Contributions of host cellular trafficking and organization to the outcomes of plant-pathogen interactions.

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Abstract

In recent years it has become increasingly apparent that dynamic changes in protein localization, membrane trafficking pathways, and cellular organization play a major role in determining the outcome of interactions between plants and pathogenic microorganisms. Plants have evolved sophisticated perception systems to recognize the presence of potentially pathogenic microorganisms via the detection of non-self or modified-self elicitor molecules, pathogen virulence factors, or the activities of such virulence factors. Dynamic changes to host cellular organization and membrane trafficking pathways play pivotal roles in detection and signaling by plant immune receptors and are vital for the execution of spatially targeted defense responses to thwart invasion by potential pathogens. Conversely, from the perspective of the pathogen, the ability to manipulate plant cellular organization and trafficking processes to establish infection structures and deliver virulence factors is a major determinant of pathogen success. This review summarizes selected topics that illustrate how dynamic changes in host cellular trafficking and organization shape the outcomes of diverse plant-pathogen interactions and addresses ways in which our rapidly expanding knowledge of the cell biological processes that contribute to immunity or infection may influence efforts to improve plant disease resistance.

Keywords:

Plant pathogen; plant disease; vesicle trafficking; actin; haustorium; papilla.

Abbreviations: ADF, actin-depolymerizing factor; ARF-GEF, adenosine diphosphate ribosylation factor-guanine nucleotide exchange factor; BFA, Brefeldin A; BIC, biotrophic interfacial complex; EHM, extrahaustorial membrane; EIHM, extra-invasive hyphal membrane; EIX, ethylene-inducing xylanase; ER, endoplasmic reticulum; ESCRT, endosomal complex required for sorting; IH, invasive hyphae; LE, late endosome; MAMP, microbe-associated molecular pattern; MAPK, mitogen-activated protein kinase; MVB, multivesicular body; NB-LRR, nucleotide binding-leucine-rich repeat; PAMP, pathogen-associated molecular pattern; PM, plasma membrane; PRR, pattern recognition receptor; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TGN, *trans*-Golgi network; TTSS, type III secretion system;

1. Introduction

Plant antimicrobial defense responses are initiated by the activation of one or more of the multitude of immune receptors encoded within a given plant genome, which collectively constitute a surveillance system to detect and respond to invasion by microorganisms [1,2]. The appropriate positioning of immune receptors to facilitate cellular surveillance along with many of the subsequent events that follow activation of plant immune receptors are directly or indirectly controlled by fundamental cell biological processes such as membrane trafficking, regulation of membrane composition and identity, and cell polarization or dynamic changes in cellular organization. These processes impact the plant immune system at multiple levels including pathogen detection, immune receptor signaling, and the execution of antimicrobial defense responses. Increasing evidence has highlighted the importance of dynamic changes in localization for the functions of both pattern recognition receptors (PRRs) that perceive conserved pathogen elicitors as well as NB-LRR (nucleotide-binding leucine-rich repeat) immune receptors that detect pathogen effectors or their activities. Similarly, the successful execution of immune responses is highly dependent on dynamic changes in host membrane trafficking pathways and cytoskeletal organization. Multiple defense outputs of the plant immune system, including local reinforcement of the cell wall and delivery of antimicrobial metabolites, require the integration of positional information with coordinated delivery of defenses to the plant-pathogen interface at the cell surface.

On the pathogen side of the interaction, invading microorganisms often employ sophisticated strategies to parasitize plants. To be successful pathogens, microorganisms must overcome, through suppression and/or evasion, plant immune responses and successfully acquire nutrients to fuel the completion of their life cycles. Considerable evidence suggests that these

objectives are accomplished primarily through the delivery of protein, small molecule, and small RNA effectors to suppress defenses and manipulate host cellular processes to the benefit of the pathogen [3-5]. Plant pathogens use a variety of strategies to infect their hosts and many successful pathogens orchestrate elaborate manipulations of host cells to accommodate infection structures that facilitate prolonged defense evasion and effective nutrient acquisition. The formation of pathogen infection structures appears to frequently require recruitment of novel, host-derived membranes or interfaces, implying considerable manipulation of host membrane trafficking events and cellular organization by the pathogen. Thus, regulation of cellular trafficking and organization is pivotal to the outcome of a given plant-microbe interaction, either immunity or infection, and the party that successfully exerts control over these processes gains the upper hand in the struggle. The role of fundamental cell biological processes in dictating the outcome of plant-pathogen interactions is currently the subject of intensive research in several key areas. This article will provide an overview of selected topics linking cell biology and plant disease with a focus on dynamic changes in host cellular trafficking and membrane and cytoskeletal organization during compatible and incompatible interactions, including the hijacking and manipulation of host cells by pathogens. Key recent findings will be highlighted for each topic area and, where available, readers will be directed to other recent reviews providing more in-depth coverage of specific topics.

2. Building host-pathogen interfaces during invasion

Plant pathogens employ a wide variety of infection strategies to interact with and parasitize their hosts. Many plant pathogens establish intimate interactions with host cells that involve the formation of specialized infection structures. Bacterial phytopathogens often

manipulate their hosts via specialized types of protein secretion apparatus such as the well-studied type III secretion system (TTSS) employed by numerous bacterial phytopathogens to deliver effectors directly into the cytoplasm of host cells (reviewed in [6]). Viral pathogens are capable of sophisticated manipulations of host membranes and cytoskeletal components to facilitate viral proliferation through the formation of virus replication factories and to promote cell-to-cell movement by manipulation of host plasmodesmata. For a recent review article covering viral manipulation of plant cell organization, readers are directed to reference [7].

Filamentous pathogens such as fungi and oomycetes often orchestrate complex manipulations of host cellular trafficking and organization. This section will highlight recent advances in our understanding of the mechanisms by which filamentous plant pathogens manipulate host cells for accommodation of pathogenic interfaces. Intracellular filamentous pathogens, those that invade living plant cells, have been observed to form an array of specialized invasion structures that vary depending on the infection strategy, typically either movement of invasive hyphae from cell to cell or differentiation of terminal specialized hyphae to parasitize single cells. These include the accommodation of specialized invasive hyphae surrounded by host-derived membranes, the development of haustorial complexes surrounded by novel host membranes, and the construction of novel host-derived interfacial structures.

2.1 Extra-invasive hyphal membrane interfaces

Many filamentous plant pathogens exhibit an intracellular infection strategy whereby invasive hyphae (IH) interact with living plant cells for all or part of the pathogen's lifecycle and move from cell to cell. Well-studied examples of this pathogenic lifestyle include the rice blast fungus *Magnaporthe oryzae* and anthracnose fungi such as *Colletotrichum higginsianum*. *M.*

oryzae invades rice leaf tissues by breaching the cuticle and cell wall through a specialized penetration structure referred to as an appressorium. Once the fungus has defeated the physical barriers imposed by the cuticle and cell wall, it elongates hyphae within invaded cells.

Intracellular IH do not breach the plant cytoplasm, but instead are surrounded by a plant-derived membrane referred to as an extra-invasive hyphal membrane (EIHM) [8]. The EIHM appears to be tightly sealed around *M. oryzae* IH based on the observations that the lipophilic dye FM4-64 stains the EIHM but does not label fungal IH membranes and that *M. oryzae* apoplastic effector proteins outline the IH and remain within the compartment bounded by the EIHM rather than dispersing within the plant apoplast [8-11]. *M. oryzae* hyphae invade neighboring host cells at sites of pit fields, clusters of plasmodesmata that connect adjacent plant cells, and are observed to undergo remarkable constriction when passing from cell-to-cell [8]. Upon initial invasion or entry into a new cell, a membrane-rich cap is observed adjacent to the IH tip and this cap subsequently develops into a specialized interface referred to as the biotrophic interfacial complex (BIC) [8,10]. The BIC structure is labeled by host plasma membrane (PM) and endoplasmic reticulum (ER) protein markers, indicating that it is host-derived and *M. oryzae* effector proteins destined for the plant cytoplasm accumulate at the BIC, implicating this specialized structure in effector delivery [9,10]. In addition to these specialized membrane structures, a recent study illustrated the importance of host vacuolar membrane continuity for biotrophic hyphal growth and the authors speculate that *M. oryzae* may manipulate host plant cells to maintain vacuolar membrane integrity during biotrophic intracellular growth [12]. The specific sources of membrane material for the BIC and EIHM and the strategies used by the fungus to manipulate plant cells to build these elaborate accommodation structures remain intriguing mysteries.

Similar to *M. oryzae*, hemibiotrophic anthracnose fungi of the genus *Colletotrichum* initially invade living plant cells via IH during a brief biotrophic phase. *Colletotrichum* IH are also observed to be surrounded by a host-derived EIHM [13-15]. However, the EIHM surrounding *Colletotrichum* IH does not appear to be sealed in the same manner as that of *M. oryzae* based on retraction of the EIHM away from the *Colletotrichum* IH, but not the *M. oryzae* IH, upon host cell plasmolysis and on diffusion of *Colletotrichum higginsianum* apoplastic effectors beyond the EIHM into the host cell wall [8, 13, 16]. Although lacking the tight seal observed for *M. oryzae* EIHM and any apparent collar or neckband as observed to seal haustoria of biotrophic pathogens, the EIHM surrounding *Colletotrichum* IH appears to be specialized and distinct from the normal host PM as the *Arabidopsis thaliana* Penetration 1 (PEN1) PM protein is present in the invaginated host PM surrounding young primary hyphae and at the neck area of more mature IH, but significantly decreased or absent from the EIHM surrounding the apices of *C. higginsianum* IH [14,17]. In another contrast with *M. oryzae*, BICs or BIC-like compartments are not observed in association with *Colletotrichum* IH. However, fluorescent fusions of several effectors revealed the accumulation of effectors at punctate interfacial bodies in the *C. higginsianum*-*Arabidopsis* interaction and in ring-shaped accumulations at the neck of biotrophic IH in the *C. orbiculare*-cucumber interaction, indicating the existence of specialized domains or structures for effector delivery by *Colletotrichum* species [16,18].

2.2 Extra-haustorial membrane interfaces

Many biotrophic and some hemibiotrophic plant pathogens parasitize their host plants by forming specialized, terminally differentiated hyphae referred to as haustoria within host cells. Similar to IH, haustoria are surrounded by a host-derived interfacial membrane, the

extrahaustorial membrane (EHM). Haustoria are generally considered to be primary sites of effector delivery and nutrient acquisition for the pathogen and, thus, the successful manipulation of host cells to accommodate haustoria is essential for infection [19-21]. EHM are contiguous with the PM of host cells, however, these interfacial membranes have been observed to exclude proteins typically found within the normal host PM, indicating specialization of the EHM either through *de novo* biogenesis of the interface or extensive remodeling of the invaginated host PM [22]. Though the source(s) of EHM in specific plant-pathogen interactions are not fully resolved, all potential models for EHM biogenesis imply sophisticated manipulation of host cellular organization by the pathogen. A major advance toward understanding the biogenesis of EHM has been the discovery of plant proteins that localize to this specialized membrane. The first such protein was the atypical resistance protein RPW8.2 that was found to localize to the EHM in *A. thaliana* cells interacting with the powdery mildew *Golovinomyces cichoracearum* [23]. Follow-up studies have demonstrated the importance of appropriate temporal control of *Rpw8.2* gene expression for delivery to the EHM and uncovered the importance of secretory vesicles labeled by the vesicle-associated membrane proteins VAMP721 and VAMP722 in EHM trafficking of RPW8.2 [24,25]. Recent investigations of cellular dynamics during invasion of oomycete haustoria have identified additional proteins targeted to EHM, such as *A. thaliana* PDL1, a plasmodesmatal protein involved in defensive deposition of callose into haustorial encasements during interaction with the downy mildew *Hyaloperanospora arabidopsidis*, as well as the synaptotagmin SYT1 and the remorin REM1.3 that label EHM subdomains during the interaction of *Nicotiana benthamiana* with the hemibiotroph *Phytophthora infestans* [26-28]. REM1.3 was implicated as a potential susceptibility factor as overexpression of this protein enhanced susceptibility to *P. infestans* whereas silencing enhanced resistance [26]. Comparative

analyses of the localizations of a number of PM and endomembrane markers during two different plant-oomycete interactions indicated that specific manipulations of host membrane dynamics to promote EHM formation differ among diverse pathosystems, indicating that pathogens have likely evolved numerous unique strategies to co-opt host trafficking processes for infection [28]. Observations of specific endomembrane compartments in the vicinity of *P. infestans* haustoria and accumulation of late endosome marker proteins in the *P. infestans* EHM suggest that the EHM is derived, at least in part, by re-routing of plant endomembrane trafficking toward the nascent host-pathogen interface [28,29]. Future studies on cellular dynamics during haustorium formation in multiple pathosystems and further dissection of the contributions of specific trafficking pathways and compartments to this process will pave the way toward a comprehensive understanding of the manipulation of plant cellular organization by haustorium-forming pathogens and facilitate studies to elucidate the roles of specific pathogen effectors in manipulating host plant cells and identify their host targets.

3. Delivery of pathogen effectors from specialized infection interfaces

The delivery of pathogen effectors to host cells is crucial for infection and, collectively, effectors are presumed to facilitate both suppression of host defense responses and acquisition of nutrients by the pathogen. Many bacterial phytopathogens deliver batteries of effectors directly to the cytoplasm of host cells through specialized secretion systems, most notably the TTSS. The molecular components of bacterial TTSS and the structural architectures of the basal body and secretion pilus have been studied in detail and recently reviewed [6]. However, relatively less is known about manipulations to the host cell wall and PM to facilitate effector delivery through the TTSS. TTSS accessory proteins such as translocators and harpins are required for effector

translocation into host cells but dispensable for effector secretion from bacterial cells. Several such proteins required for effector translocation exhibit membrane or lipid binding activity, such as HrpN from *Erwinia amylovora*, HrpF from *Xanthomonas campestris* pv. *vesicatoria*, Hpa1 from *X. oryzae* pv. *oryzae*, HrpZ1 from *Pseudomonas syringae* (multiple pathovars), and PopA1 from *Ralstonia solanacearum* [30-34]. HrpF, HrpZ1, and PopA1 have been found to exhibit pore-forming activities in membranes, suggesting a potential role for these proteins in creating a conduit within the host PM to facilitate effector delivery [30-32, 35]. HrpZ1 interacted with the PM lipid phosphatidic acid and the presence of phosphatidic acid was required for pore-forming activity of HrpZ1 in artificial membranes [36], whereas PopA1 showed affinity for sterols and sphingolipids *in vitro* [32], suggesting that some harpins may exploit specific host membrane lipids for integration into the host PM. Harpins may also target to the host PM by exploiting specific PM proteins. In addition to lipid-binding activity, HrpZ1 from *P. syringae* pv. *phaseolicola* has also been reported to bind peptides with a specific motif as well as a protein from bean [37]. HrpN was found to interact with an apple integral membrane protein, HIPM (HrpN-interacting protein from *Malus*), and its *Arabidopsis* orthologue AtHIPM [38], while Hpa1 has been shown to interact with the *Arabidopsis* aquaporin AtPIP1;4 [39], potentially suggesting that some harpins may utilize host PM proteins for membrane recruitment. In addition to a likely role in forming pores in host membranes to facilitate effector translocation, some harpins have also been proposed to play a role in aiding the secretion pilus to traverse the plant cell wall. This notion is based on the observation that the harpins HrpW1 from *P. syringae* pv. *tomato* and HrpW from *E. amylovora* contain domains characteristic of pectate lyases, enzymes involved in the degradation of the cell wall polymer pectin [40,41]. However, the role for these harpins in aiding the pilus to traverse the host cell wall has been challenged by findings of a

detailed mutational study indicating that the *P. syringae* pv. *tomato* harpins containing pectate lyase domains, HrpW1 and HopAK1, likely function in the same effector translocation process as the HrpK1 translocator rather than a more upstream process such as cell wall loosening or modification [42]. For more in-depth reviews of bacterial type III secretion, readers are directed to references [6,43].

In contrast to bacterial pathogens, filamentous fungal and oomycete pathogens are not known to construct direct conduits to allow for effector delivery to the host cytoplasm. A major breakthrough in the study of effectors from filamentous pathogens was the discovery of the RxLR amino acid motif commonly present in oomycete effectors and similar to a motif found in effectors of the malarial parasite *Plasmodium falciparum* [44,45]. The discovery of such motifs has facilitated the cataloging of large numbers of potential effectors from numerous oomycete phytopathogens and has led to a growing body of research aimed at characterizing the mechanisms of delivery of filamentous pathogen effectors to their target host cells. Subsequent to the discovery of the conserved RxLR motif, this motif was implicated in the translocation of the *Phytophthora infestans* Avr3a effector into host plant cells [46]. In this study, mutant versions of Avr3a with an altered RxLR motif were stably expressed in *P. infestans* and were observed to be secreted from haustoria, but were unable to trigger HR on host leaves carrying the cognate *R3a* resistance gene, suggesting that the RxLR motif is required for translocation into host cells but dispensable for secretion from the oomycete haustorium [46]. These observations prompted many subsequent studies aimed at elucidating the nature of translocation of secreted RxLR effectors across the host PM. Several studies have provided evidence that oomycete effectors may be taken up by plant cells independently of any pathogen-derived factors or interface [47-50]. These studies have generally relied on two types of observations to reach the

conclusion of pathogen-independent uptake of effectors. The first is the observation of re-entry into plant cells of heterologously expressed effectors carrying a signal peptide for secretion from the plant cell. These experiments involve the observation of either effector-triggered HR or intracellular fluorescence of effector-fluorescent protein fusions as evidence for re-entry of the effector into the plant cell after secretion. The second type of experimental observations in support of pathogen-independent entry are based on delivery of purified effectors or effector-fluorescent protein derivatives into the apoplast of plant tissues or by bathing of root tissues and observation of their subsequent uptake by plant cells, using similar readouts as above. However, whether these observations are sufficient to conclude that oomycete effectors are capable of entering host cells independently of pathogen machinery remains a point of active debate. Both experimental methods have drawbacks that hinder the interpretation of results and have been challenged in recent commentaries. The cell re-entry method relies on trafficking of the heterologously expressed effector through the plant secretory system for delivery to the apoplast, where it is then available for re-entry. However, escape from the secretory system into the plant cell cytoplasm cannot be ruled out in these experiments. Similarly, the uptake of purified effectors by plant cells has been challenged by a study focused on the *P. infestans* Avr3a and *P. sojae* Avr1b effectors that reported either no uptake or no difference between uptake of RxLR leader-fluorescent protein fusions compared to the control fluorescent protein, depending on the assay and cell type utilized [51]. However, another study indicated uptake of Avr1b and other effector RxLR lead-fluorescent fusions above background, non-specific uptake of control fluorescent proteins [50]. Additionally, RxLR mutants of Avr1b that impair translocation to the host when expressed in *P. sojae* also impair entry in pathogen-independent assays, providing further support for the notion of pathogen-independent effector translocation [47,52]. Another

potential consideration that is not addressed by cell re-entry or uptake experiments is the likelihood that the membrane interfaces through which filamentous pathogen effectors are translocated differ in their protein and/or lipid composition from the typical host PM, as these experiments evaluate effector passage through the PM of host cells rather than through membranes with the composition of the EHM or EIHM. Whether a unique membrane composition at these plant-microbe interfaces influences effector translocation has not yet been addressed. In addition to pathogen-independent translocation, evidence for a potential role of phospholipid binding by effectors, in some cases mediated by the RxLR motif, in their host translocation has emerged recently [48,49]. The role, if any, of phospholipid binding in effector translocation into host cells is similarly disputed, as some studies have failed to support binding of phospholipids by the RxLR domain or specific RxLR effectors and/or have proposed that phospholipid binding may have a role within the pathogen or host cell rather than contributing to translocation [53-56]. Nonetheless, a recent study indicated that transient expression and secretion of phosphatidyl-inositol-3-phosphate-binding proteins by *Theobroma cacao* leaves reduced infection by the oomycete pathogens *P. tropicalis* and *P. palmivora*, suggesting that it may be possible to exploit lipid binding activity of filamentous pathogen effectors for novel biotechnological strategies to control diseases regardless of the specific role of such lipid-binding activity in effector delivery or function [57]. For more in-depth review and discussion of effector delivery by filamentous pathogens, readers are directed to several recent comprehensive reviews and commentaries [58-63].

4. Effector modulation of host cellular trafficking and cytoskeletal organization

Effectors delivered to plant cells by phytopathogens are presumed to carry out a wide range of functions to create an environment favorable for pathogen proliferation and reproduction. The mechanistic details of effector function in pathogenicity are only beginning to be uncovered and are the subject of considerable contemporary research efforts. Relative to the large numbers of prospective effector proteins identified from the analysis of pathogen genomes, only very few effectors have been studied in detail and, undoubtedly, our present understanding of effector function is far from comprehensive. However, a major and long-hypothesized role for effectors is clearly to thwart the activation and execution of plant antimicrobial defense responses. Here, we will focus on effectors that promote disease by interfering with plant cellular trafficking or organization.

The *Pseudomonas syringae* pv. *tomato* effector HopM1 appears to suppress plant defense responses, in part, by interfering with host endomembrane trafficking events. HopM1 was initially proposed to interfere with cell surface antibacterial immune responses associated with deposition of callose [64]. Subsequent investigations identified a number of *Arabidopsis* proteins that interacted with an N-terminal portion of HopM1 in yeast two-hybrid assays, including the adenosine diphosphate ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) AtMIN7 (*Arabidopsis thaliana* HopM interactor 7) [65]. ARF-GEFs regulate, through nucleotide exchange, ARF GTPases that are involved in transport vesicle formation, vesicle coat recruitment, and vesicle-cytoskeleton interactions [66]. Thus, ARF-GEFs are key regulators of endomembrane trafficking events. Remarkably, HopM1 was shown to promote disappearance of AtMIN7 in both yeast and plant cells that was dependent on proteasome function and associated with increased AtMIN7 ubiquitination, indicating that HopM1 promotes proteasomal degradation of the AtMIN7 ARF-GEF [65]. Both AtMIN7 and HopM1 localized to *trans*-Golgi

network (TGN) compartments in plant cells, suggesting that AtMIN7 regulates trafficking events at this subcellular compartment and implying a role for TGN trafficking in defense [67].

Additionally, HopM1-mediated destruction of AtMIN7 was found to interfere with both pattern-triggered and effector-triggered immune responses and *P. syringae*-induced degradation of AtMIN7 was blocked by activation of effector-triggered immunity, suggesting a central role for AtMIN7-associated trafficking events in antibacterial defense [67].

In another example of effector interference with host vesicle trafficking functions, the *Phytophthora infestans* RxLR effector Avr1 was found to interact with the potato Sec5 protein, a component of the multi-protein exocyst complex involved in vesicle tethering prior to their fusion with target membranes [68,69]. Silencing of Sec5 in *Nicotiana benthamiana* leaves impaired defenses against *P. infestans*, disrupted secretion of the pathogenesis-related PR1 protein, and reduced callose deposition elicited by inoculation with non-virulent *Pseudomonas syringae* mutant bacteria, suggesting a role for Sec5 in defense-associated trafficking events. Additional support of a role for the exocyst complex in plant antimicrobial immune responses comes from a number of recent mutant studies implicating other exocyst complex subunits in defense as well as a recent report indicating that loss-of-function mutants of the *Arabidopsis* exocyst *Exo70B1* subunit exhibit defense activation that is dependent on an atypical TIR-NB (Toll-Interleukin1 receptor-nucleotide binding) immune receptor, suggesting a model whereby exocyst complex function may be guarded by plant immune receptors [70-72]. In contrast to HopM1 destabilization of AtMIN7, Avr1 appears to stabilize Sec5 in plant cells, though it is not yet clear how this activity may interfere with exocyst complex function or vesicle targeting [68].

In addition to interfering with regulators and executors of host vesicle trafficking, pathogen effectors have also been implicated in disrupting host cytoskeleton function. Recent

evidence indicates that numerous phytopathogen effectors target the plant microtubule network to interfere with host defense responses. The *Pseudomonas syringae* effector HopZ1a possesses acetyltransferase activity and was found to bind to and acetylate plant tubulins and to alter microtubule networks by decreasing microtubule density [73]. Pharmacological disruption of microtubules increased the multiplication of *P. syringae* pv. *tomato*, consistent with a positive contribution of the microfilament network to antimicrobial defenses, and HopZ1a was found to interfere with secretion and to block callose deposition induced by the bacterial flagellin-derived flg22 peptide [73]. Whether HopZ1a disrupts microtubule networks via direct acetylation of tubulin or by acting on another host protein remains unclear. Interestingly, another acetyltransferase effector, AvrBst from *Xanthomonas campestris*, was recently reported to interact with and acetylate an *Arabidopsis* protein, ACIP1 (acetylated interacting protein 1), that contributes to antimicrobial immunity and shows partial co-localization with microtubules [74]. Whether AvrBst similarly contributes to pathogen virulence by disrupting the microtubule cytoskeleton remains to be determined. Another *P. syringae* effector, HopE1, has also recently been found to target microtubules. This effector interacts with host calmodulin and, subsequently, the microtubule-associated protein MAP65, causing dissociation of MAP65 from microtubules [75]. MAP65 contributes to antimicrobial defense and, similar to HopZ1, HopE1 interferes with defense-associated secretion [75]. The actin cytoskeleton of plant cells has also recently been implicated as a target of a *P. syringae* effector. HopW1 from *P. syringae* pv. *maculicola* was found to co-immunoprecipitate actin when expressed transiently in *N. benthamiana* [76]. When delivered by *P. syringae* pv. *tomato* DC3000, HopW1 increased disruption of the actin cytoskeleton during infection and promoted increased bacterial multiplication on *Arabidopsis*. Transient expression of HopW1 alone in *N. benthamiana* leaves

was sufficient to cause actin filament disruption and inducible expression in *Arabidopsis* altered actin-dependent host protein targeting and disrupted endocytosis. Taken together, these recent findings indicate that disruption of host vesicle trafficking and interference with host cytoskeleton function may be common functions of pathogen effectors to block plant defenses and promote infection. The ongoing intensive focus on pathogen effector function will likely uncover additional strategies used by phytopathogens to target these processes and may provide new insights into plant vesicle trafficking regulation and cytoskeletal organization and function [77,78].

5. Dynamic host processes in perception of pathogens by plant immune receptors

To protect themselves from pathogenic microorganisms, plants have evolved an immune system that relies on immune receptors to detect the presence of microbial invaders and initiate signaling to activate appropriate defense responses. Plant immune receptors generally perceive two categories of pathogen-derived signals to activate defense responses, either conserved elicitor molecules characteristic of potentially pathogenic microorganisms termed pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) or pathogen virulence factors (effectors) either directly or indirectly through the consequences of their virulence activities [2]. Perception of PAMPs typically occurs at the cell surface whereas perception of virulence effectors typically occurs within the cell, thus, delivery and placement of immune receptors in appropriate locations at appropriate times within plant cells is crucial for immune system function. Additionally, recent evidence indicates that dynamic localization of immune receptors plays an important role in both activation and attenuation of plant immune responses.

5.1 Pattern recognition receptor dynamics

The *A. thaliana* PRR Flagellin-Sensing 2 (FLS2) is a receptor-like kinase that detects bacterial flagellin, via a conserved domain represented by the flg22 peptide, to activate plant pattern-triggered immune responses [79,80]. FLS2 is constitutively present within the plasma membrane at the surface of *A. thaliana* cells and undergoes continuous cycling between the cell surface and endocytic compartments, a process that is disrupted by the trafficking inhibitor Brefeldin A (BFA) [81]. In addition to continuous recycling of the receptor through endomembrane compartments, FLS2 also undergoes distinct ligand-stimulated endocytosis whereby the receptor is internalized into endomembrane compartments and depleted from the PM upon treatment with flg22 [82]. Upon removal of the flg22 stimulus, FLS2 signal recovers at the PM, though recovery is blocked by the protein biosynthesis inhibitor cycloheximide, indicating that replenishment of FLS2 in the PM is accomplished via *de novo* synthesis and delivery of new receptor and implying that endocytosed activated FLS2 is degraded rather than recycled back to the cell surface [82]. Ligand-induced endocytosis of FLS2 is insensitive to BFA, indicating that continuous endocytic cycling and ligand-induced endocytosis of FLS2 occur through distinct trafficking processes. Detailed studies on the trafficking of FLS2 in *A. thaliana* and *N. benthamiana* after ligand stimulation have revealed that FLS2 initially traffics to a compartment labeled by both the SYP61 TGN marker and the RabF2b (aka ARA7) multivesicular body (MVB)/late endosome (LE) marker, indicating a unique compartment that may be an intermediate between the TGN and LE [83]. At later timepoints after elicitation, FLS2 is primarily observed at LE compartments labeled by RABF2b and RABF1 (aka ARA6), suggesting progression of the activated receptor from a TGN-related compartment to the LE and, presumably, subsequent delivery to the vacuole [81,83]. Several components regulating

endomembrane trafficking have been implicated in trafficking of activated FLS2 through the endomembrane system. These include the RAB GTPase RABA6a that appears to contribute to sorting or delivery of FLS2 into RABF2b-positive LE compartments from SYP61/RABF2b-labeled TGN compartments and RABA4c that may be involved in delivery of FLS2 to TGN compartments [83]. Additionally, loss of RABF2b function blocked FLS2 endocytosis [81]. The endosomal complex required for sorting (ESCRT) subunits VPS37-1 and VPS28-2 were required for normal levels of FLS2 endocytosis and *vps37-1* mutants exhibited reduced FLS2 accumulation within the lumen of MVBs, suggesting a role for the ESCRT complex in sorting of activated FLS2 into the MVB lumen [84]. Recent studies in both *A. thaliana* and *N. benthamiana* have also implicated dynamin-related proteins (DRPs) in ligand-induced FLS2 endocytosis, suggesting that this process occurs, at least in part, by clathrin-mediated endocytosis [85,86]. The extent to which FLS2 signals to activate immune responses from endosomal compartments, as opposed to the PM, remains unclear. The available evidence at present suggests that the majority of FLS2 immune signaling occurs from the PM, however, there are indications that signaling events leading to specific responses may be initiated from endosomal compartments after FLS2 endocytosis. In the *Arabidopsis drp2b* mutant that is impaired in FLS2 endocytosis, activation of reactive oxygen production, MAPK signaling, and callose deposition remain intact, however, upregulation of the *PR1* (*Pathogenesis Related 1*) gene is impaired [86]. Similarly reactive oxygen production, MAPK signaling, and callose deposition remained intact in the *vps37-1* ESCRT complex subunit mutant, but flg22-induced closure of stomata was impaired, providing further support for a requirement for FLS2 endocytic trafficking in the activation of some immune response outputs [84]. Although detailed cell biological studies have not been performed for most identified plant PRRs, another example of a plant PRR that undergoes

ligand-induced endocytosis is the tomato receptor LeEIX2 that perceives the ethylene-inducing xylanase (EIX) from the fungus *Trichoderma viride*. LeEIX2, a receptor-like protein that lacks an intracellular kinase domain, undergoes endocytic trafficking to a late endocytic compartment upon detection of EIX [87]. In contrast to FLS2, impairment of LeEIX2 endocytosis disrupts all known signaling outputs from this PRR, highlighting the need for additional cell biological interrogations of PRR trafficking and signaling to determine whether the trafficking events occurring after FLS2 activation are generalizable to other PRRs [88]. For in-depth review of endocytic trafficking of PRRs, readers are directed to references [89,90].

5.2 NB-LRR immune receptor dynamics

NB-LRR proteins represent a second major class of plant immune receptors. While PRRs typically function at the cell surface to detect pathogen signals in the extracellular space, NB-LRRs generally detect pathogen virulence effectors, either directly or indirectly through consequences of effector virulence function. As most effectors are delivered to host cells, NB-LRR receptors function at various intracellular locations and research into the mechanisms of effector detection and subsequent signaling by NB-LRRs has highlighted the importance of dynamic processes in NB-LRR function and defense activation. A growing number of NB-LRR immune receptors have been reported to localize to both the cytoplasm and nucleus [91-99]. Interestingly, recent evidence suggests that activated NB-LRRs may engage distinct signaling components in the nucleus and cytoplasm to activate specific defense outputs. In the case of the barley MLA10 NB-LRR that confers resistance to powdery mildew, exclusion of the protein from the nucleus by addition of a nuclear export signal resulted in a failure to reduce pathogen growth but retention of the ability to elicit cell death [91,96]. In contrast, adding a nuclear

localization signal to MLA10 abrogated the ability of MLA10 to trigger cell death but did not impair effective restriction of pathogen growth [91]. A similar scenario has been reported for the *Arabidopsis* RPS4 protein that, in concert with another NB-LRR, RRS1, detects AvrRps4 from *Pseudomonas syringae*, PopP2 from *Ralstonia solanacearum*, and an unknown effector from the fungus *C. higginsianum* [94,100,101]. Sequestration of AvrRps4 in the nucleus blocked RPS4-mediated cell death, but not restriction of bacterial multiplication whereas exclusion of AvrRps4 from the nucleus compromised effective antibacterial defense but only moderately impacted cell death [102]. Intriguingly, the RPS4 partner NB-LRR RRS1 features a C-terminal WRKY DNA binding domain that is characteristic of plant WRKY transcription factors involved in pathogen defense [103]. Recent results strongly support an “integrated decoy” model whereby the RRS1 WRKY domain serves as a decoy to detect pathogen effectors that target WRKY transcription factors and activates defense signaling in concert with RPS4 [104,105]. While many NB-LRRs appear to accumulate in the nucleus, it is clear that nuclear localization is not necessarily required for NB-LRR signaling as NB-LRRs have been reported to localize to a variety of subcellular compartments including the PM, Golgi and endosomal compartments, and the tonoplast and nuclear localization does not appear to play a role in signaling from a number of these immune receptors [106-110]. Understanding how signaling from NB-LRR immune receptors located at diverse subcellular compartments is integrated to activate specific defense responses remains a considerable challenge for future research in this area. For a more in-depth review on NB-LRR localization and function, readers are directed to reference [111]

6. Impacts of host cellular trafficking and membrane organization on defense outputs

Our understanding of the defensive outputs of the plant immune system that effectively limit the growth and multiplication of microbial pathogens remains limited. A major aspect of the plant immune response that has received considerable attention is local reinforcement of the plant cell wall through the recruitment of physical and chemical defenses to the host-pathogen interface. An overview of our present understanding of the dynamic processes and cellular trafficking pathways contributing to focal immune responses is presented in Fig. 1. Forward genetic screens and follow-up investigations have revealed molecular components that contribute to the recruitment of local defenses at the cell surface. In *Arabidopsis*, PEN1, PEN2, and PEN3 contribute to penetration resistance against powdery mildew fungi. The *PEN1* gene encodes a SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) involved in vesicle fusion events that localizes to the PM [112-114]. Upon attempted penetration by nonadapted powdery mildews, PEN1 accumulates at the penetration site and this accumulation has been shown to occur outside the cell, within cell wall reinforcing papillae [112,115]. PEN3, a PM-localized ABC (ATP-binding cassette) transporter, and PEN2, an atypical myrosinase, define an additional, distinct defense pathway contributing to penetration resistance [116,117]. PEN2 is reported to localize to peroxisomes and mitochondria, both of which are observed to accumulate near powdery mildew penetration sites [22,116,118]. However, it appears that the mitochondrial pool of PEN2 is sufficient for its function in penetration defense [118]. PEN2 cleaves an indolic glucosinolate secondary metabolite and this pathway is hypothesized to generate a toxic defensive metabolite [119,120]. Furthermore, the PEN3 transporter is proposed to transport the product of the PEN2 pathway, as well as potentially other defensive metabolites, at the host-pathogen interface to impair fungal ingress. Like PEN1, PEN3 accumulates at powdery mildew penetration sites within papillae in the extracellular space [117,121].

Studies on the trafficking of PEN1 and PEN3 upon defense activation have provided new insights into regulation of cellular trafficking during the plant immune response. Recruitment of PEN1 to penetration sites is blocked by BFA and dependent on the function of the ARF-GEF GNOM [122]. Additionally, PEN1 undergoes continuous cycling between the PM and TGN and *de novo* synthesis of PEN1 is not required for its accumulation at penetration sites, suggesting that the recycling pool of PEN1 may be diverted to the host-pathogen interface upon pathogen attack [123,124]. The observation that TGN SNAREs of the SYP4 family contribute to defense against powdery mildew invasion supports a role for TGN trafficking in this defense response [125]. Additionally, the TGN protein KEG (Keep On Going) is required for export of the defense-related proteins C14 and PR1 and KEG is degraded upon infection by an adapted powdery mildew, suggesting that pathogens may target TGN trafficking to interfere with defenses [126].

In contrast to PEN1, delivery of PEN3 to penetration sites is insensitive to BFA, but sensitive to inhibitors of the actin cytoskeleton, suggesting that PEN1 and PEN3 are recruited to the host-pathogen interface through distinct trafficking mechanisms [121]. However, similar to PEN1, *de novo* synthesis of PEN3 is not required for accumulation at penetration sites, indicating that existing PEN3 is also diverted to the host-pathogen interface. Focal accumulation of both PEN1 and PEN3 is induced by perception of PAMPs such as flg22 and chitin, a PAMP from fungal cell walls, suggesting that targeted delivery of defenses is a component of the pattern-triggered immune response and implying that pattern recognition receptors can convey spatial information to initiate defense recruitment to sites of pathogen detection at the cell surface [121]. The appearance of PEN1 and PEN3, along with FM4-64-labelled membrane material, outside the cell within papillae has prompted the development of a model whereby defense components are

delivered to the host-pathogen interface through fusion of MVBs with the PM to release extracellular vesicles, or exosomes [127]. Recent observations indicated an increase in the numbers of MVBs and extracellular paramural membrane vesicles during infection by *P. syringae*, suggesting a potential role for MVB-related trafficking in defense against other pathogens [128]. Although definitive evidence that PEN1, PEN3, or other defense components are delivered to the host-pathogen interface via MVBs is still lacking, the observations that MVBs accumulate and potentially fuse with the PM at sites of papilla deposition provide compelling support for this model [129-131]. For a detailed review of host membrane trafficking in plant-microbe interactions, readers are directed to reference [132].

Given the importance of dynamic changes in cellular trafficking and organization for plant immune responses, cytoskeletal organization is expected to play a fundamental role in plant immunity. Indeed, the role of the actin cytoskeleton in defense against pathogens that directly penetrate the cell wall has received considerable attention [133]. The contribution of the actin cytoskeleton, and specifically of actin-associated myosin motors, to the execution of antimicrobial immune responses at the plant cell surface was recently addressed in a study that uncovered a key role for *Arabidopsis* myosins XI in these responses [134]. Pharmacological perturbation of myosins and genetic analysis of myosin gene families indicated that myosins XI contribute to organellar movements and endocytic cycling involved in penetration defense and reinforced the notion that PEN1 delivery to pathogen penetration sites is dependent on endomembrane cycling of existing protein [134]. Additionally, recent evidence has expanded our knowledge of the role of the actin cytoskeleton in plant immunity to include contributions to both pattern-triggered and effector-triggered plant immune responses and signal transduction. *Arabidopsis* Actin-Depolymerizing Factor 4 (ADF4), a protein that regulates actin cytoskeleton

dynamics by severing filamentous actin, was found to be required for effector-triggered immune responses initiated by perception of the *Pseudomonas syringae* effector AvrPphB [135]. Interestingly, ADF4 was not required for responses triggered by two other *P. syringae* effectors, AvrB and AvrRpt2, and loss of ADF4 function disrupted hypersensitive response programmed cell death, but did not block the restriction of bacterial multiplication upon perception of AvrPphB. *Arabidopsis adf4* mutants accumulated lower levels of RPS5, the cognate resistance protein mediating AvrPphB recognition, and were also impaired in pattern-triggered immune signaling responses including flg22-induced activation of MAP kinases and induction of the *FRK1* (*flagellin-induced receptor kinase*) gene, implicating actin dynamics in signaling during the pattern-triggered immune response [136]. ADFs have also been shown to play a role in the interaction of wheat (*Triticum aestivum*) with the stripe rust fungus *Puccinia striiformis* f. sp. *tritici*. In this pathosystem, both positive and negative impacts on defense have been attributed to specific ADF family members in wheat, with TaADF3 negatively regulating penetration resistance against this fungus and TaADF7 positively regulating ROS production and HR cell death [137,138]. In accordance with a role for the actin cytoskeleton in pattern-triggered immune responses, actin filament density was found to increase rapidly in response to several PAMPs including flg22, elf86 (derived from bacterial elongation factor Tu) and chitin [139,140]. In addition to changes in filament abundance, PAMP perception alters the dynamic behavior of actin arrays and individual filaments [141]. Regulation of actin dynamics upon PAMP detection appears to involve inhibition of the actin regulatory capping protein as *Arabidopsis cpb-1* mutants phenocopy PAMP effects on actin dynamics and do not exhibit further changes in actin dynamics or organization in response to PAMPs [141]. For a comprehensive discussion of the role of actin in plant-microbe interactions, readers are directed to reference [142]. Continued

investigations into the PAMP responses and signaling pathways affected by cytoskeleton remodeling should complement other cell biological studies to illuminate specific mechanisms and cellular compartments engaged during the plant pattern-triggered immune response.

7. Concluding remarks and future perspectives

The impacts of dynamic cellular processes including endomembrane trafficking, cytoskeletal reorganization, and cell polarization on the struggle between plants and their pathogenic microbes have become more apparent over the last decade. An emerging theme is the existence of a complex power struggle between plant cells and invading pathogens whereby plant cells attempt to maintain control of cellular trafficking pathways and divert them toward defensive processes and, in contrast, pathogens attempt to hijack plant cellular trafficking to promote parasitism and evade defense responses. Many key questions remain to be investigated. How is spatial targeting of defenses at the cell surface achieved? What are the rate limiting steps governing local delivery of defenses? How do PRRs coordinate cytoskeletal reorganization and redirection of endomembrane trafficking pathways for defensive purposes? What cargos are delivered to the host-pathogen interface? How do pathogens orchestrate the construction of elaborate infection structures? Given the central role of specific plant trafficking pathways in defense and disease, it is likely that these pathways are repeatedly targeted by diverse pathogens to promote infection. Indeed, recent evidence suggests that effectors from diverse pathogens likely converge on a limited number of host targets [143,144]. The centrality of cellular trafficking pathways to effective defense suggests that these pathways are likely to be monitored for integrity, potentially at many points, through guarding by resistance proteins. Continued research to define mechanisms by which pathogens interfere with cellular trafficking in the

elaboration of defenses may lead to promising new strategies to tip the balance in favor of plant cells maintaining control over their cellular trafficking processes, and thus, more effectively resisting infection. Such discoveries should play a key role in future efforts to improve disease tolerance in crop plants and concomitantly increase agricultural productivity.

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Figure Legend

Figure 1. Simplified model of dynamic cellular processes during the plant pattern-triggered immune response. The left panel illustrates steady-state cells where defenses have not been activated. PRRs and defense-associated proteins such as PEN1 and PEN3 continuously cycle from the cell surface to the TGN and back. MVBs mature from the TGN and deliver cargo to the vacuole for degradation. Actin cytoskeleton dynamics are modulated by the activities of ADFs and CP. The right panel illustrates changes in cellular dynamics upon pathogen detection through PAMP perception. Activated PRRs are endocytosed and trafficked to the vacuole through unique TGN/LE and MVB compartments. Endomembrane cycling of PEN1 and PEN3 is diverted toward the site of pathogen detection at the cell surface that is presumed to be marked by an as yet unknown spatial targeting signal. The divergence of endomembrane trafficking pathways during the pattern-triggered immune response highlights the presumed importance of as yet unknown cargo sorting processes at the TGN and MVBs. PEN1, PEN3, and other defense components are hypothesized to be exported in the nascent papilla on exosomes derived from fusion of MVBs with the PM. Changes in actin filament density and dynamics occur due to inhibition of ADFs and CP. Abbreviations: ADF – Actin Depolymerizing Factor; CP – Capping Protein; MVB – Multivesicular Body; LE – Late Endosome; PAMP – Pathogen-Associated Molecular Pattern; PRR – Pattern Recognition Receptor.

Figure