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A Highway for War and Peace: The Secretory Pathway in Plant–Microbe Interactions

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ABSTRACT **Secretion of proteins and other molecules is the primary means by which a cell interacts with its surroundings. The overall organization of the secretory system is remarkably conserved among eukaryotes, and many of the components have been investigated in detail in animal models. Plant cells, because of their sessile lifestyle, are uniquely reliant on the secretory pathway to respond to changes in their environments, either abiotic, such as the absence of nutrients, or biotic, such as the presence of predators or pathogens. In particular, most plant pathogens are extracellular, which demands a robust and efficient host secretory system directed at the site of attack. Here, we present a summary of recent advances in our understanding of the molecular details of the secretory pathway during plant–microbe interactions. Secretion is required not only for the delivery of antimicrobial molecules, but also for the biogenesis of cell surface sensors to detect microbes. The deposition of extracellular material is important in the defense against classical bacterial pathogens as well as in the so-called 'non-host' resistance. Finally, boosting the protein secretion capacity is vital for avoiding infection as well as for achieving symbiosis, even though in the latter case, the microbes are engulfed in intracellular compartments. The emerging evidence indicates that secretion provides an essential interface between plant hosts and their associated microbial partners.**

Key words: **protein secretion; defense; salicylic acid; NPR1; symbiosis; Arabidopsis; legume; endoplasmic reticulum; SNARE.**

INTRODUCTION

In 221 BC, the emperor Qin Shi Huang united China. To defend his vast northern border, he built two unprecedented structures. The first was the Great Wall. The second was a military highway that linked the Wall with the heart of the empire. It is hard to exaggerate the strategic importance of this so-called 'Qin Zhi Dao'. In times of war, the transportation artery allowed the emperor's army to cover the 700-km distance to the border in a matter of days. Although the Great Wall has always been world-renowned, the existence of the military highway was not widely known until recently. Similarly, in plant research, the cell wall has long been recognized as a physical barrier against potential invaders. On the other hand, despite early studies implying the importance of the secretory pathway in plant–microbe interactions (Snyder and Nicholson, 1990; Walther-Larsen et al., 1993), the molecular mechanisms involved have only been revealed in recent years. In this review, we would like to give a bird's-eye view of the current understanding of the role the secretory pathway plays in defense as well as symbiosis. For more in-depth discussions on the subject, we recommend several other reviews (Huckelhoven, 2007; Kwon et al., 2008a; Sup Yun et al., 2008; Frei dit Frey and Robatzek, 2009; Ivanov et al., 2010; Saijo, 2010).

THE INFRASTRUCTURE

The secretory pathway consists of intracellular membrane structures: the endoplasmic reticulum (ER), the Golgi apparatus, and vesicles. Proteins destined for the extracellular space or the vacuole often contain signal peptides (SPs) at their amino termini. Following initial translation in the cytosol, the SP sequence is captured by the signal peptide recognition particle, which is itself recognized by a receptor located on the ER membrane. After this ER 'docking' step, translation resumes and the nascent polypeptide is transferred into the ER lumen through a protein channel termed the translocon complex. Upon translocation, the SP is removed by the signal peptidase complex (SPC), leaving the mature polypeptide ready to fold into its functional conformation. Protein folding in the ER is facilitated by the ER-relative of chaperones and their related proteins, including the HSP70-class BiP, the HSP90-class GRP94/

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SPD (Ishiguro et al., 2002), and the HSP40-family of ER-Js (Jin et al., 2009). In addition, secreted proteins, such as pathogenesis-related (PR) proteins, contain intramolecular disulfide bridges to maintain their conformation in the harsh extracellular environment (Kitajima and Sato, 1999). Correct disulfide bond formation is achieved through the action of protein disulfide isomerases (PDIs) and oxidoreductases. Finally, cell-surface proteins (such as receptors) in animals and plants are often glycosylated. In the ER, short chains of glycan moieties are conjugated to an Asn residue in target proteins by the oligosaccharyltransferase (OST) complex. A glycosylated protein is repeatedly processed by glucosidase I/II and UDP-glucose—glycoprotein glucosyltransferase (UGGT)—and then folded by the calcium-binding chaperones calreticulin (CRT) and calnexin (CNX). This CRT/UGGT cycle continues until the substrate matures and is released to BiP.

Cargo proteins that pass the quality control of the ER are then delivered to the Golgi apparatus, where additional modifications, notably to the sugar moieties, can occur. Vesicles containing the final products fuse to the plasma membrane or other cellular destinations. Vesicle trafficking at each stage is mediated by specific SNARE complexes. Each SNARE complex comprises a vesicle membrane-localized VAMP, a soluble SNAP protein, and a target membrane-localized syntaxin. Their interactions bring the two membranes together and drive fusion between the two lipid bilayers.

MILITARY USE

Maintaining Surveillance

Just as the Great Wall would be useless without sentinels, the plant would be ignorant of an impending invasion without cell-surface receptors that recognize pathogen-associated molecular patterns (PAMPs). Over the last decade, a number of such pattern recognition receptors (PRRs) have been identified, such as FLS2 (which recognizes bacterial flagellin; Gomez-Gomez, L. & Boller, T., 2000), EFR (recognizing bacterial elongation factor EF-Tu; Zipfel et al., 2006), CERK1 (recognizing fungal chitin fragments; Miya et al., 2007), and XA21 (recognizing bacterial protein AX21; Song et al., 1995). These receptors share several structural features: a signal peptide at the amino terminus, followed by extracellular LRR or LysM repeats, a *trans*-membrane domain, and an intracellular kinase domain. The presence of signal peptides indicates the receptor proteins need to go through the protein secretory pathway for proper biogenesis and localization. The extracellular regions are responsible for recognizing PAMPs at specific regions, such as those defined by the peptides flg22 and elf18, and are frequently glycosylated. The kinase domains are activated following ligand binding, triggering an oxidative burst, MAP kinase signaling, and callose deposition, ultimately leading to PAMP-triggered immunity.

Prolonged activation of EFR results in stunted growth and loss of anthocyanin (blue pigment) accumulation. Genetic screens were performed by two research groups to isolate mutants that are aberrant in elf18-induced anthocyanin production and growth suppression. As expected, many loss-of-function alleles of EFR were identified. Another class of mutants contains no changes in the *EFR* gene, yet exhibits altered localization, accumulation, or function of the EFR protein. Four publications in 2009 reported that these mutants are impaired in the protein quality-control mechanism in the ER. Table 1 summarizes the mutant genes and their effects on the EFR protein.

In most cases, mutations in these ER quality-control proteins resulted in reduced accumulation of the mature EFR protein. However, in mutants for which such experimental data were not available, a defect in EFR biogenesis could be inferred: for example, in the *erd2b* mutant, the CRT3 protein, which is required for EFR accumulation, was almost undetectable (Li et al., 2009). Either directly or indirectly, these mutations all affected the glycosylation pattern of EFR, highlighting the importance of the quality-control mechanism to the functioning of this cell-surface protein.

Table 1.

Gene	Protein localization/function	Effect on EFR	Reference
CRT3	ER-resident chaperone, glycoprotein folding	Little or no protein	(Li et al., 2009);(Saijo et al., 2009)
UGGT	Protein glycosylation	Little or no protein	(Li et al., 2009)
ERD2b	Receptor for ER retention signal	N/A*	(Li et al., 2009)
GII b subunit	Glucosidase II, subunit for ER retention	Decrease in protein levels and increase in protein size	(Lu et al., 2009)
GII a subunit	Glucosidase II, catalytic subunit	No obvious effect on protein abundance	(Lu et al., 2009)
STT3A	Oligosaccharyltransferase (OST) complex subunit	Little or no protein	(Saijo et al., 2009);(Nekrasov et al., 2009)
AtSDF2	Component of ER complex with BiP and ERdj3B	Decreased protein levels, sensitive to endoglycosidase H	(Nekrasov et al., 2009)
ERdj3B	ER-resident HSP40	N/A	(Nekrasov et al., 2009)

* CRT3 accumulation is impaired in *erd2b* mutants.

The aforementioned genetic screens might have been designed to reveal components and regulators of the elf18/EFR signaling pathway (Zipfel and Robatzek, 2010). Instead, they led to the surprising discovery of receptor biogenesis as a major mode of regulation of PAMP signaling. Two lines of evidence indicate that the observed effect is highly specific to EFR: (1) most mutants show no obvious morphological defects (Li et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009), and (2) the mutations have little effect on the function of FLS2, even though it is a glycoprotein structurally similar to EFR.

It is intriguing that ER proteins important for EFR biogenesis have a minor or no role in the function of FLS2. (The *sdf2* and *erdj3b* mutations result in somewhat reduced output from FLS2.) One possible explanation is that FLS2 maturation is less dependent on ER quality control than EFR. Alternatively, FLS2 may rely on a distinct subset of ER-resident proteins for its proper folding and modification. Consistent with the latter interpretation, the EFR-defective *elfin* and *psl* mutants were also intact in CERK signaling. Genetic screens for flg22-signaling components have been performed using a similar strategy as for the elf18-signaling mutants. Whether the ER quality-control genes will be found among those flg22-insensitive mutants has yet to be determined. The first genes identified in the screens include *FLG22 INSENSITIVE 3* (*FIN3*), which encodes ETHYLENE INSENSITIVE 2 (EIN2) that regulates *FLS2* at the transcriptional level through EIN3 and EIL1 transcription factors (Boutrot et al., 2010).

Structurally and functionally similar to EFR and FLS2, the rice glycoprotein XA21 recognizes a conserved protein secreted by the blight pathogen *Xanthomonas oryzae* (Lee et al., 2009). The XA21 protein exists in two partitions: the ER and the plasma membrane. Some of the XA21 proteins, presumably of the ER fraction, associate with BiP3 (Park et al., 2010). Interestingly, increasing the amount of BiP3 in the cell decreased the total level of the XA21 protein and compromised XA21-mediated resistance. This unexpected observation revealed that the chaperone machinery that facilitates protein folding and secretion can also serve to attenuate PAMP signaling by affecting PRR stability.

Additionally, biochemical studies showed the rice CERK1 interacts with HSP90 and its cochaperone Sti1 in the ER en route to the plasma membrane (Chen et al., 2010). In tobacco, N protein-mediated virus resistance also requires the up-regulation of multiple ER components, in part through the receptor-like kinase IRK (Caplan et al., 2009). Therefore, the ER is involved in the biogenesis of a wide array of receptor-like membrane proteins involved in defense responses.

Whether the correct glycosylation pattern serves solely as a marker for the quality-control CRT3/UGGT cycle or also contributes to the functioning of the mature protein is another active area of research. Recently, a point mutation in a conserved N-glycosylation site in EFR was found to abolish its ligand binding, even though the mutant protein was properly localized to the plasma membrane (Haweker et al., 2010). This finding supports the theory that the sugar chain modification may directly affect protein function.

Supplying the Troops

The involvement of the secretory pathway in the plant immune response makes logical sense, since most plant pathogens, whether eukaryotic or prokaryotic, are extracellular. The colonization of plants by fungal pathogens starts with the germination of fungal spores on the plant epidermis. The germination tube then forms an appressorium, which attempts to penetrate the plant cell wall. If this attack is successful, the appressorium differentiates into branched feeding structures, called haustoria, in close contact with the host plasma membrane. This intimate host–fungal interaction can be highly specific: *Blumeria graminis* f. sp. *hordei*, the causal agent of powdery mildew on barley, cannot penetrate *Arabidopsis*, suggesting that *Arabidopsis* may have mechanisms to arrest the fungal infection at an early stage. Therefore, *Arabidopsis* is known as a 'non-host' of *B. g. hordei*.

Genetic studies revealed that the non-host resistance of *Arabidopsis* to *B. g. hordei* depends on PEN1 (also known as AtSYP121), a plasma-membrane-localized syntaxin (Collins et al., 2003). The PEN1–SNAP33–VAMP721/722 complex, which normally plays a role in development, is co-opted for disease resistance to direct exocytosis at the site of fungal penetration (Kwon et al., 2008b). PEN1-mediated exocytosis contributes to the formation of papillae, the buildup of cell wall material as a reinforced physical barrier underneath the fungal penetration site. Other cargos may include chemicals toxic to the fungus, although the exact nature of the compounds delivered is unknown.

In addition to fungi, bacterial pathogens of plants also proliferate in the intercellular space. A major defense mechanism against these pathogens involves the secretion of the antimicrobial PR proteins, whose synthesis is under the control of the salicylic acid (SA) signaling pathway. The secretion of PR proteins is unlikely to occur via PEN1, as the *pen1* mutation has no effect on the outcome of infection by the pathogenic bacterium *Pseudomonas syringae* (Kwon et al., 2008b). Instead, experiments in tobacco showed that PR protein secretion is mediated by another syntaxin, SYP132 (Kalde et al., 2007).

In a microarray experiment to characterize transcriptional changes induced by SA and the transcriptional regulator NPR1, we detected the up-regulation of a number of protein secretory pathway genes. They include not only components of the ER-localized protein import and processing machinery, such as the translocon, BiP, GRP94, PDIs, CRTs, and CNXs (Figure 1A), but also components up- and down-stream of the ER (Wang et al., 2005). Further genetics studies showed that the increased secretory capacity transports PR proteins. Compromising these pathway components led to defects in PR protein secretion and impaired defense response. Finally, the coordinated induction of protein secretory genes seems

to be achieved by an unknown transcription factor through a novel *cis*-element in their promoter regions.

The differential requirement for PEN1/SYP121 and SYP132 in disease resistance predicts that these two sub-classes of syntaxins recognize distinct vesicles. Whereas SYP132 mediates the delivery of PR proteins, PEN1/SYP121 is likely to be responsible for depositing cell-wall-reinforcing materials and possibly for secreting antifungal toxins. It also remains to be tested whether the VAMP partners on the secretory vesicles (and potentially the SNAPs) are different. More evidence for the multifaceted roles of syntaxins in defense comes from a study of SYP122, the closest relative to PEN1 in *Arabidopsis*: when *SYP122* was knocked out together with *PEN1*, the double mutant activated multiple disease-resistance pathways, suggesting that this pair of syntaxins normally suppresses defense responses separate from penetration resistance (Zhang et al., 2007).

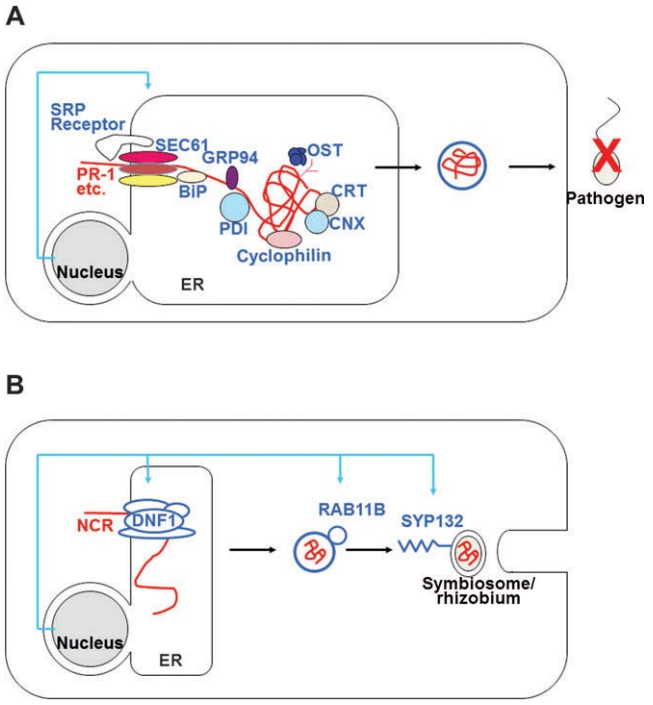

Figure 1. Boosting Protein Secretion during Defense and Symbiosis.
(A) During defense against bacterial pathogens in *Arabidopsis*, the master regulator, NPR1, not only induces the production of antimicrobial pathogenesis-related proteins such as PR-1, but also directs the expansion of protein import and folding capacities in the ER.
(B) In nitrogen-fixing symbiosis, the host *Medicago truncatula* uses another set of antimicrobial proteins, the NCR peptides, to control the developmental program of the symbiont. The efficient delivery of NCR peptides is achieved through a nodule-specific secretory pathway. The small GTPase RAB11B is associated with protein trafficking (de Graaf et al., 2005). Blue lines in both panels indicate transcriptional up-regulation. BiP, luminal binding protein (ER-resident HSP70); CNX, calnexin; CRT, calreticulin; GRP94, glucose-responsive protein 94 (ER-resident HSP90); OST, oligosaccharyltransferase; PDI, protein disulfide isomerase; PR-1, pathogenesis-related protein-1; SEC61, translocon complex; SRP, signal recognition particle.

CIVILIAN FUNCTIONS

Most of the microbes associated with plants are beneficial: 80% of land plants are engaged in arbuscular mycorrhizal (AM) symbiosis with beneficial fungi. Legume species can forge an additional symbiosis with nitrogen-fixing bacteria in a specialized root organ called the nodule. In this symbiosis, the bacteria are delivered into the host cytoplasm through an 'infection thread' (IT). Once inside, the bacteria reside in a membrane-bound structure, symbiosome (Figure 2A), where the bacteria convert atmospheric nitrogen into ammonium in exchange for host photosynthates.

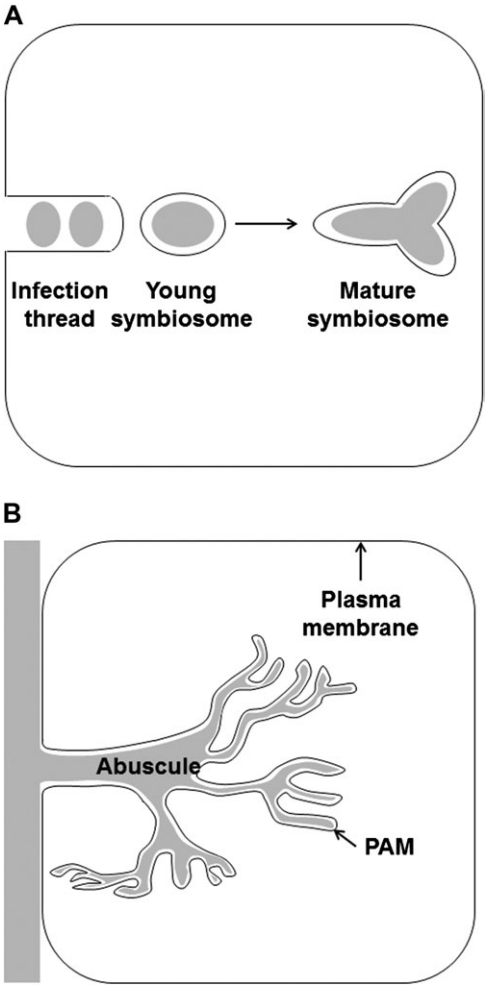

Figure 2. Microbial Symbioses in the Root.
(A) In nitrogen-fixing symbiosis, the bacteria are delivered by the IT (infection thread) but are then detached from the IT to form a completely surrounded symbiosome. Young symbiosomes are spherical, but, in *Medicago truncatula*, mature symbiosomes are often branched.
(B) In the more ancient AM (arbuscular mycorrhizal) symbiosis, the host and the fungus produce an intimate and highly branched structure (the arbuscule), where nutrients are exchanged across the interface. The peri-arbuscular membrane (PAM), although physically continuous with the plasma membrane, has a distinct makeup of proteins.

Helping the Neighbors

The two abovementioned forms of symbiosis are evolutionarily related, with AM symbiosis being more prevalent and more ancient. In this interaction, the fungus acquires soil nutrients for the plant, mainly phosphorus, in exchange for fixed carbon from the host. Such a transaction is accomplished through a highly branched fungal penetration structure (the arbuscule), surrounded by the host membrane (Figure 2B). Aside from signaling molecules (Akiyama et al., 2005) and nutrients, the identities of other molecules, particularly proteins, secreted by the host into the plant–fungal interface are largely unknown. However, it is known that many integral membrane proteins from the host are localized to this peri-arbuscular membrane (Javot et al., 2007; Pumplin and Harrison, 2009). Although not 'secreted' in the strict sense, these proteins would be inserted into the ER membrane and travel through the secretory pathway for proper targeting. Recent cell biology studies have visualized the ER and the Golgi in close proximity to the arbuscules, hinting at active protein secretion throughout the course of the plant–fungal interaction (Pumplin and Harrison, 2009).

Keeping Peace at Home

Because intracellular lifestyles are rare among plant-associated microbes, the organellar identity of the nitrogen-fixing symbiosome has long been a matter of debate. This issue is not trivial, as it relates to how the host tolerates and maintains intracellular bacteria. Although the manner of bacterial uptake superficially resembles endocytosis, nascent symbiosomes do not acquire endosomal markers (Limpens et al., 2009). In contrast, the plasma membrane marker SYP132 labels the symbiosome membrane immediately after the release of the bacteria into the host cytoplasm and persists throughout the life of the symbiosome (Catalano et al., 2007; Limpens et al., 2009). This observation argues that the symbiosome, at least in part, has the unusual property of being an extracellular compartment within the cell; however, the biological role of SYP132 on the symbiosome membrane is unknown. Similarly, a number of symbiosome-localized host proteins were shown to contain signal peptides. However, it was unclear whether the delivery of these proteins to the symbiosome requires the protein secretory pathway.

Further evidence for the requirement of protein secretion during nitrogen-fixing symbiosis came from forward genetic studies. The *Medicago truncatula dnf1* mutant forms ineffective nodules (Starker et al., 2006). Although bacteria can be released into the *dnf1* cytoplasm, their development is soon arrested. That *DNF1* encodes a subunit of the signal peptidase complex establishes that symbiosome development and function require an intact protein secretory pathway (Wang et al., 2010). Co-up-regulated with *DNF1* during nodule formation are genes encoding additional components of the protein secretory pathway, further supporting the importance of this pathway in nitrogen-fixing symbiosis (Figure 1B).

The cloning of *DNF1* did not immediately reveal the identities of the cargo proteins directly responsible for symbiosome development. It remained possible that the effect of *DNF1* was indirect. This concern was addressed by the work of Van de Velde et al. (2010) on a large family of *Medicago truncatula* proteins that are induced in nodules, have cleavable signal peptides, contain a number of cysteines at regular intervals, and are delivered to the bacteria inside the symbiosome (Figure 1B). In the *dnf1* mutant, these so-called nodule-specific, cysteine-rich (NCR) peptides retain their signal peptides, and are consequently mis-localized, indicating they are cargo proteins of the nodule-specific secretory pathway. The NCRs are related to an ancient class of antimicrobial proteins known as defensins and can block bacterial division both *in planta* and in culture. During the symbiosis with *M. truncatula*, the bacteria keep duplicating their genomes, but, due to the presence of NCRs, no longer undergo cytokinesis. This results in endoreduplication—a developmental dead-end for the bacteria. This could benefit the host because terminally differentiated bacteria remain metabolically active, but lack the potential to multiply excessively. The unexpected involvement of antimicrobial mechanisms in symbiosis suggests that nitrogen-fixing symbiosis is akin to a domestication process, in which one partner (the host plant) dominates. It is worth noting that *dnf1* nodules do not contain an appreciably higher bacterial titer, which argues that the secretion of additional host proteins is required to promote bacterial growth.

Investigating the expression profile of DNF1 led to the discovery that multiple components of the protein secretory pathway are coordinately up-regulated during symbiosis (Maunoury et al., 2010; Wang et al., 2010). Some functions seem to be shared with the *Arabidopsis* defense-associated protein secretory pathway controlled by NPR1, such as translocon subunits, PDIs, and cyclophilins. There are also some differences. For example, CRT and CNX are not up-regulated in nitrogen-fixing symbiosis, indicating that secretory proteins processed by the DNF1 pathway may not require glycosylation.

MANAGEMENT

Transcriptional Control of Protein Secretion during Defense and Symbiosis

It is intriguing that, in both nitrogen-fixing symbiosis and defense, the protein secretory pathway is up-regulated at the transcriptional level (Figure 1). Is there a common regulatory mechanism? In the case of bacterial resistance, there is evidence that the transcriptional cofactor NPR1 facilitates the recruitment of an unknown protein to the novel *cis*-element (GAAGAAGAA) present in the promoter regions of protein secretory pathway genes (Wang et al., 2005). An enrichment of the same element has not been noticed in the genes involved in nitrogen-fixing symbiosis, and the corresponding *trans*-factor(s) for symbiosis is also unknown. It thus remains to be determined whether the similarity in transcriptional up-regulation results from evolutionary conservation or convergence.

Delivery of Secretory Material to the Site of Microbial Interaction

The common requirement for secreted proteins in defense and symbiosis poses important challenges to the host: when accommodating a nitrogen-fixing bacterium, the cell has two potential target membranes: the plasma membrane and the newly built symbiosome membrane. How are materials for symbiosis, such as the NCR peptides, delivered to the symbiosome but not the extracellular space? Similarly, in both forms of fungal interactions (symbiotic and pathogenic), the deposition of membrane proteins, cell wall materials, and presumably secreted proteins are all clearly restricted to the site of contact. How does the host maintain a distinct interface that is physically continuous with the rest of the plasma membrane?

Although, at present, we do not fully understand the targeting specificity of secretion in plant–microbe interactions, it is an issue of cell polarity. Therefore, future studies could benefit from knowledge gained from the work on other polarized systems, such as cilia, epithelial membranes, and cell division in budding yeast (Lew, 2005; Mellman and Nelson, 2008; Wallingford, 2010). Establishing and maintaining polarity require two functions: the marking of the target membrane domains and the differential delivery of secretory vesicles destined for these domains. The presence of a microbe could serve as the initial signal that recruits membrane markers such as PEN1. The vesicles themselves are defined by the cargo molecules and presumably their interactions with vesicle coat proteins. Directional trafficking of the vesicles almost certainly requires the cytoskeleton and associated motor proteins. Different forms of plant–microbe interactions share common features: the structural similarity between a pathogenic fungal interaction (appressorium) and a symbiotic one (arbuscule) is obvious. The structures to accommodate fungal and bacterial symbionts are also strikingly similar: an incipient arbuscule may be comparable to an IT for rhizobium (Harrison, 1998). Moreover, a branch of the arbuscule bears uncanny resemblance to a symbiosome (Figure 2). Beneath these superficial similarities may lie common cell polarity machinery and advances in one field could present insights into the other. For example, characterizing the peri-arbuscular membrane biochemically is technically challenging, but studies of the symbiosome membrane are relatively straightforward (Hernandez et al., 1996) and could facilitate hypothesis-driven research in the former.

CONCLUDING REMARKS

It has become increasingly clear that many different forms of host–microbe interactions have much in common at the molecular level. Symbioses and diseases (or the defense against them) may merely represent the extremes of a continuum of interactions connected by shared mechanisms, where the devil is in the details of the cellular machinery, such as the specific molecules being delivered to the interface. Even more tantalizing is the discovery that some molecules participating in host–microbe interactions, including secreted and secretion-mediating proteins, are also involved in plant reproduction, specifically the interaction between the male (pollen) and female (pistil) components of the same species (Kachroo et al., 2001; Samuel et al., 2009; Kessler et al., 2010). As both types of processes rely on direct cell-to-cell contact, plants appear to govern all these biotic interactions through the protein secretory pathway.

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