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Membrane Trafficking in Plant Immunity

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Abstract

Plants employ sophisticated mechanisms to interact with pathogenic as well as beneficial microbes. Of those, membrane trafficking is key in establishing the rapid and precise response. Upon interaction with pathogenic microbes, surface-localized immune receptors undergo endocytosis for signal transduction and activity regulation while cell wall components, antimicrobial compounds, and defense proteins are delivered to pathogen invasion sites through polarized secretion. To sustain mutualistic associations, host cells also reprogram the membrane trafficking system to accommodate invasive structures of symbiotic microbes. Here, we provide analysis of recent advances in understanding the roles of secretory and endocytic membrane trafficking pathways in the plant immune activation. We also discuss strategies deployed by adapted microbes to manipulate these pathways to subvert or inhibit plant defense.

INTRODUCTION

Being sessile organisms, plants are constantly exposed to environmental challenges, including a great number of potential pathogenic microbes. Remarkably, plant disease is the exception rather than the rule. The cuticle layer, the plant cell wall, and the secreted antimicrobial compounds provide effective barriers on the plant surface to prevent penetration and growth of most pathogens. Even after these pre-formed barriers are breached, pathogens will face multiple layers of innate immune responses. The first layer of plant innate immunity employs plasma membrane (PM)-bound receptor-like kinases (RLKs) or receptor-like proteins (RLPs), collectively termed pattern recognition receptors (PRRs). The extracellular domains of PRRs detect microbe-associated molecular patterns (MAMPs) which can be evolutionarily conserved (e.g. flagellin and chitin) or species-specific (e.g. ethylene-inducing-xylanase produced by saprophytic ascomycete *Trichoderma viride*) (Boller and Felix, 2009). After MAMP perception, the intracellular domain of the PRR initiates signal transduction and activates pattern-triggered immunity (PTI). PTI consists of a complex set of cellular responses, including a burst of small signaling molecules, such as

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Ca^{2+} and reactive oxygen/nitrogen species (ROS/RNS), and activation of signaling cascades mediated by mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs). These responses regulate the activities of a large spectrum of protein targets and promote downstream production of defense proteins, phytohormones, and secondary metabolites to confer resistance to a broad spectrum of pathogens (Bigeard et al., 2015). PTI together with the pre-formed barriers comprise the so-called “basal resistance” of a plant species to nonadapted pathogens. However, adapted pathogens evolved a myriad of effector proteins that are released into the intercellular space or injected into the cytoplasm of host cells and cause effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Basal resistance of susceptible hosts infected by effectors-carrying pathogens is substantially compromised by ETS. Pathogen effectors, in turn, have driven the evolution of the second layer of the innate immunity in plants, termed effector-triggered immunity (ETI). ETI employs intracellular nucleotide-binding domain, leucine-rich repeat (NLR) receptors to detect the activities of specific pathogen effectors at various subcellular locations (Cui et al., 2015; Jones et al., 2016). ETI triggers a strong and robust defense response, manifested in a dramatic transcriptome reprogramming, which often leads to programmed cell death (PCD) in the infected tissue. In the non-infected distal tissues, synthesis of the phytohormone salicylic acid (SA) triggers enhanced broad-spectrum resistance to secondary infection, an immune mechanism known as systemic acquired resistance (SAR) (Durrant and Dong, 2004). PTI and ETI bear significant differences in the activation mechanism (Gu et al., 2016; Tsuda et al., 2009; Wang et al., 2014b); however, there is a continuum in their defense outcome and the two immune responses share part of the signaling network (Tao et al., 2003; Thomma et al., 2011).

Targeted protein transport and coordinated membrane dynamics are involved in almost every key step of these plant immune mechanisms. For instance, ligand-bound PRRs are incorporated into endosomes during PTI for activity regulation (Ben Khaled et al., 2015); intracellular membrane vesicles undergo directional movement during basal defense for targeted delivery of defense proteins and compounds to pathogen invasion sites (Bednarek et al., 2010); and chloroplasts extend their membranes to form stromule structures for proximate release of ETI signals and pro-defense molecules to the nucleus (Caplan et al., 2015; Gu and Dong, 2015). These trafficking events are mediated by a large group of regulatory proteins whose function determines the selectivity, directionality, speed and intensity of the immune responses. Some of the membrane trafficking regulators themselves are also subject to transcriptional/posttranslational regulation and significant activity changes in response to multiple forms of immune signals (Ben Khaled et al., 2015; Wang et al., 2005). The central importance of membrane trafficking in immunity is highlighted by the fact that it is a favorite target of pathogen effectors. Manipulations of critical trafficking regulators have been shown to be the key for successful pathogen infection in many cases (Ben Khaled et al., 2015; Toruno et al., 2016). Here, we review the fundamental role of two major membrane trafficking pathways, the secretory and the endocytic pathways, in the regulation and execution of plant immunity.

MEMBRANE TRAFFICKING IN PLANTS

Plants contain two major classes of membrane trafficking pathways: (1) the secretory pathway that transports newly synthesized proteins from the endoplasmic reticulum (ER) to the PM or the extracellular space, and (2) the endocytic pathway that recycles surface localized proteins between the PM and early endosomes, as well as transports internalized cargo to the vacuole through late endosomes. The membrane trafficking process in both pathways involves budding of vesicles from the donor membrane, and subsequent transport, tethering and fusion of vesicles to the target membrane (Inada and Ueda, 2014). Each of these steps is coordinated by a specific set of regulatory machinery. First, the donor membrane is deformed by ADP ribosylation factors (ARFs) or coat protein complexes (CPCs), such as COPI, COPII, and clathrin. Second, budded membrane is wrapped around at the neck by the large GTPase Dynamin-related proteins (DRPs) that catalyze membrane scission and generate transport vesicles. Third, newly formed transport vesicles undergo targeted transport, tethering, and docking to the target membrane. This step is mainly regulated by Rab proteins. Activated Rab proteins associate with different types of transport vesicles and interact with tethering factors present on target membranes to mediate specific vesicle tethering (Rutherford and Moore, 2002). For example, tethering of late endosome to the vacuole depends on the interaction between Rab7 present on the late endosomes and tethering complex Homotypic Fusion and Protein Sorting (HOPS) present on the vacuole and pre-vacuolar compartments (Niihama et al., 2009; Rojo et al., 2001; Rojo et al., 2003; Seals et al., 2000). Finally, soluble N-ethylmaleimide-sensitive-factor attachment protein receptors (SNAREs) mediate the membrane fusion between tethered vesicles and target membranes. The framework and principal components of membrane trafficking pathways are conserved among eukaryotic lineages and are suggested to have been established before the last common eukaryotic ancestor (Dacks et al., 2009). However, Rab GTPases, tethering factors, and SNARE subfamilies are all remarkably expanded in plants, suggesting functional diversification (Fujimoto and Ueda, 2012).

ROLES OF THE SECRETORY PATHWAY IN IMMUNITY

The secretory pathway is an integral component of the plant immune mechanism. It contributes to basal resistance by transporting PRRs to the PM, delivering defense proteins and metabolites with antimicrobial activities to the extracellular space, and mediating focal deposition of callose to produce localized cell wall thickening at pathogen invasion sites (Wang et al., 2016). It also plays an essential role during the establishment of SAR. SA and Non-expresser of pathogenesis-related genes 1 (NPR1)-mediated induction of defense genes is coordinated with the upregulation of both the ER-associated protein folding/modification machinery and the secretory pathway (Wang et al., 2005). Such a mechanism ensures proper modifications and timely export of ER-accumulated defense proteins to the extracellular space. In fact, *Arabidopsis* Pathogenesis-related Protein 2 (PR2) is completely retained in the ER when over-expressed in healthy plants, but is exported into the apoplast upon SA treatment or pathogen infection (Zavaliev et al., 2013). On the other hand, many adapted pathogenic as well as symbiotic microbes evolved the ability to rewire the host secretory pathway as an effective strategy for successful invasion (Toruno et al., 2016), underlining the essential role the pathway in immune activation.

I. Vehicles and tracks for secretory trafficking during defense

Although secretion is clearly required for defense activation, we have little understanding of the nature of vehicles that carry the defense cargo and how they achieve targeted secretion. Here we provide evidence for functional links between defense-related secretory trafficking and three types of membrane vesicles, the *trans*-Golgi network/early endosome (TGN/EE), the multivesicular body (MVB), and the exocyst positive organelle (EXPO) (Figure 1).

i. Trans-Golgi network/early endosome—In the canonical secretory pathway, properly folded proteins are transported from the ER to Golgi and subsequently to TGN/EE vesicles which then fuse with the PM to deliver the soluble cargo to the extracellular space or the membrane cargo to the PM. In plants, the TGN/EE serves as a key sorting station at the intersection of secretory and endocytic pathways (Dettmer et al., 2006; Viotti et al., 2010). The E3 ubiquitin ligase Keep On Going (KEG) localizes to the TGN/EE and is essential for TGN/EE-mediated extracellular sorting of Pathogenesis-Related protein 1 (PR1) and papain-like Cys protease C14. Loss of KEG leads to vacuolar, rather than apoplastic, accumulation of these antimicrobial proteins (Gu and Innes, 2012). An adapted powdery mildew pathogen specifically degrades KEG during infection, presumably to disturb TGN/EE-mediated secretion of defense molecules (Gu and Innes, 2012). The *Arabidopsis* Resistance to Powdery Mildew 8.2 (RPW8.2) is a key protein in resistance against non-adapted powdery mildew fungal pathogens and is specifically targeted to the extrahaustorial membrane (EHM), the part of host PM that encases the haustorium, the fungal feeding structure (Wang et al., 2009). It has been shown that targeted delivery of RPW8.2 to EHM is mediated by TGN/EEs and requires the TGN/EE-resident SNARE protein VAMP721/722 (Asaoka et al., 2013; Kim et al., 2014). These findings suggest that the TGN/EE plays a critical role in sorting and delivering of PM-localized and extracellular defense proteins during immune activation.

ii. Multivesicular body—MVBs are considered as late endosomes in plants. The limiting membrane of MVB invaginates to form vesicles within its lumen. These intraluminal vesicles can be delivered to the extracellular space as defense cargo-containing exosomes upon fusion with the PM (Contento and Bassham, 2012). MVBs have been suggested as carriers of defense molecules and responsible for exosome accumulation during fungal invasion in barley based on electron microscopy observations (An et al., 2006a; An et al., 2006b). This hypothesis is supported by the recent finding that infection in *Arabidopsis* by the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) promotes biogenesis of MVBs and exosome-like paramural vesicles (Wang et al., 2014a). This process requires MAPK (MPK3/6)-dependent phosphorylation of LYST-Interacting Protein 5 (LIP5), a positive regulator of MVB biogenesis. A loss of LIP5 function largely compromised basal resistance to *Pst* DC3000, suggesting an essential role of induced-MVB biogenesis and exosome accumulation in basal resistance (Wang et al., 2014a). The Thordal-Christensen group demonstrated in barley that MVBs are trafficked to the penetration site prior to the formation of the cell wall apposition called papilla during infection by the barley powdery mildew pathogen *Blumeria graminis*. They further showed that the MVB-resident ARF GTPase, ARFA1b/1c, is critical for callose deposition and penetration resistance (Bohlenius et al., 2010). Callose deposition is mediated by a callose

synthase that is encoded by *Powdery Mildew Resistance 4* (*PMR4*). PMR4 localizes to the PM and intracellular vesicles (Ellinger et al., 2013; Vaten et al., 2011; Xie et al., 2012), but redistributes to attempted penetration sites and EHM during fungal infection in *Arabidopsis* (Meyer et al., 2009). The involvement of MVBs in callose deposition indicates that PMR4 redistribution may occur through MVB-mediated exocytosis that targets fungal invasion sites. Consistently, a recent study in *Nicotiana benthamiana* showed that during infection by the oomycete pathogen *Phytophthora infestans,* which causes late blight, trafficking of late endosomes is diverted toward the EHM, where some immune-related late endosome cargo, such as RLKs, accumulate (Bozkurt et al., 2015).

iii. Exocyst positive organelle—Another type of vesicles that is likely to carry defense cargo and contributes to the secretory defense is the double-membrane organelle, EXPO, which is uniquely labeled by components of the exocyst complex, including Exo70A1, Exo70B1 and Exo70E2 (Wang et al., 2010). The exocyst complex is required for the tethering of secretory vesicles to the PM at the early stage of exocytosis (Hsu et al., 2004). EXPOs are distinct from TGN/EEs and MVBs, and mediate nonclassical protein secretion (Wang et al., 2010). Exo70B1 and Exo70B2 have been reported to be necessary for PTI induction and resistance against various pathogens in *Arabidopsis* (Pecenkova et al., 2011; Stegmann et al., 2012; Stegmann et al., 2013). The barley exocyst component HvExo70F-like was also reported to be critical for penetration resistance against *B. graminis* (Ostertag et al., 2013). Intriguingly, Exo70B1 was recently found to be guarded by the NLR receptor, TN2, and a loss of Exo70B1 function activates TN2-mediated ETI in *Arabidopsis*. This finding suggests that adapted pathogens may have evolved effectors to target the plant exocyst complex to suppress basal defense (Zhao et al., 2015). Indeed, the RXLR-effector AVR1 produced by *P. infestans* was recently shown to target the potato exocyst component Sec5. Silencing of *Sec5* homologs in *N. benthamiana* disrupted SA-induced PR-1 secretion and pathogen-induced callose deposition (Du et al., 2015).

Both MVBs and EXPOs have been proposed to fuse with the PM and release exosomes to the extracellular space. Plant exosomes were recently purified from the apoplastic fluid of *Arabidopsis* leaves and their contents were highly enriched with proteins functioning in abiotic and biotic stress responses (Rutter and Innes, 2017). It was proposed that these defense molecules discharge when the exosome membrane bursts (Wang et al., 2010); but how this process is regulated in plants is still unclear.

iv. Actin cytoskeleton—The cytoskeleton provides tracks for the transport of secretory vesicles, and is a key factor in regulating directional secretion. In plants, movement of membrane organelles relies heavily on the dynamic actin network and the actin motor protein myosin XI (Cai et al., 2014). Rapid rearrangement of actin cytoskeleton toward pathogen penetration sites is one of the earliest cellular responses during defense activation (Day et al., 2011). The Staiger group showed that inhibition of the actin depolymerization factor and the capping protein is key for PAMP-triggered actin remodeling in *Arabidopsis* during PTI (Henty-Ridilla et al., 2014; Li et al., 2015). Meanwhile, the Wei group showed that simultaneous knocking out four myosin XI homologs in *Arabidopsis* prevents actin reorganization and polarized delivery of membrane vesicles and defense compounds, leading

to impaired penetration resistance and enhanced susceptibility to both adapted and nonadapted fungal pathogens (Yang et al., 2014).

II. Regulators of secretory trafficking during defense

As discussed above, vesicle trafficking is controlled by specific sets of regulatory machinery, including ARF and Rab small GTPases, tethering factors, and SNAREs. Extensive studies have pointed to intimate involvements of these trafficking regulators throughout the process of secretion-dependent immune activation.

i. Small GTPases—The *Arabidopsis* genome encodes eight Rab subfamilies (A–H) (Rutherford and Moore, 2002). RabA1 group members are involved in transport between the TGN/EE and the PM (Asaoka et al., 2013). A dominant-negative form of RabA1b inhibited the PM targeting of Flagellin-Sensitive 2 (FLS2), the plant receptor for flagellin, suggesting a role for secretion in presentation of PRRs at the PM (Choi et al., 2013). Expression of another Rab, RabA4c, is significantly up-regulated in response to various biotic stresses, implying a positive role in immunity. Consistently, overexpression of RabA4c caused complete penetration resistance to the powdery mildew fungal pathogen *Golovinomyces cichoracearum*. This enhanced resistance is due to increased callose deposition at penetration loci and requires direct interaction between RabA4c and the callose synthase PMR4 at the PM (Ellinger et al., 2014). RabE1d is distributed on both Golgi and PM, suggesting a role in regulating post-Golgi secretion. Indeed, expression of a constitutively active form of RabE1d activated secretion of proteins, including the antimicrobial protein, PR1, and enhanced resistance against *Pst* DC3000 (Speth et al., 2009). Interestingly, a *Pst* DC3000 effector protein, AvrPto, was found to specifically interact with only the RabE subfamily members, including RabE1d, suggesting that AvrPto targets RabE-dependent secretory events to compromise basal resistance (Speth et al., 2009). Similarly, p27 replication protein from red clover necrotic mosaic virus hijacks Golgi-localized ARF1 and blocks cellular trafficking, which allows an efficient viral RNA replication in tobacco BY-2 cells (Hyodo et al., 2014; Hyodo et al., 2013). A barley Golgi-localized ARF GTPase activating protein (ARF-GAP) was also identified as a target of fungal effectors and the *Arabidopsis* homolog of this ARF-GAP is required for penetration resistance to the nonadapted powdery mildew fungus *Erysiphe pisi* (Schmidt et al., 2014).

ii. Tethering factors—The tethering factor Conserved Oligomeric Golgi (COG) was reported to contribute significantly to basal immune responses. The COG complex resides on Golgi and is essential for retrograde trafficking at Golgi (Ungar et al., 2006; Vasile et al., 2006). Transient gene silencing of multiple individual components of the COG complex in barley resulted in significantly enhanced susceptibility to *B. graminis*. Consistently, a COG-interacting Rab protein, HvYPT1-like, is also required for barley penetration resistance against *B. graminis* (Ostertag et al., 2013).

The exocyst complex is the essential tethering factor for exocytosis and plays an essential role in secretory defense as discussed above. In addition it also facilitates symbiotic interactions. For example, Exo70I was shown to be required for symbiotic association with arbuscular mycorrhizae (AM) in *Medicago truncatula*. Exo70I was detected exclusively in

AM-infected plants and specifically mediated formation of the periarbuscular membrane by deposition of the host membrane around the invasive fungal arbuscule hyphae (Zhang et al., 2015). Another tethering complex, HOPS, coordinates the establishment of the *Rhizobium*–legume symbiosis. In *Medicago* root nodules, expression levels of two vacuole-localized HOPS components are down regulated. This leads to host vacuole contraction to accommodate symbiosome expansion (Gavrin et al., 2014).

iii. SNAREs—SNARE complexes execute the final membrane fusion. A SNARE complex comprises four SNAREs: three with a central glutamine residue in the SNARE motif (Q-SNAREs) usually reside on the target membrane and are also referred as t-SNAREs; and one with a central arginine (R-SNARE) in the SNARE motif usually resides on the transport vesicle, thus is referred as a v-SNARE. The v-SNARE and t-SNAREs bridge and fuse the transport vesicle and the target membrane (Saito and Ueda, 2009). After the membrane fusion, the SNARE complex is dissociated and recycled by the N-ethylmaleimide-sensitive factor (NSF) and the soluble NSF attachment protein (α-SNAP).

The SYP1 group Q-SNAREs mainly localize to the PM and its member PENETRATION1 (PEN1)/SYP121 is important for penetration resistance against *B. graminis* in *Arabidopsis*. PEN1 forms a SNARE complex with SNAP33 and VAMP721/722 to direct focal secretion of defense molecules (Collins et al., 2003; Kwon et al., 2008). Another SYP1 member, SYP123, accumulates at the tip of growing root hairs and is necessary for polarized trafficking of cell wall components and induced systemic resistance (ISR) triggered by rhizobacteria in *Arabidopsis* (Rodriguez-Furlan et al., 2016). SYP132 appears to be central for multiple defense responses because silencing of a SYP132 ortholog in *N. benthamiana* resulted in impaired basal resistance, ETI and SAR (Kalde et al., 2007). The SYP4 group Q-SNAREs (SYP41/42/43) localize to TGN/EEs. The *Arabidopsis syp42 syp43* double mutant is defective in both secretory and vacuolar transport pathways and fails to restrict hyphal branching of *E. pisi* (Uemura et al., 2012). The SYP7 group is a plant-specific subfamily of SNAREs and a member of this group from *Arabidopsis* was shown to localize to the PM, suggesting a role in the secretory pathway (Suwastika et al., 2008). Virus-induced gene silencing of all SYP71 homologs in wheat resulted in susceptibility to the stripe rust disease caused by the fungus *Puccinia striiformis* (Liu et al., 2016). In contrast to the above examples, the *Arabidopsis* Golgi-localized SNARE protein, MEMB12, plays a negative role in defense. During bacterial infection, MEMB12 activity is down-regulated by Argonaute 2-regulated and miRNA393*-mediated silencing, which contributes to secretion of PR1 and antibacterial immunity (Zhang et al., 2011).

Evidence suggests that SNAREs have also been co-opted in establishing compatible interactions with pathogenic nematodes. In a resistant soybean cultivar *Rhg1*, hyperaccumulation of a dysfunctional variant of α-SNAP disrupted vesicle trafficking at the feeding site of soybean cyst nematode (SCN) and resulted in demise of the biotrophic interface (Bayless et al., 2016). The *Rhg1* locus provides one of the most important soybean resistant mechanisms to the SCN.

ROLES OF THE ENDOCYTIC PATHWAY IN IMMUNITY

The endocytic trafficking pathway is at the intersection of host immunity and microbial pathogenesis. It is employed not only by the host to regulate immune responses but also by adapted oomycete and fungal pathogens to deliver their effector proteins to promote virulence (Kale and Tyler, 2011).

I. Constitutive endocytosis

Diverse types of PM proteins, including PRRs, undergo constitutive endocytosis. In the absence of its ligand, FLS2 in *Arabidopsis* distributes in dedicated PM nanodomains and constitutively recycles between the PM and endosomal compartments via a brefeldin A (BFA)-sensitive endocytic pathway (Beck et al., 2012; Bucherl et al., 2017). BFA is a fungal toxin that blocks the activity of plant ARF-GEFs and inhibits recycling of endosomes. MIN7 is such an ARF-GEF target of BFA in *Arabidopsis* and localizes to the TGN/EE (Tanaka et al., 2009). Blocking constitutive endocytosis by either BFA treatment or disruption of MIN7 allowed a *Pst* DC3000 strain lacking the functionally redundant effectors AvrE and HopM1 to proliferate, suggesting that constitutive endocytosis contributes to plant basal resistance and that the virulence function of these effectors is to inhibit constitutive endocytosis (Nomura et al., 2006). HopM1 also targets MIN7, but unlike BFA, it does so through proteasome-mediated degradation (Nomura et al., 2006). The biological consequence of this degradation has recently been shown to be the establishment of an aqueous apoplast environment essential for bacterial virulence (Xin et al., 2016). How the HopM1-mediated degradation of MIN7 and inhibition of constitutive endocytosis lead to the “water-soaking” condition in the apoplast is an interesting question. The answer may come from a seemingly unrelated study by Zhou et al. (2015) which showed that induction of plant defense by SA in darkness caused a severe loss in plant fresh weight that was not observed in plants grown under a light-dark cycle. This likely involves SA-mediated repression of water transport genes, including several that encode aquaporins, a defense strategy against pathogens like *Pst* DC3000 (Zhou et al., 2015). It is possible that TGN/EE-localized MIN7 affects distribution of PM aquaporins through membrane recycling and HopM1 may affect apoplast water availability by perturbing trafficking of these water transporters. Supporting this hypothesis, the TGN/EE-localized SNARE complex SYP61-SYP121 has been shown to coordinate the delivery of aquaporin PIP2;7 to the PM and modulates the membrane water permeability (Hachez et al., 2014).

II. Clathrin-mediated endocytosis

In contrast to constitutive endocytosis of the ligand-free FLS2, the ligand-bound FLS2 is internalized from PM nanodomains into clathrin-coated vesicles (CCVs) through clathrin-mediated endocytosis (CME), in a BFA-insensitive manner (Beck et al., 2012; Mbengue et al., 2016). This process is mediated by three clathrin heavy chains (CHCs) and three clathrin light chains (CLCs), which self-polymerize to form a triskelion on the PM to pinch off CCVs. CME appears to be a common trafficking pathway for plant defense signaling as it is also required for internalization and activity of other PRRs, such as EF-TU RECEPTOR (EFR), the danger peptide receptor PEPR1, and likely the lysin motif-containing receptor-like kinase LYK5 (Erwig et al., 2017; Mbengue et al., 2016; Ortiz-Morea et al., 2016). In

addition, the PM-localized receptor-like protein Cf-4 that confers race-specific resistance against the leaf mold fungal pathogen *Cladosporium fulvum* was shown to harbor a canonical clathrin-binding motif YXXΦ and undergo endocytosis upon recognition of the pathogen effector Avr4 in a transient expression assay (Postma et al., 2016). Receptor internalization is thought to be critical for both signal transduction and quenching (Ben Khaled et al., 2015; Geldner and Robatzek, 2008; Murphy et al., 2009).

Dynamins are important regulators of CME and catalyze the scission and release of CCVs from the PM. Mutating *Dynamin-Related Protein 2B* (*DRP2B*) in *Arabidopsis* impaired ligand-induced FLS2 endocytosis and led to enhanced early PTI responses including increased cytosolic Ca^{2+} and ROS production but compromised late PTI outcomes including *PR1* expression and resistance to *Pst* DC3000 *hrcC*$^{-}$ (Smith et al., 2014). This phenotype suggests a complicated role of CME-mediated internalization in the activation and termination of FLS2 signaling. Recently, it was shown that the *P. infestans* effector AvR3a perturbs PTI responses partly by targeting DRP2B (Chaparro-Garcia et al., 2015), supporting a positive role of CME in PTI activation. The increased ROS production in the *drp2b* mutant may be due to altered endocytosis and enhanced activity of NADPH/respiratory burst oxidase protein D (RbohD), a major source of ROS during plant-microbe interactions (Hao et al., 2014).

Evidence suggests that the host CME is heavily exploited by adapted powdery mildew pathogens during compatible interaction. Three out of four characterized *Arabidopsis enhanced disease resistance* (*edr*) mutants against *G. cichoracearum* carry mutations in genes that are potentially involved in CME. *EDR3* encodes Dynamin-Related Protein 1E (DRP1E) (Tang et al., 2006). EDR4 forms a complex with EDR1 and Clathrin Heavy Chain 2 (CHC2) and accumulates at pathogen penetration sites. Both *edr4* and *chc2* mutants displayed similar resistance phenotypes, and *edr4* was shown to be impaired in endocytosis measured by uptake of the endocytic tracer dye FM4-64 (Wu et al., 2015). One possibility is that *edr* mutants impair host endocytosis and affect delivery of fungal pathogen effectors. Alternatively, they may counteract pathogen-mediated manipulation of host CME.

III. Late stages of endocytosis

Activated FLS2 is eventually sorted to intraluminal vesicles within the MVB before being targeted to the central vacuole for degradation and signal quenching (Beck et al., 2012; Choi et al., 2013). Formation of intraluminal MVB vesicles is accomplished by sequential recruitment of the Endosomal Sorting Complex Required for Transport (ESCRT) complexes, including ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. Subunits of ESCRT-I, VPS37-1 and VPS28-2, in *Arabidopsis* are required for MVB sorting of FLS2 and critical for flg22-triggered stomatal closure to prevent bacterial entrance (Spallek et al., 2013). This result suggests a role for the late-stage endocytic trafficking of FLS2 in defense activation in addition to signal quenching and receptor degradation. A loss of function mutation in *AMSH1*, which encodes the ESCRT-III-associated deubiquitinating enzyme, leads to up-regulation of SA signaling and increased pathogen resistance (Katsiarimpa et al., 2013). However, the mechanism that links ESCRT and SA signaling is not clear yet.

CONCLUSIONS AND FUTURE PERSPECTS

Extensive genetic and biochemical studies of different plant-microbe interaction systems uncovered many key membrane trafficking regulators as essential players in controlling proper plant responses to pathogenic or beneficial microbes. Figure 1 summarizes the major membrane trafficking events initiated by a plant cell under pathogen attack and how they may be manipulated by pathogen effectors to promote virulence. Despite the large body of experimental evidence accumulated to date, our knowledge of the mechanisms that govern the interplay between immunity and membrane trafficking is still fragmental. To achieve a better understanding of the role of membrane trafficking pathways during immune signaling, we envision that future research should focus on the following important aspects:

1. A major question is how the secretory and endocytic pathways in host cells are remodeled to meet immunity-associated challenges. More specifically, what are the signals and molecular transducers that direct the reprogramming of intracellular trafficking events, and are there defense-specific membrane trafficking regulators for this process? Determining the nature and dissecting the molecular composition of endomembrane vesicles involved in immune signaling is one way to answer these questions. For example, the Innes group identified two E3 ubiquitin ligases, KEG and ATL1, that are involved in resistance against powdery mildew pathogens (Serrano et al., 2014; Wawrzynska et al., 2008). These E3 ligases were later demonstrated to be components of the TGN/EE and regulate multiple immune-related membrane trafficking events (Gu and Innes, 2011, 2012; Serrano et al., 2014). In addition, their E3 ligase activity is likely regulated through phosphorylation by the EDR1 kinase (Serrano et al., 2014). EDR1 is recruited to the TGN/EE by KEG and ATL1 (Gu and Innes, 2011; Serrano et al., 2014), and has been shown to play an important role in multiple stress responses, including resistance against powdery mildew pathogens (Christiansen et al., 2011; Frye and Innes, 1998).

2. Given the fundamental importance of secretion for defense, it is necessary to understand the role of different types of secretory vesicles (TGN/EEs, MVBs and EXPOs) in the execution of distinct immune outcomes. We also need to further investigate the dynamics of exosomes in the apoplastic space, the regulatory mechanism of the exosome membrane burst, and potentially diverse functions of exosome contents.

3. The interplay between membrane trafficking and SA signaling is also intriguing. SA not only modulates the secretory pathway to ensure the extracellular delivery of defense proteins (Wang et al., 2005), but also inhibits clathrin-mediated endocytosis independent of SA-induced defense signaling and transcriptome reprogramming (Du et al., 2013). However, the potential immune function and molecular mechanism of this inhibition is not clear yet. In addition, up-regulation of SA signaling has been frequently observed in mutants defective in both secretory and endocytic trafficking pathways (e.g., *edr1, edr2, edr4*, *syp42 syp43*, *amsh1*, and *syp121 syp122*), suggesting an intrinsic link between membrane trafficking homeostasis and SA signaling. Exploring the molecular details of this

connection may shed light on the novel roles of SA signaling in membrane trafficking regulation and/or elucidate unknown mechanism of membrane trafficking in regulating SA-dependent immunity.

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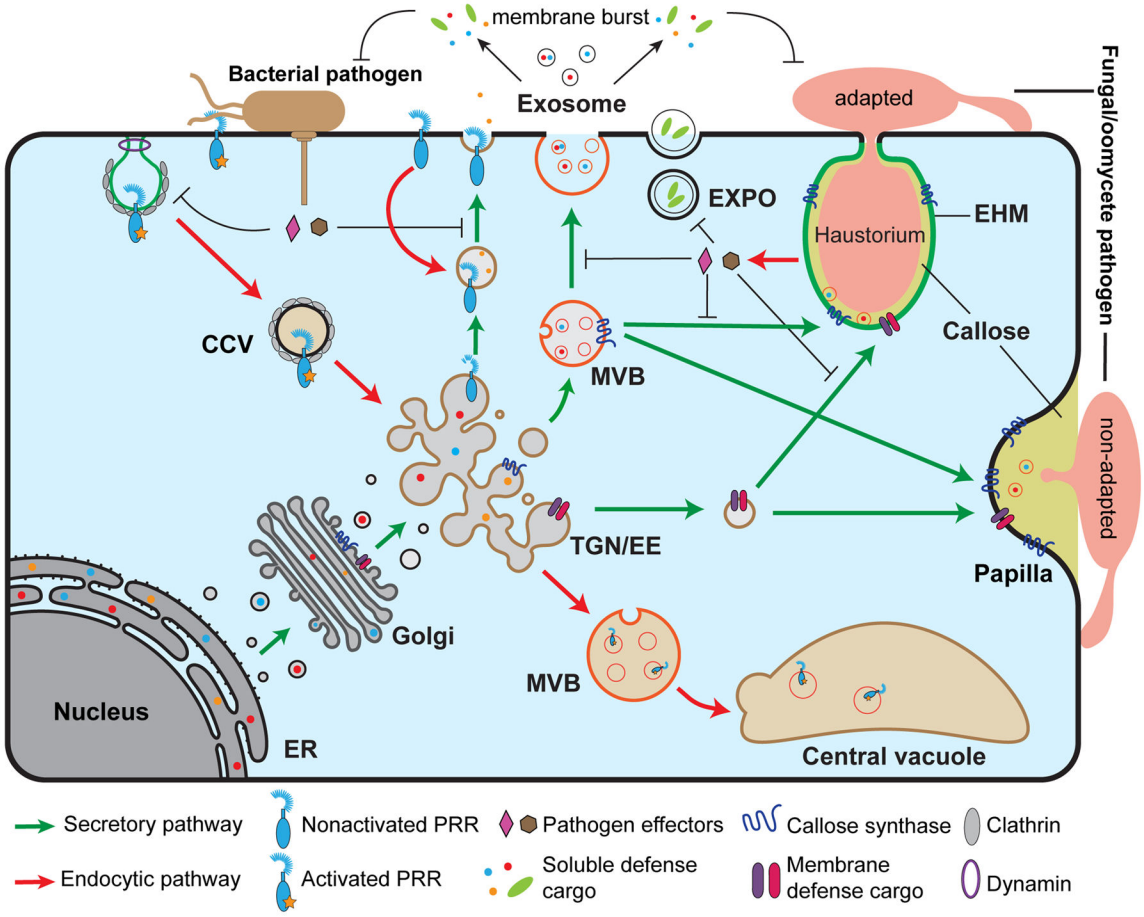

Figure 1. Membrane trafficking during plant immune responses

Defense molecules are synthesized in the ER and transported via Golgi to the sorting hub TGN/EE. Vesicles derived from the TGN/EE, including MVBs, fuse with the PM/EHM to deliver soluble cargo to the extracellular space and membrane cargo to the PM/EHM. MVBs and EXPOs contribute to paramural accumulation of exosomes, whose membrane burst in the extracellular space discharges contents with anti-microbial activities. Effector proteins secreted by pathogens into host cells inhibit multiple steps of the secretory trafficking pathways to block defense activation. PM-localized immune receptors, such as PRRs, undergo constitutive endocytosis before activation. Once activated, these receptors are internalized into clathrin coated vesicles (CCVs) and undergo clathrin-mediated endocytosis (CME). The CME sorts activated receptors to the central vacuole through TGN/EEs and MVBs. Both constitutive endocytosis and CME contribute to basal immunity and are targeted by pathogen effectors. Arrows indicate steps of the secretory (green) and the exocytic (red) pathways.