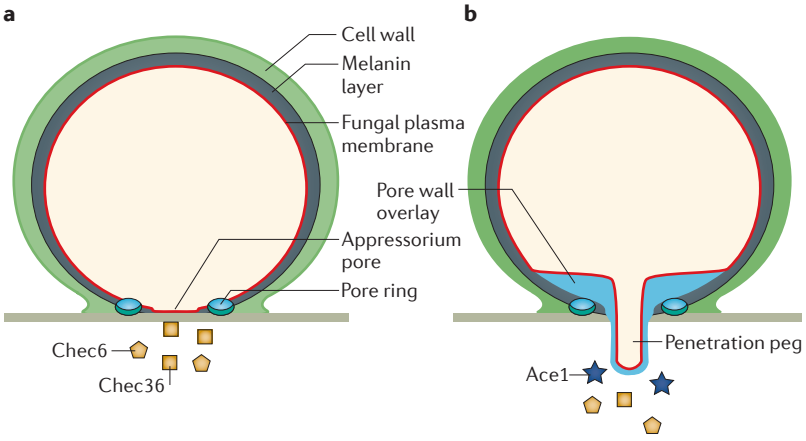


Figure 3 | **Focal effector secretion in melanized appressoria.** Melanized appressoria power penetration by building enormous turgor pressure[5,49]. The melanin layer is deposited between the plasma membrane and the cell wall, where it blocks the passage of molecules larger than water. The melanin functions as a barrier to the efflux of appressorium solutes, and water entering the appressorium generates pressure to force the penetration peg through the leaf cuticle. **a** | The diagram shows an appressorium after melanization, but before penetration. The appressorium pore, sealed against the cuticle by a pore ring, is a region in which the fungal plasma membrane is in direct contact with the cuticle for hours before penetration. An abundance of vesicles near the pore meant that it was suggested to function as a window for communication with the underlying host[49]; now it seems that *Colletotrichum higginsianum* orchestrates focal secretion of effectors through this pore, which is ~200 nanometres in diameter[48]. **b** | A bilayered pore wall overlay is laid down over the pore shortly before penetration peg emergence. The peg cell wall is continuous with the pore wall overlay. Effector secretion before and during penetration has been shown for *C. higginsianum* (Chec6 and Chec36) and implicated for *Magnaporthe oryzae* (Ace1). It is unknown if the secreted effectors travel into the host cell below, but it is an interesting working hypothesis.

belongs to a family of cysteine protease inhibitors that are conserved in the order Pucciniales[6,93,94]. This protein was recently reported to aggregate into amyloid-like filaments *in vitro*[95]. Immunoelectron microscopy localized Rtp1 to EHMx protuberances that extend into the host cytoplasm. The exact role for this interesting protein remains to be discovered.

AVR effector recognition

To trigger HR and block infection, plant *R* gene products recognize AVR effectors either by direct binding (ligand–receptor model) or by indirect detection of a modification of the effector's virulence target or a decoy of that target (guard or decoy model)[20]. Indirect recognition occurs for *C. fulvum* Avr2 and tomato CF-2, which is mediated by protease RCR3 (REF. 76), and for *P. infestans* Avr2 and the *Solanum demissum* R2 protein, which is mediated by a putative plant phosphatase BSL1 (REF. 96). Direct interaction has been shown for the *P. infestans* effector Avrblb1 and the coiled-coil domain of Rpi-blb1 (REF. 97), for four out of five rice blast AVR–R protein pairs[17] (involving Avr-Pita[98], Avr-Pik/km/kp[99], Avr1-Co39 (REF. 100) and Avr-Pia[100]), and for two out of four flax rust gene pairs[101] (involving *AvrL567* (REFS 102,103) and *AvrM*[104]). So far, direct binding between AVR and R proteins is more common for filamentous pathogen effectors than for the bacterial effectors.

Both the mechanism of interaction between AVR and R proteins and the virulence functions of AVR proteins affect *AVR* gene evolution in nature[20]. Virulence functions for most AVR effectors are unknown (FIG. 1; TABLE 1), although some have sequence motifs or structures that suggest their function: metalloproteinase for Avr-Pita[66], serine protease inhibitor for AvrP123 (REF. 105) and carboxypeptidase inhibitor for Avr9 (REF. 21). Field population studies showed that each cloned flax rust *AVR* gene shows evidence for diversifying selection (that is polymorphism with high rates of non-synonymous nucleotide substitutions that alter the amino acid sequences of proteins) on at least one host plant[93,101,103]. This presumably results from loss of recognition in response to deployed *R* genes while maintaining virulence function. *AvrL567* is present as a complex multigene family in *Melampsora lini* isolates from cultivated flax, *AvrP123* and *AvrP4* occur as complex multigene families in *M. lini* isolates from wild flax (*Linum marginale*), and *AvrM* and *AvrP4* are present as complex multigene families from *Melampsora larici-populina* isolates from poplar, which perhaps reflects the *R* gene composition of these host plants. A different strategy is implicated for rice blast effectors, as only *AVR-Pik/km/kp* shows evidence for classical diversifying selection among field isolates[99]. Instead, blast *AVR* genes are often deleted in response to deployment of the corresponding rice *R* gene[10,17,106,107]. Recent insight that *AVR-Pita* has experienced multiple examples of translocations to different, often subtelomeric, chromosomal regions in various field isolates has led to the interesting hypothesis that *AVR* genes retained in the population as a whole are regained by individual isolates through parasexual recombination[108]. Field population studies suggest that diverse AVR effectors for which no virulence function has been discovered in the laboratory do have a role.

Translocation motifs

The best-studied amino acid motif in oomycete effectors is the RXLR motif, which is found in effectors from the haustoria-forming oomycetes, *Phytophthora* spp. and the downy mildews[109–111]. Many RXLR proteins have a dEER (double glutamic acid and arginine) sequence within 25 amino acids downstream of the RXLR. The Crinkler motif (also called the CRN or LXLFLAK motif) occurs more broadly in oomycetes[73]. Both motifs reportedly function as signals for translocation into the host cytoplasm, although questions about this remain[12,14,25,26,110–112]. Genome sequencing has revealed new effector protein families with conserved amino-terminal motif sequences in other oomycetes, for example, the YXSL[RK] motif for *Pythium ultimum* and CHXC for *Albugo* spp.[12].

Bioinformatic analyses have not identified putative translocation motifs for most fungal effectors[10,14]. Many powdery mildew effector candidates contain a short Y/F/WXC motif within 30 amino acids of the signal peptide[32,113]. Recent structure and evolution studies of barley powdery mildew effector candidates indicate that this motif might derive from an ancestral ribonuclease that is suggested to have provided a stable structural fold as a template for effector diversification[114]. Some

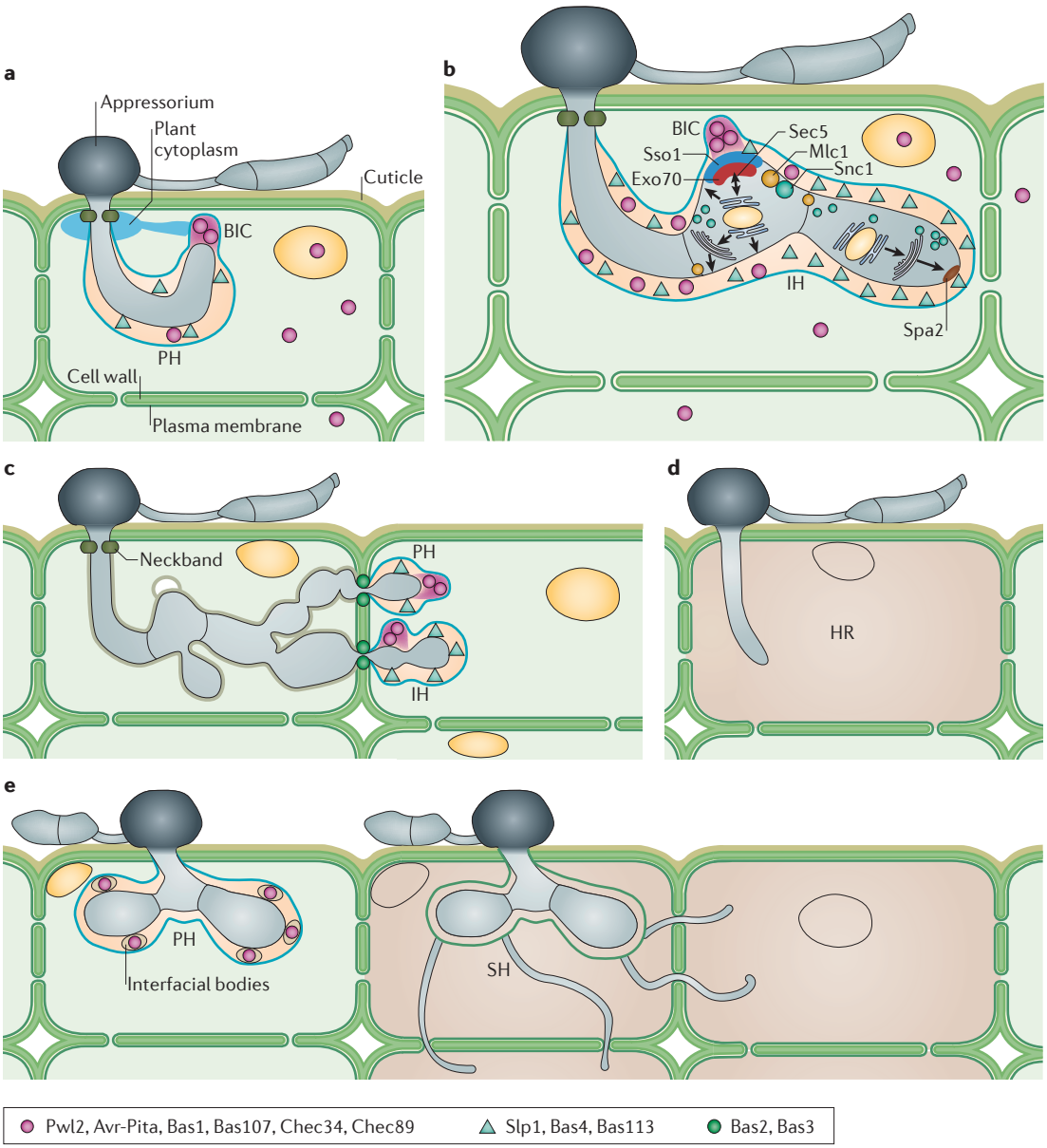


Figure 4 | **Effector secretion and accumulation during live cell invasions.** Schematic diagrams are not drawn to scale. **a**–**c** | Extended biotrophy by *Magnaporthe oryzae*. **a** | At 22–25 hours post inoculation (hpi), fluorescent cytoplasmic effectors are secreted and accumulate in the biotrophic interfacial complex (BIC) outside tips of primary hyphae (PH). Some BIC-localized proteins are translocated into the cytoplasm of invaded rice cells (some also move into uninvaded cells). Apoplastic effectors are retained in the extrainvasive hyphal matrix (EIHMx) without host translocation. **b** | At 26–30 hpi, the primary hypha differentiates into bulbous invasive hyphae (IH) that fill the first invaded rice cells and then move into living neighbouring cells. The BIC remains beside the first bulbous IH cell, which is also differentiated from other IH cells in that it contains concentrated foci of fluorescent protein secretion machinery components normally only associated with growing cells. These are myosin motor regulatory component Mlc1 (MGG_09470.6), v-SNARE Snc1 (MGG_12614.6), t-SNARE Sso1 (MGG_04090.6) and exocyst component Exo70 (MGG_01760.6). Polarisome component Spa2 (MGG_03703.6) is restricted to growing IH cells. Blast effectors targeting distinct host compartments may require specialized secretory processes: apoplastic effectors (Slp1, Bas4 and Bas113) follow the conventional, brefeldin A (BFA)-sensitive, Golgi-dependent secretion pathway, whereas cytoplasmic effectors (Pwl2, Avr-Pita, Bas1 and Bas107) follow a non-conventional, BFA-insensitive secretion pathway involving exocyst and SNARE proteins. **c** | At 36–40 hpi, IH produce highly constricted hyphal pegs that cross plant cell walls and reinitiate the hyphal differentiation and BIC development processes. Some effectors, including Bas2 and Bas3, accumulate at points of cell-to-cell passage. **d** | PH differentiation and fluorescent protein accumulation fails to occur if avirulence (AVR) effector recognition triggers the hypersensitive response (HR). **e** | Localized biotrophy by *Colletotrichum higginsianum*. Bulbous biotrophic primary hyphae secrete effectors that accumulate in randomly distributed interfacial bodies. The fungus switches to necrotrophic secondary hyphae (SH) while still in the first invaded cell and kills neighbouring host cells before invasion.

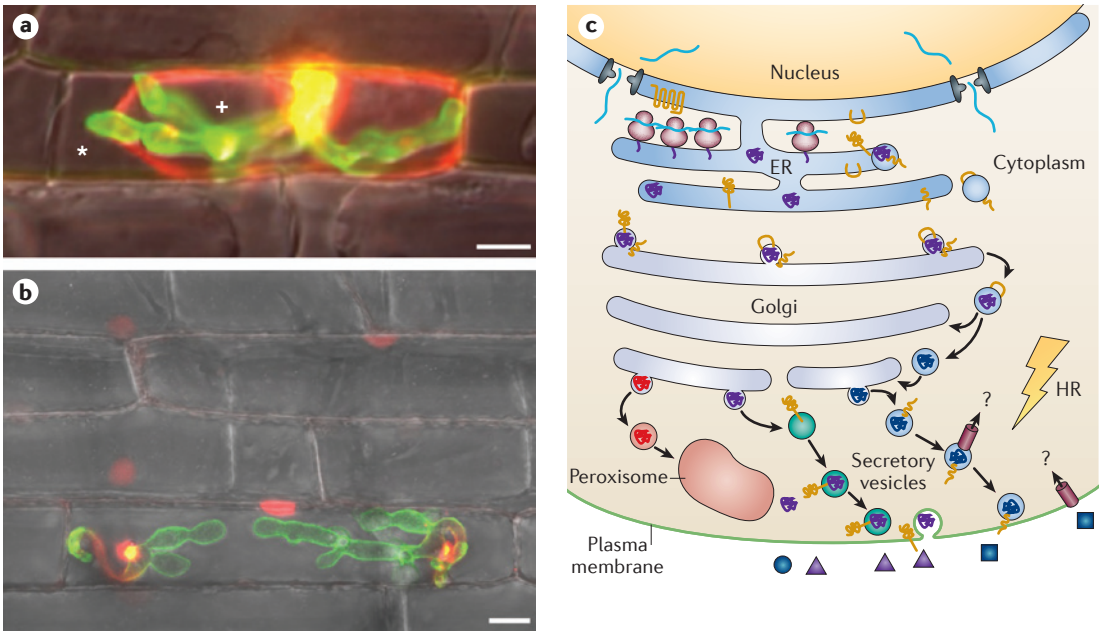


Figure 5 | **Pathogen-dependent and pathogen-independent effector translocation assays.** **a** | Epifluorescence microscopy image of *Magnaporthe oryzae* secreting the cytoplasmic effector Pwl2 linked to tdTomato, a tandem dimer of monomeric red fluorescent protein (mRFP) (a 68.3 kDa fusion protein), and apoplastic Bas4 linked to GFP after plasmolysis. Pwl2 fluorescence (red) is observed in the cytoplasm surrounding the dark vacuole (+). Neither Pwl2 nor Bas4 (green) is observed in the apoplast (*), even in conditions resulting in overexposed biotrophic interfacial complex (BIC) fluorescence (yellow). Effector fusion proteins with tdTomato fail to spread from cell to cell, consistent with size exclusion limits in plasmodesmata. This image shows merged DIC with red and green fluorescence channels. Scale bar represents 10 µm. **b** | Projected confocal microscopy image of *M. oryzae* secreting the cytoplasmic effector Pwl2 (red) linked to mRFP and a nuclear localization signal (a 44.5 kDa fusion protein), together with apoplastic Bas4 (green) linked to GFP. Pwl2 preferentially accumulated in the BIC (inner layer; yellow) and was translocated into the rice cytoplasm (red; host nuclei). Pwl2 also moved ahead into adjoining uninvaded cells. Bas4–GFP was retained in the extrainvasive hyphal matrix (EIHMx), verifying the intactness of the extrainvasive hyphal membrane (EIHM). Scale bar represents 10 µm. **c** | Diagram representing the pathogen-independent assay. Transient expression of avirulence (AVR) effectors by plant cells without a signal peptide will activate the hypersensitive response (HR) in the cytoplasm of a resistant plant. But with a signal peptide, the effector will enter and follow the secretory pathway. If proper control experiments are performed to ensure efficient effector entry into the endoplasmic reticulum (ER), induction of the HR by an AVR effector with a signal peptide indicates this effector has crossed the plant plasma membrane (or vesicular membranes before secretion). How this process occurs is unknown. Part **a** is reproduced, with permission, from REF. 54 © (2010) American Society of Plant Biologists. Image in part **b** courtesy of C. H. Khang, Kansas State University, USA.

candidate rust effectors share the YXC motif, although the location of this motif is more dispersed in these proteins[6,93]. Functional characterization for the YXC motif is lacking.

Translocation of cytoplasmic effectors

Microscopic translocation assays during pathogen invasion. Three cytoplasmic effectors have been localized to the cytoplasm of infected host cells using immunofluorescence light microscopy and/or immunolocalization electron microscopy: *U. fabae* Rtp1 (REF. 92), *M. lini* AvrM[115] and *U. maydis* Cmu1 (REF. 88).

The ability to transform some pathogens enables the direct observation of secretion of fluorescent effector proteins during *in planta* infection. For *M. oryzae*, translocation of fluorescent effectors into the rice cytoplasm is routinely observed using a variety of effector proteins fused to various versions of green or red fluorescent proteins[54,60]. Translocated cytoplasmic effector fluorescence is weak compared to BIC fluorescence, but it can be clearly differentiated from host autofluorescence. Imaging of fluorescent effectors in the host cytoplasm is facilitated by plasmolysis (BOX 1; FIG. 5a) or by concentrating the translocated fusion protein in the rice nucleus by addition of a nuclear localization signal (NLS; either a peptide NLS or an entire histone protein) (FIG. 5b). Using these translocation assays, the BIC-localized AVR effectors Pwl2 and AvrPiz-t and many additional Bas proteins have been visualized in the rice cytoplasm, implying translocation[23,54,60,68]. By contrast, fluorescent versions of apoplastic Bas4 protein were not observed in the rice cytoplasm, even with an added NLS signal[54]. Bas4 protein only occurred in the rice cytoplasm at sites where infection failed and the EIHM had broken[54,64].

M. oryzae effectors that were translocated into the rice cell showed different localization patterns. Some effectors accumulate in the rice cytoplasm and nucleus. One, Bas107, localized to the rice nucleus without an artificially added NLS[23,60]. Other effectors, including Bas2 and Bas3, accumulated around hyphae where they

Plasmolysis
Shrinkage of the protoplast away from the plant cell wall as a result of the loss of water through osmosis.

Box 2 | **Questions for future research**

- Do effectors that are secreted by appressoria before penetration make their way through the plant surface into host cells? What roles do they have in penetration and plant invasion?
- Do filamentous plant pathogens deliver virulence factors by secreting exosomes into the biotrophic interface? What role do fungal and/or plant exosomes have in building the extrahaustorial matrix (EHMx)? What role do plant exosomes have in delivering defence responses?
- How are some *Magnaporthe oryzae* effectors targeted to the biotrophic interfacial complex (BIC)-associated alternative secretion pathway, and how does this secretion pathway work? Have other filamentous plant pathogens evolved specialized secretion mechanisms for different classes of effectors?
- What are the modes of action of effectors at the biochemical level and what are the structural bases for the interactions? How can we improve high-throughput biological screens to define targets for the hundreds of candidate effector proteins identified through genome sequencing?
- Are filamentous pathogen effectors translocated across the plant membrane by a pathogen-independent mechanism? Does plant endocytosis have a role in uptake? How can discrepancies in lipid binding and translocation assays be clarified for RXLR effectors in oomycetes?
- Do motifs or signals target fungal effectors for translocation across the host cytoplasm and, if so, what is the mechanism?
- How can understanding the cell biology at the biotrophic pathogen–plant interface contribute to durable resistance to plant diseases that threaten the world's supply of food, fibre and biofuels?

crossed the plant cell wall[23,64] (FIG. 4c). It is possible that these effectors block apoplastic plant defences in a similar way to *U. maydis* Pep1 (REF. 55), or that they may have a role in the mechanism used for crossing.

Some translocated *M. oryzae* effectors moved into surrounding uninvaded cells and were clearly visible up to three to four cells away from the invaded cell, presumably to prepare host cells before pathogen entry[54] (FIG. 5b). Cell-to-cell effector movement was confirmed for the *U. maydis* Cmu1 effector when the protein was transiently expressed in maize cells[88]. For both *M. oryzae* and *U. maydis* effectors, cell-to-cell movement of effectors depended on host cell type (for example, no movement into guard cells was observed), which is consistent with symplastic movement through plasmodesmata (BOX 1). For rice blast, effector fusion proteins larger than 45 kDa are translocated into rice cells, but they fail to spread from cell to cell[54] (FIG. 5a), which is consistent with the idea that there is a size exclusion limit on proteins that move through plasmodesmata[54]. These results identify plasmodesmata as a new interaction interface for effectors.

M. oryzae is so far the only filamentous pathogen that has been shown to deliver fluorescent effector fusion proteins into host cells using fluorescence microscopy of natural infection. Attempts to show translocation during infection with *P. infestans*, *U. maydis* and *C. higginsianum* tranformants expressing fluorescent effectors have not succeeded[48,88,111]. Perhaps these pathogens translocate lower effector quantities and the fluorescence is too dilute for observation. It is also possible that *M. oryzae* uses a different translocation system than the other pathogens, one that translocates large fusion proteins.

Pathogen-independent translocation assays. How effector proteins from eukaryotic filamentous pathogens cross plant membranes into living host cells is an important question. There are reports in both oomycete and fungal systems that translocation of effectors across the plant plasma membrane occurs by a pathogen-independent process that does not require pathogen components such as the bacterial type III secretion system[69,109–111,115]. Pathogen-independent assays involve the transient expression of AVR effectors in plant cells with and without their signal peptides (FIG. 5c). Without a signal peptide, the AVR effector protein remains in the host cytoplasm and induces the HR in the presence of its corresponding R protein. With its signal peptide, the effector protein enters the plant secretory system (FIG. 5c). An AVR effector inside the secretory system should not induce the HR unless it is translocated back across the plant membrane into the host cytoplasm. This assay requires efficient entry of the AVR effector into the ER, as any mislocalization of the protein to the host cytoplasm would trigger the HR. A control to indicate efficient protein delivery into the ER involves incorporation of an HDEL ER amino acid retention signal at the C terminus of the effector protein, which traps the effector in the ER. A complementary assay involves the direct treatment of host tissue with purified fluorescent fusion proteins and observation of its uptake[110]. N-terminal domains from oomycete RXLR effectors and N-terminal domains that lack amino acid motifs from fungal effectors seem to be active in promoting effector translocation using these HR-induction and fluorescence protein uptake assays[12,14,115]. Conflicting results regarding the function of the RXLR motif in binding phosphatidylinositol-3-phosphate and in mediating translocation have been obtained using the same effector systems[25,26]. Beyond reconciling conflicts for individual effectors, there is no reason to expect that diverse fungal and oomycete pathogens have evolved a single effector translocation mechanism. Understanding translocation mechanisms for eukaryotic filamentous pathogens remains an important challenge for the field of plant–pathogen interactions.

Future challenges

We have barely begun to understand the cellular biology of the complex sets of effectors produced by fungi and oomycetes (BOX 2). Effectors are subject to remarkable temporal and spatial targeting during biotrophic invasion, beginning before the pathogen even enters the plant. Recent results suggest that fungi use distinct secretion systems to target effectors[34,60], as has long been known to occur for bacterial pathogens.

Live-cell imaging of individual pathogen encounter sites requires careful documentation and quantification of outcomes at individual sites, because of inherent biological variability in the interaction between the pathogen and the host (the pathogen does not always succeed even in interaction with a fully susceptible host)[54]. The addition of large fluorescent proteins to effectors may change their activities or translocation properties. In addition, the inherent limits of microscopy present

challenges to increasing our understanding of the *in planta* targeting and functions of pathogen effectors. For light and fluorescence microscopy, these include limits to resolution, photobleaching and phototoxicity. For electron microscopy, the limitations include cell structure and membrane preservation and static views (that is, an inability to view live cells). Higher resolution light microscopy[116–119] as well as TEM methods such as HPF-FS[34,39] that more effectively preserve membranes at biotrophic interfaces are beginning to be applied to define vesicular trafficking at the interface and the host compartments that are targeted by effectors. Correlative light and electron microscopy (CLEM)[120] offers the potential to obtain precise ultrastructural views of features identified through live-cell imaging. For CLEM, cellular features of interest are located in fixed tissue by fluorescence microscopy and directly sectioned for electron microscopy. New techniques are also needed to image infection sites that are deeper inside the plant tissue than epidermal cells.

The identification of virulence targets for the hundreds of candidate effectors identified by genome sequencing remains a major challenge, partly because functional redundancy seems to be common and because high-throughput cell biological assays are lacking. The establishment of reliable transformation protocols for obligate biotrophic rusts and mildews would facilitate studies of these systems, although new techniques such as host-induced gene silencing (HIGS) have aided the analysis of powdery mildew effector candidates[42].

Filamentous eukaryotic pathogens of cultivated crops remain major food security threats, and incorporation of *R* genes into these crops provides the best route for sustainable disease control. High-throughput effectoromics methods for screening the large sets of effector gene candidates available from genome sequencing are already being used to identify new *AVR* gene–*R* gene pairs that might be useful to agriculture[7,21]. Understanding *in planta* effector targeting and function is crucial if we are to design durable methods of disease control.

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