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Microreview

Vesicle trafficking in plant immune responses

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Summary

In plants, perception of pathogen-associated molecular patterns at the surface is the first line of defence in cellular immunity. This review summarizes recent evidence of the involvement of vesicle trafficking in the plant's immune response against pathogens. I first discuss aspects of ligand-stimulated receptor endocytosis. The best-characterized pattern-recognition receptor (PRR), FLS2, is a transmembrane leucine-rich repeat receptor kinase that recognizes bacterial flagellin. FLS2 was recently shown to undergo internalization upon activation with its cognate ligand. An animal PRR, TLR4 that mediates perception of bacterial-derived lipopolysaccharides, similarly exhibits ligand-stimulated endocytosis. The second focus is *N*-ethylmaleimide-sensitive factor adaptor protein receptor (SNARE)-mediated immunity involving syntaxins and their cognate partners. One of the genes involved in basal immunity in *Arabidopsis*, *PEN1*, encodes a syntaxin that focally accumulates at fungal penetration sites, raising the possibility that induced exocytosis is important for active defence. Pathogen-triggered endocytic and exocytic processes have to be balanced to ensure host cell homeostasis. Thus, understanding how phytopathogens have evolved strategies to exploit host cell vesicle trafficking to manipulate immune responses is currently an area of intense study.

Introduction

Plants depend on innate immune systems to defend themselves against potentially infectious pathogens that grow epiphytically on their surfaces. No acquired immune system is known for plants and they lack a circulatory system. However, a large repertoire of immune receptors that mediate local responses help trigger systemic defence effectively protecting plants from pathogen invasion (Chisholm *et al*., 2006). The plant immune system is comprised of two definable layers. The first is expressed principally at the cell surface and involves perception of pathogen-associated molecular patterns (PAMPs). PAMPs are microbial molecules that are highly conserved throughout whole classes of microbes and are essential for microbial life and therefore difficult to dispense with. For example, lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, and flagellin protein that forms part of the bacterial motility organ, act as PAMPs in animals and plants. Perception of PAMPs is mediated by cognate pattern-recognition receptors (PRRs). In animals, PRRs of the Toll-like receptor (TLR) family stimulate inflammatory responses in innate immunity. These receptors are single transmembrane proteins containing an extracellular leucine-rich repeat (LRR) domain and an intracellular tail interacting with signalling proteins. In plants, PAMPs are also recognized by LRR-containing transmembrane receptors that trigger a plethora of immune responses including the generation of reactive oxygen species (ROS), nitric oxide, the plant stress hormone ethylene, activation of a mitogen-activated protein kinase cascade as well as changes in gene expression (Felix *et al*., 1999; Zeidler *et al*., 2004; Zipfel *et al*., 2004). Among those genes that are upregulated after PAMP perception is a high proportion that code for receptors potentially involved in immunity, thereby probably priming the host for microbial attack (Zipfel *et al*., 2004). This pre-invasive PAMP-triggered immunity leads to dramatic cellular reprogramming and the production of antimicrobial compounds, callose deposition and cell wall thickening (Chisholm *et al*., 2006).

In order to invade a plant, phytopathogenic bacteria and fungi have evolved strategies to subvert host immunity and they actively penetrate plant tissues. Bacteria such as *Pseudomonas syringae* swim towards openings in the plant tissue (for example stomata, hydathodes, wound sites) and enter the apoplastic space of plant tissues. In this sense bacterial flagellin can be considered a virulence factor. Spores of infectious fungi germinate and form an appressorium, a fungal structure that exerts high pressure on the plant cell walls allowing the fungal hyphae to enter and invaginate cell membranes. At this stage,

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pathogens need either to bypass or suppress PAMP-triggered immune responses to proliferate and finally colonize plant tissues. This appears to be achieved through the secretion of pathogen-produced effectors, many of which are proteins. Phytopathogenic bacteria inject a series of effector proteins via their type III secretion system (TTSS) into the plant cytoplasm (Grant *et al*., 2006). Several of such effectors have been identified that downregulate PAMP-triggered immune signalling, thereby suppressing host defences. Some fungal effectors are released through an exocytic process in the plant apoplast where they counteract plant-produced antimicrobial compounds, other effectors are secreted into the plant cytoplasm (O'Connell and Panstruga, 2006).

Plants have evolved a second surveillance layer that operates at the post-invasive level. Several pathogen-derived effector proteins are known as avirulence (Avr) factors that are recognized by cognate immune receptors known as resistance proteins in a microbial-strain/plant-cultivar specific manner (Chisholm *et al*., 2006). This effector-triggered immunity results in a potentiation of PAMP-triggered immune responses leading to locally restricted plant cell death. Intriguingly, RIN4 is a negative regulator of PAMP-triggered immunity and interacts with intracellular immune receptors mediating effector-triggered immunity, thereby potentially connecting both pathways (Kim *et al*., 2005). In addition, cell-autonomous defence responses stimulate systemic acquired resistance (SAR), a pre-formed awareness towards pathogen attack in distant tissues (Dong, 2004).

There is increasing evidence that dynamic cellular processes play an important role in both pre- and post-invasive defence responses. Cytoskeleton rearrangements and nuclear relocation have been observed in response to fungal pathogens (Schmelzer, 2002). Also during *Erysiphe cichoracearum* infection, the cytoplasm and organelles including peroxisomes migrate towards penetration hyphae (Koh *et al*., 2005). PEN2, a glycosyl hydrolase, localizes to peroxisomes that focally accumulate in response to fungal penetration (Lipka *et al*., 2005). Moreover, *Agrobacterium*-derived VirE2 has the capability to interact with plant lipid rafts, which are membrane microdomains enriched in sterols and sphingolipids (Duckely *et al*., 2005). Potato virus X, in contrast, induces formation of endoplasmic reticulum-originated vesicles that associate with actin filaments (Ju *et al*., 2005). Recent evidence also points to induced vesicle trafficking in endocytic and exocytic processes as an important component of plant innate immune response.

Pattern-recognition receptor endocytosis

In plants, PRRs can be grouped into two classes. Single transmembrane spanning proteins that contain a short cytoplasmic tail are referred to as receptor-like proteins (RLP) and those that contain a serine/threonine kinase domain are called receptor-like kinases (RLKs). In *Arabidopsis*, these comprise large multigene families with about 200 RLPs and 600 RLKs (Shiu *et al*., 2004; Tor *et al*., 2004). To date, only three PRRs have been functionally defined in plants. Tomato LeEix is an RLP that recognizes fungal-derived xylanase (Ron and Avni, 2004), the *Arabidopsis* RLK that perceives the bacterial elongation factor EF-Tu, EFR (Zipfel *et al*., 2006) and the RLK flagellin receptor, FLS2, which is best characterized and described in more detail below.

Ligand-stimulated endocytosis of FLS2

Arabidopsis FLS2 mediates flagellin perception by recognizing the elicitor-active epitope, flg22, which corresponds to the most conserved domain of the flagellin protein and physically interacts with FLS2 (Felix *et al*., 1999; Chinchilla *et al*., 2006). Mutant plants lacking the *FLS2* gene exhibit an enhanced susceptibility to virulent *P. syringae* infection when these are inoculated on the leaf surface. This highlights the importance of the FLS2 pathway in pre-invasive defence (Zipfel *et al*., 2004). Studying dynamic changes at the subcellular level by transgene expression of FLS2 fused to the green fluorescent protein (GFP), FLS2 was found to enter an endocytic pathway (Robatzek *et al*., 2006). Upon flg22 addition, membrane resident FLS2-GFP rapidly accumulated in intracellular vesicles that are likely trafficked for degradation. This is reminiscent of ligand-induced receptor endocytosis in animals where dynamin was shown to interact with phospholipids and a number of proteins associated with the cytoskeleton (Konopka *et al*., 2006). Dynamin encodes a large GTPase involved in 'pinching off' coated pits to form clathrin-coated vesicles (CCVs). The *Arabidopsis* dynamin-like protein 1 was implicated in plasma membrane vesiculation (Hong *et al*., 2003). However, a role for dynamin-like proteins in receptor-mediated endocytosis has not yet been demonstrated in plants.

Surface receptor endocytosis has been well studied in animals, but is a newly emerging field in plant science. Some signalling events in the epidermal growth factor receptor pathway require receptor endocytosis (Sorkin and Von Zastrow, 2002). Similarly, flg22-triggered responses and FLS2 endocytosis appear to be linked (Robatzek *et al*., 2006). Receptor endocytosis involves the exposure of sorting motifs to cytosolic proteins (Kurten, 2003). FLS2 contains a PEST-like motif in its kinase domain which implicates ubiquitination, and more specifically, monoubiquitination as an endocytic signal (Shih *et al*., 2000). Mutation of the PEST-like motif was found to compromise FLS2 endocytosis and downstream signalling but not flg22-triggered ROS production

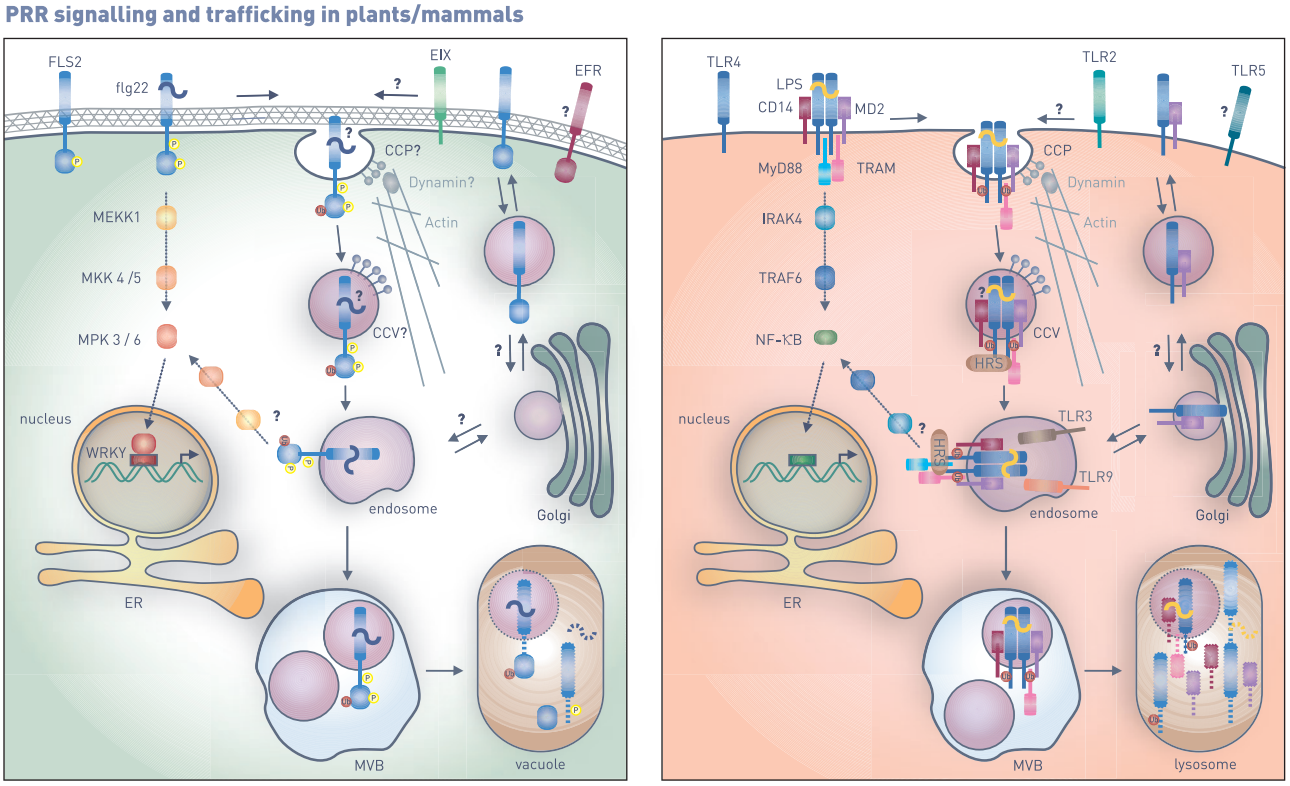

Fig. 1. Model of flg22-mediated internalization of FLS2 compared with LPS-triggered TLR4 endocytosis. Upon flg22 binding to cell membrane resident FLS2, a MAP kinase cascade is activated that transmits the signal into the nucleus involving WRKY transcription factors (Chisholm *et al.*, 2006). Concomitantly, FLS2 is internalized into endosomal compartments, potentially comprising clathrin-coated pits (CCPs) and CCVs, involving the plant's cytoskeleton (actin and dynamin). FLS2 accumulates via endosomes to MVBs and is targeted to vacuoles for lysosomal degradation (Robatzek *et al.*, 2006). Endocytosis of FLS2 likely requires FLS2 phosphorylation (P), and ubiquitination (ub). There is evidence for cell membrane FLS2 recycling in its flg22-unbound stage. Similarly, in response to LPS, mammalian cell membrane resident TLR4 activates the MyD88/IRAK4 kinase pathway resulting in nuclear translocation of NF-ĸB, and TLR4 is endocytosed via CCVs. The interacting factor MD2 is co-endocytosed together with LPS and TLR4 (Husebye *et al.*, 2006). TLR4 is ubiquitinated (ub) in response to LPS, interacts with the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), and is finally targeted to the lysosome. Distinct TLRs appear to be surface localized, whereas others localize to endosomes. PAMP-triggered receptor endocytosis is embedded into the global cellular vesicle trafficking system linking the organelles endoplasmic reticulum, Golgi and vacuoles with the plasma membrane.

(Robatzek *et al.*, 2006). Taken together, I suppose that FLS2-mediated flg22 signalling might be initiated at the plasma membrane, but continue from intracellular compartments. In addition, non-flg22-triggered FLS2 might constitutively recycle at the cell membrane. Figure 1 summarizes current knowledge of flg22 signalling and induced FLS2 endocytosis together with findings from general vesicle trafficking in plants.

Similarities between FLS2 and TLR endocytosis

In mammals, the first line of innate immune responses involves the perception of PAMPs mediated by TLRs (Akira *et al.*, 2006). TLRs that recognize lipids and proteins localize and initiate signalling at the cell membrane whereas those that localize to endosomal compartments intercept nucleic acids (Barton *et al.*, 2006). While in animals ligand-induced endocytosis of surface receptors is well known it has only recently been demonstrated for PAMP-sensing immune receptors as shown for the LPS receptor, TLR4. LPS perception by the binding protein CD14 triggered relocation of TLR4 into endosomes (Husebye *et al.*, 2006). As depicted in Fig. 1, LPS was co-internalized with TLR4 and its interacting partner MD2. Significantly, intracellular TLR4 could still interact with the downstream TRAM kinase suggesting that TLR4 signals from endosomes. Non-LPS-triggered TLR4 was recycled in vesicles together with MD2. Furthermore, LPS enhanced TLR4 ubiquitination which likely promotes receptor degradation. Mechanistically, TLR4 endocytosis was found to be primarily important for attenuating LPS signalling transmitted from the cell membrane because its inhibition led to an increase in the LPS response (Latz *et al.*, 2002; Husebye *et al.*, 2006).

TLR4 contains three tyrosine-based tetrapeptide motifs YxxΦ, a known endocytic-sorting signature. The YxxΦ motif mediates contact to μ-adaptins thereby initiating assembly of the clathrin trisekelion, vesicle budding and

formation of CCVs (Honing *et al*., 2005). TLR4 endocytosis is dependent on dynamin and clathrin, suggesting that CCVs are formed during this process. The hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) localizes to endosomal clathrin coats and was found to interact with TLR4. Hrs also binds to the endosome-associated complex that is required for transport (ESCRT-I, -II, -III), thereby regulating endosomal sorting of transmembrane proteins via multivesicular bodies (MVBs) to lysosomes (Bache *et al*., 2003). In *Arabidopsis*, there are homologues for all ESCRT components present (Winter and Hauser, 2006). However, their potential functions and whether the FLS2 vesicles belong to the CCV-type are unclear.

Further evidence of PAMP-triggered endocytosic processes in plants

Pathogen-associated molecular patterns such as fungal xylanase, bacterial EF-Tu and flagellin bind to cell membranes where they initiate immune responses (Ron and Avni, 2004; Chinchilla *et al*., 2006; Zipfel *et al*., 2006). In addition, they can be processed by the endocytic machinery, as observed by the internalization of bacterial LPS and exopolysaccharides (Romanenko *et al*., 2002; Gross *et al*., 2005). Similar to TLR4/LPS endocytosis in mammals, LPS internalization in plants likely occurs through receptor-mediated endocytosis. However, its cognate PRR is yet unknown.

Some insights can be gained from analysis of the tomato LeEix2 receptor that recognizes xylanase and triggers cell death (Ron and Avni, 2004). Its cytoplasmic tail contains the YxxΦ motif that when mutated abolishes LeEix2-mediated cell death, indicating that LeEix2 endocytosis is involved in xylanase signalling. Also, the EFR receptor contains the YxxΦ motif within its cytoplasmic juxtamembrane region, suggesting endocytic processes may be involved in EF-Tu signalling (Zipfel *et al*., 2006). Endocytosis seems not only to play a role in PAMP-triggered immunity but also in effector-triggered immunity. For example, tomato Ve which conditions resistance to *Verticillium* infection, and the Cf4 and Cf9 RLPs, conferring Avr4- and Avr9-mediated resistance to *Cladosporium fulvum*, also posses the YxxΦ signature in their cytoplasmic tails (Kawchuk *et al*., 2001). Thus, immune receptor endocytosis may well be a general phenomenon in plant defence signalling and regulation.

Exocytosis and plant immunity

The plant endomembrane system exhibits a much higher degree of complexity than that of mammals or yeast. Plant cells contain several Golgi stacks per cell, distinct storage and lytic vacuoles and different vesicle trafficking routes to those in mammals (Jurgens, 2004). This is accompanied by an increased complexity of genes coding for proteins regulating vesicle trafficking. Whereas 26 genes in yeast encode *N*-ethylmaleimide-sensitive factor adaptor protein receptors (SNAREs) about 60 such genes are found in *Arabidopsis* (Sanderfoot *et al*., 2000). At the target membrane, a syntaxin and a synaptosome-associated protein (SNAP-25) together form a t-SNARE complex that can associate with vesicle-anchored synaptobrevins (v-SNAREs). This interaction generates a stable SNARE complex that facilitates vesicle docking at the target membrane and finally membrane fusions essential for secretory pathway function (Parlati *et al*., 2000). It is thought that distinct interactions give rise to different SNARE complexes resulting in targeted trafficking of specific cargo vesicles.

PEN1-mediated immunity

The importance of SNARE proteins in plant immunity has been demonstrated by elegant genetic studies combined with fluorescence microscopy. The powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) infects barley successfully but fails to colonize *Arabidopsis* plants. Mutant *Arabidopsis* lacking *penetration 1*, *pen1* (the barley homologue of *required for mlo resistance 2*, *ror2*) allowed enhanced penetration of *Bgh* but restricted further growth (Collins *et al*., 2003). Thus, PEN1 contributes to pre-invasive immunity against *Bgh* but not to post-invasive immunity. *AtPEN1* and *HvROR2* encode plasma membrane resident syntaxins. In addition, immunity in barley requires the function of the SNAP-25-like protein HvSNAP34, which forms a binary complex with HvROR2. Both PEN1 and HvROR2 were found to relocate and accumulate at fungal penetration sites (Bhat *et al*., 2005). The plant-specific seven transmembrane protein mildew *resistance locus o* (*mlo*), which interacts with its specific syntaxin counterpart, also localizes at fungal appressoria. This suggests a fungal-induced formation of a plasma membrane microdomain reminiscent of lipid rafts.

Involvement of VAMPs encoding v-SNAREs is currently not well understood in plants. AtVAMP7 has been identified as a suppressor of ROS-mediated cell death in yeast and *Arabidopsis* (Levine *et al*., 2001). Its so-called ‘longin’ domain is important for subcellular sorting (Uemura *et al*., 2005). Recently, members of the AtVAMP72 family were shown to form a ternary SNARE complex together with PEN1 and AtSNAP33 (the homologue of *HvSNAP34*) *in vitro* (C. Kwon and P. Schulze-Lefert, pers. comm.). This provides first evidence for a ternary plant SNARE complex, likely to be involved in exocytic vesicle trafficking contributing to pre-invasive immunity (as depicted in Fig. 2).

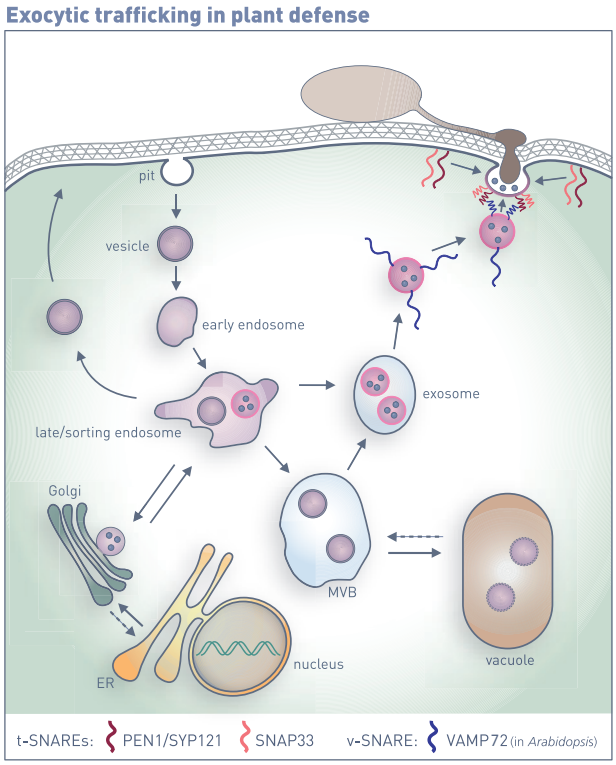

Fig. 2. Model of SNARE-mediated vesicle trafficking in basal immunity. In response to fungal attack, focal accumulation of the syntaxin PEN1/AtSYP121 and the SNAP-25-like protein AtSNAP33 is triggered (Collins *et al.*, 2003; Bhat *et al.*, 2005). They form a binary t-SNARE complex marking the target membrane. Vesicles labelled with cognate v-SNAREs of the AtVAMP72 subfamily are trafficked to the target membrane where they form a ternary complex and facilitate vesicle fusion (C. Kwon and P. Schulze-Lefert, pers. comm.). Thereby compounds exhibiting antimicrobial activities are delivered and secreted to combat fungal ingress. Exocytosis is in balance with endocytic processes in order to ensure cell integrity.

Further evidence of exocytic processes in plant immunity

Recently, a specific plasma membrane resident syntaxin, NbSYP132 (SYP for syntaxin of plants), was identified as a novel component of effector-triggered immunity against bacterial pathogens in *Nicotiana benthamiana* (M. Kalde and S. Peck, pers. comm.). NbSYP132-silenced plants were impaired in the secretion of pathogenesis-related (PR) proteins, implicating NbSYP132 as the cognate t-SNARE for exocytosis of vesicles containing antimicrobial PR proteins in this species. NbSYP132 also plays a role in PAMP-triggered immunity and SAR indicating that NbSYP132-dependent secretion defines a critical step of active resistance against bacterial pathogens in plants.

Pathogen-induced *AtSNAP33* expression correlated with that of the secreted *PR1* gene product (Wick *et al.*, 2003). Transcription of *PR* genes is under the control of Non-expressor of Pathogenesis-Related genes (NPR1), a key molecule of effector-triggered immunity and SAR (Dong, 2004). Gene expression analysis revealed that NPR1 also co-ordinately regulates transcription of genes encoding proteins involved in the secretory pathway (Wang *et al.*, 2005). Null mutants in some of these genes displayed a reduced secretion of PR1 and enhanced susceptibility to virulent *P. syringae*. Thus, it may be that upon bacterial infection, the secretory pathway is co-ordinately upregulated in order to prepare the cell for immune-related exocytosis.

Interference of PAMP signalling through effectors

Phytopathogens have evolved various strategies to counteract PAMP-triggered immune responses in plants. Gram-negative bacteria inject effectors into the host cytoplasm via the TTSS that forms a pilus penetrating the cell membrane. *P. syringae* expresses about 30 such effectors that support infection and promote pathogen growth (Petnicki-Ocwieja *et al.*, 2002). Recently, effectors have been reported that are able to downregulate PAMP signalling pathways by targeting shared components of distinct PAMP pathways. The TTSS-delivered HopPtoD2 protein exhibits tyrosine phosphatase activity that inactivates MAP kinase cascades (Espinosa *et al.*, 2003). AvrPto and AvrPtoB suppress flg22-induced callose deposition and gene transcription, most likely upstream of the MAP kinase pathway (Hauck *et al.*, 2003; He *et al.*, 2006; Janjusevic *et al.*, 2006; de Torres *et al.*, 2006). Determination of the AvrPtoB structure revealed it to be an ubiquitin E3 ligase that likely mimics and thereby interferes with the host ubiquitin machinery or possibly targeting key defence proteins for degradation.

Vesicle trafficking targeted by pathogen-derived effectors/compounds

Intracellular trafficking of vesicles is also a potent target for pathogen-produced effector proteins and compounds. For example, Brefeldin A (BFA), a compound from the fungus *Alternaria carthami*, promotes plant disease through interference in the formation of Golgi-derived vesicles (Driouich *et al.*, 1997). Some evidence suggests that powdery mildew fungi manipulate the host vesicle trafficking system to establish its haustorial feeding structure by targeting the plant-specific MLO protein (Consonni *et al.*, 2006).

A recent report shows that the secreted effector protein HopM1 from *P. syringae* localizes to plant endomembrane fractions (Nomura *et al.*, 2006). In a search for host cell targets, *Arabidopsis* MIN7 was identified as a HopM1 interacting protein. HopM1 triggered proteasomal degradation of AtMIN7 as part of its virulence function. AtMIN7

encodes an adenosine diphosphate ribosylation factor (ARF) guanine nucleotide exchange factor (GEF). ARF-GEF proteins are major regulators of vesicle formation and intracellular trafficking, and are targeted by BFA. Not surprisingly, BFA was found to phenocopy HopM1 (Nomura *et al*., 2006). AtMIN7 is highly related to GNOM which localizes to endosomes and controls polarized trafficking of the auxin efflux carrier PIN1 to the cell membrane (Geldner *et al*., 2003). Mutants lacking AtMIN7 were reduced in polarized callose deposition in response to non-pathogenic bacteria (Nomura *et al*., 2006). This suggests that *P. syringae* pv. *tomato* circumvents retrieval of antimicrobial compounds through modulation of vesicle trafficking by targeted ARF-GEF degradation. Effector-mediated interference of vesicle trafficking has been similarly observed during intracellular *Salmonella* infection of mammalian cells (Kuhle *et al*., 2006).

Concluding remarks and perspectives

Plant immunity is composed of several layers that constitute robust pre- and post-invasion defences. Recent results have highlighted the importance of intracellular vesicle trafficking in resistance and the significance of endocytic and exocytic processes. It appears that in both animal and plant cells activated PAMP receptors are subject to endocytosis. Studies reported that attenuation of TLR signalling is mostly achieved through its interactions with negative regulators (Liew *et al*., 2005). However, TLR4 was recently found to be internalized in response to LPS which may attenuate signalling (Husebye *et al*., 2006). In plants, responses to PAMPs such as flagellin are typically transient and this is probably important for maintaining host cell integrity. This might be at least partially achieved through internalization of receptors such as FLS2. As FLS2 is in physical contact with its ligand (Chinchilla *et al*., 2006), flg22 might be co-internalized as does TLR4 LPS. In this way the activated PRRs and elicitor-active PAMPs would be disposed of. One point of interest is that TLRs are recruited to microdomains in response to PAMPs, thereby establishing a localized recognition interface with distinct signalling capacities (Triantafilou *et al*., 2004). Moreover, lipid raft formation has been connected with immune receptor internalization (Stoddart *et al*., 2002). Whether lipid rafts are also involved in PAMP perception in plants and precede the formation of endocytic compartments remains to be addressed. It seems likely that further PRRs such as EFR and LeEix may be targeted for endocytosis. However, presence of the YxxΦ motif suggests a pathway involving adaptin complexes, which differs to that of FLS2. It will be of interest to establish whether flagellin perception in animals also triggers internalization of its cognate receptor, TLR5.

Upon pathogen attack, plant cells induce components of the secretory pathway to facilitate exocytic vesicle trafficking. Cell membrane resident syntaxins and SNAPs focally accumulate and form a lipid-raft-like microdomain in response to fungal ingress, building up a binary t-SNARE complex. Vesicles labelled with cognate v-SNAREs traffic towards the target membrane, eventually forming a ternary SNARE complex and driving vesicle/cell membrane fusion. Such polarized vesicle trafficking may allow delivery of plant antimicrobial cargo to the site of pathogen invasion. However, besides PR1, the content of secretory vesicles is elusive. Identifying the repertoire of vesicle compounds that may be specific to a certain pathogen is a future challenge. We also need to more fully understand dynamic processes and interactions that are likely to connect different layers of immunity.

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