THE ROLE OF PLANT MEMBRANE PROTEINS IN LEGUME-RHIZOBIA SYMBIOSIS

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ABSTRACT

Symbiotic nitrogen fixation occurs when rhizobia bacteria infect the roots of legume plants, resulting in the formation of a specialized organ called a nodule. In this mutualistic symbiosis, bacteria provide the plant with nitrogen, and the plant provides the bacteria with carbon sources. This metabolic exchange occurs inside the root nodules where bacteria reduce (or “fix”) molecular dinitrogen to ammonia, a form of nitrogen that plants can use to synthesize amino acids and proteins.

Legume-rhizobia symbiosis initiates with plant recognition of the bacterial signaling molecule Nod Factor (NF). After plant perception of NF, bacteria infect plant roots through host-derived infection threads and are eventually endocytosed into host cells. Inside the host cell, the bacteria remain surrounded by a host membrane and differentiate into their nitrogen-fixing form. A great deal is known about NF perception and signaling, but the mechanisms by which infection threads form and by which bacteria are endocytosed into host cells remain elusive.

To identify genes required for infection or bacterial endocytosis, I used a candidate gene approach in the model legume *Medicago truncatula*. I searched the *M. truncatula* genome for regions with homology to endocytosis and membrane shaping genes in plants and other organisms. I identified two *M. truncatula* flotillin-like genes, FLOT2 and FLOT4, which are up-regulated in response to the *M. truncatula* symbiont, *Sinorhizobium meliloti*. Flotillins in animals have been implicated in actin polymerization, maintenance of cell-cell contacts, membrane trafficking and pathogenesis. I silenced FLOT2 and FLOT4 using RNAi and amiRNAs and found a non-redundant requirement for both genes in symbiosis. When FLOT4 was silenced, infection threads typically aborted in root hairs. FLOT2-silenced plants formed fewer nodules and infection threads. This work implicates plant flotillins in legume-rhizobia symbiosis and suggests that flotillins in plants and animals may have a common function.

NF recognition in *M. truncatula* requires the receptors NFP and LYK3. Each receptor is required for separate downstream responses: NFP is necessary for all
known responses to bacteria, while *LYK3* is required for infection. A leucine-rich repeat receptor-like kinase *DMI2* acts downstream of *NFP*. Using GFP-tagged proteins, I localized the symbiotic receptor kinases *DMI2* and *LYK3*. Both proteins had punctate distributions associated with root hair plasma membranes. After bacterial inoculation, both *LYK3*:GFP and *DMI2*:GFP were present on intracellular vesicles. *LYK3*:GFP persisted in infected cells and localized to infection thread membranes. The *DMI2*:GFP signal was nearly absent by one day post inoculation. These data are consistent with a role for *LYK3* in infection and a role for *DMI2* in signaling.

I found that like *LYK3* and *DMI2*, GFP-tagged *FLOT4* is associated with the plasma membrane and has a patchy distribution. Upon inoculation with *S. meliloti*, *FLOT4*:GFP puncta become more diffuse and redistribute to form a cap at the tips of elongating root hairs. I investigated the dependence of *FLOT4* distribution on NF perception and signaling via symbiotic receptors *NFP*, *LYK3*, and *DMI2*. I found that *FLOT4*:GFP has a decrease in puncta density specific to the putative dead kinase allele of *LYK3*, *hcl-1*. I co-expressed *LYK3*:GFP and *FLOT4*:mCherry and found that in buffer-treated root hairs, there is little co-distribution of the two proteins, and tagged *LYK3* puncta are dynamic while *FLOT4* is relatively stable. After inoculation, I found an increase in *LYK3*:GFP and *FLOT4*:mCherry co-localization and that *LYK3*:GFP shares the stable distribution shown by *FLOT4*:mCherry. The similarities in mutant phenotypes, protein localization, and protein dynamics suggest that *FLOT4* and *LYK3* may be components of a shared complex.

This work indicates that protein arrangement within plant membranes is complex and is altered by perception of a symbiotic bacterium. This work suggests that redistribution of plant membrane proteins upon signal perception may serve to compartmentalize cellular processes.
PREFACE

Publication of chapters and role of author


The transgenic *Medicago truncatula* lines used in Chapters 3 and 4 were generated by Brendan Riely using a protocol developed by David Tricoli in Doug Cook’s Lab at University of California, Davis. I performed all experiments and generated all remaining materials.
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CHAPTER 1: Introduction and contributions

Overview of legume-rhizobia symbiosis

Plants in the family Fabaceae (legumes) form symbiotic relationships with Gram negative α-proteobacteria including the genera Sinorhizobium, Rhizobium, Mesorhizobium, and Bradyrhizobium. These bacteria live in association with plant roots inside morphologically unique structures called nodules. The interaction between plant and bacterium is viewed as mutualistic: the bacteria reduce (or “fix”) molecular dinitrogen to ammonia, a form of nitrogen that plants can utilize; in exchange, the plants supply the bacteria with carbon sources. Through this intimate metabolic partnership, both plant and bacteria benefit.

Of all biologically-available fixed nitrogen in soil, about half is formed via biological fixation (Zahran, 1999); nearly half of biological nitrogen fixation is symbiotic (Werner and Newton, 2005). Naturally occurring forms of nitrogen are insufficient to sustain agriculture at current levels of production and as a consequence, use of synthetic ammonium fertilizer is necessary. Industrial fertilizer production is costly and requires large inputs of fossil fuel. In 2008, nitrogen fertilizer production was the eighth largest source of atmospheric carbon dioxide (E.P.A., 2010). Post-production field application of synthetic fertilizers results in pollution in the form of additional greenhouse emissions and fertilizer runoff. By understanding symbiotic nitrogen fixation, biological nitrogen fixation may be improved and reduce the need for synthetic nitrogen fertilizer.

In addition to its importance to agronomic sustainability, symbiotic nitrogen-fixation provides a genetically tractable framework to study host-microbe interactions. Several rhizobia-legume pairs have both genetic and molecular tools available including microarrays, mutant libraries, and genome sequences. Model rhizobia-legume pairs include (1) Medicago truncatula, a diploid relative of alfalfa, and its bacterial symbiont Sinorhizobium meliloti and (2) Lotus japonicus and its symbiont Mesorhizobium loti. Experiments in these model systems have elucidated some of the
genetic and molecular mechanisms by which bacteria communicate with and infect eukaryotes.

Studies of legume-rhizobia symbioses have described developmental events during the establishment of symbiosis. Symbiosis initiates with a signal exchange between legumes and bacteria. Plant roots secrete flavonoids, which cause induction of bacterial genes required for synthesis of a lipochitooligosaccharide called Nod Factor (NF) (Peters et al., 1986). NFs act as species-specific signals to promote plant nodule development (Debellé et al., 2001; Oldroyd et al., 2001b). In response to bacteria or purified NF, specialized plant root epidermal cells, called root hairs, grow to form a curl around a colony of bacteria (or spot of applied NF). Bacteria then enter the plant root through plant-derived intracellular structures called infection threads.

Bacteria divide and penetrate nodules inside infection threads until eventually they are released into host cells via an endocytosis-like event. Inside the plant host cell, bacteria remain surrounded by a host-derived membrane and replicate only a few times. Following a series of differentiation events, the bacteria (now called “bacteroids”) synthesize nitrogenase enzyme allowing them to fix nitrogen.

**Bacterial NF production and plant NF recognition**

Purified NF is sufficient to induce plant morphological and transcriptional changes. Within minutes of NF application, the root hair plasma membrane depolarizes (Ehrhardt et al., 1992). Within the first hour after NF treatment, there are detectible oscillations in nuclear calcium levels (called “calcium spiking”) in root hairs and epidermal cells (Ehrhardt et al., 1996). A number of phenotypic changes occur in root hairs following calcium spiking. First, actively elongating root hairs stop growing and swell. The root hairs then reinitiate tip growth and grow to form a curl around a bacterial colony. These events coincide with transcriptional changes that are largely NF-dependent (Mitra et al., 2004a). When applied to some species such as *M. sativa* (alfalfa), NF is sufficient to induce nodule formation, although these nodules are not occupied by bacteria (Debellé et al., 2001).
The structure for NF and genes involved in its biosynthesis have been well characterized (reviewed in Spaink et al., 1998). The NF backbone is a tetramer of β-1,4-linked N-acetyl glucosamine (Roche et al., 1991a). Species-specific decorations on the chitin backbone of *S. meliloti* NF include: (1) a C16:2 N-acylation on the terminal non-reducing N-acetyl glucosamine residue, (2) an C6-O-acetylation on the terminal non-reducing glucosamine, and (3) a C6-sulfate modification on the reducing residue. Modified forms of *S. meliloti* NF alter host specificity and elicit altered host responses. *NodH* mutants lack the sulfate modification and elicit no measurable plant response (Roche et al., 1991b; Ehrhardt et al., 1995; Schultze et al., 1995). In contrast *nodF* mutants (which have a modified acyl chain; Shearman et al., 1986; Demont et al., 1993) and *nodL* mutants (which lack O-acetylation; Downie, 1989) trigger root hair deformation but have reduced infection (Ardourel et al., 1994). *NodFL* double mutants are completely unable to infect (Ardourel et al., 1994). *NodH* mutants fail to induce calcium spiking on *M. sativa* while *nodFL* double mutants are able to trigger calcium spiking (Wais et al., 2002). The observation that altering NF structure either abolishes all plant responses, or specifically abolishes infection, led to a two-receptor model in host NF perception where: (1) a less stringent “signaling” receptor controls calcium spiking and transcription and (2) a highly stringent “entry” receptor controls bacterial entry into root hairs (Ardourel et al., 1994).

The two-receptor model is supported by host genetics in *M. truncatula* which have identified two putative NF receptor loci *NFP* and *LYK3*. Both encode LysM-family receptors; LysM receptors binds chitin (Iizasa et al., 2010) so these are good candidates for binding the chitin backbone of NF. Loss of function mutations in *NFP* (in *Medicago truncatula*) and *NFRI/NFR5* (homologues in *Lotus japonicus*) result in complete insensitivity of plants to NF (Wais et al., 2002; Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). *LYK3* mutants (also called *hcl*) undergo root hair deformation in response to NF but do not form root hair curls (mutants were originally called *hcl* for their root *hair curling* defect) or infection threads (Catoira et al., 2001; Limpens et al., 2003; Smit et al., 2007). *Hcl* mutants undergo few or no cortical cell divisions (Catoira et al., 2001). Weaker mutant alleles of *LYK3* and *LYK3* RNAi
plants form infection threads that fail to penetrate the root cortex (Limpens et al., 2003; Smit et al., 2007). Based on these observations, NFP is alleged to encode the low stringency receptor which controls calcium spiking and gene expression, and LYK3 encodes the high stringency receptor which controls bacterial entry into root hairs (Smit et al., 2007).

Forward genetics in the model legumes *M. truncatula* and *Lotus japonicus* have identified what are likely the major components of the NFP signaling pathway. The NFP receptor signals through a Leucine-rich repeat receptor-like kinase encoded by *DMI2* (in *M. truncatula*) and *SymRK* (*L. japonicus*) which are necessary for calcium spiking in their respective species (Endre et al., 2002; Stracke et al., 2002). Also necessary for calcium spiking are a putative nuclear ion channel *DMI1* (in *M. truncatula*) and *CASTOR/POLLUX* (*L. japonicus*) (Ané et al., 2004; Riely et al., 2007; Charpentier et al., 2008) and the nucleoporins *Nup85* and *Nup133* (*L. japonicus*) (Kanamori et al., 2006; Saito et al., 2007). Calcium spiking likely controls activity of a calcium/calmodulin-dependent protein kinase (CCaMK) encoded by *DMI3* (*M. truncatula*) and *Sym15* (*L. japonicus*) (Levy et al., 2004; Mitra et al., 2004b; Tirichine et al., 2006). Gain of function alleles of *CCaMK* in both *L. japonicus* and *M. truncatula* are sufficient for nodule organogenesis in the absence of bacteria (Gleason et al., 2006; Tirichine et al., 2006). In response to NF signal transduction, CCaMK phosphorylates two GRAS-family transcription factors NSP1 and NSP2 which form heterodimers that directly bind the promoters of NF-responsive genes (Catoira et al., 2000; Oldroyd and Long, 2003; Kaló et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Murakami et al., 2006; Hirsch et al., 2009). *NSP1* and *NSP2* are necessary for all known NF-dependent transcriptional changes (Mitra et al., 2004a). A downstream transcription factor *ERN* is required for a subset of NF-dependent gene expression and CCaMK-dependent nodule formation (Middleton et al., 2007). These genes likely represent the majority of components required for NF signal perception at the host cell membrane and NF-induced signal transduction to activate gene expression and nodule organogenesis.
Crosstalk between the NF signaling and entry pathway likely occurs, as plants must coordinate infection with nodule formation. Crosstalk is supported by the phenotypes of several mutants. *M. truncatula NIN* encodes a putative transcriptional regulator (Schauser *et al.*, 1999; Borisov *et al.*, 2003; Schauser *et al.*, 2005) and is required for CCaMK-dependent nodule organogenesis and infection events but not NF-dependent transcriptional changes suggesting that it may coordinate signals through both NF receptors (Marsh *et al.*, 2007). *L. japonicus* CCaMK is able to phosphorylate CYCLOPS (Yano *et al.*, 2008), a protein of unknown function which contains a nuclear localization signal and a coiled-coil domain. CYCLOPS is dispensable for CCaMK-dependent nodule organogenesis but not for infection and may represent a branch point in NFP-signaling (Capoen and Oldroyd, 2008). The mechanisms by which *NIN, CYCLOPS* and other genes mechanistically enable crosstalk between nodule formation and infection pathways are a focus of ongoing work.

**Plant requirements for infection**

The genetic requirements for infection and invasion are not as well defined as early signaling events. A number of Fix' mutants (which form nodules but do not support nitrogen fixation) have been characterized based on microscopic observation of the progress of bacterial infection and monitoring nitrogen fixation (Utrup *et al.*, 1993; Schauser *et al.*, 1998; Morzhina *et al.*, 2000; Starker *et al.*, 2006). These mutants can be divided into two major groups (1) infection mutants that form nodules but have few infection threads and no bacterial release or (2) invasion mutants that are able to support bacterial infection and release but not nitrogen fixation. Genes required for later symbiotic events are still in early stages of characterization; it unclear how they fit together in pathways and how later events are affected by early signal transduction.

Genes required for infection are potential components of the LYK3 entry pathway. The E3-ubiquitin ligase PUB1 negatively regulates infection likely by targeting LYK3 for degradation (Mbengue *et al.*, 2010). A second putative E3-ubiquitin ligase LIN1 is required for infection, suggesting that protein degradation
both positively and negatively regulates infection (Kiss et al., 2009). A plant-specific gene RPG encodes a nuclear-localized protein with a long coiled-coil domain and is required for infection thread polar growth (Arrighi et al., 2008). Infection thread growth in L. japonicus requires the genes NAP1 and PIR1, which are involved in actin reorganization (Yokota et al., 2009). A plant-specific membrane protein, remorin, is also required for infection (Lefebvre et al., 2010). While all of these genes appear to be required for normal infection, how they work co-operatively and integrate signals from the LYK3-dependent NF perception pathway remains to be seen.

After bacteria are endocytosed into their legume host cells, several genes involved in protein secretion and vesicle trafficking are required for symbiosome maturation. DNF1 encodes a nodule-specific signal peptidase required for secretion of antimicrobial cysteine-rich peptides which control bacterial differentiation (Van de Velde et al., 2010; Wang et al., 2010). The syntaxin SYP132 localizes to symbiosome membranes (Catalano et al., 2007), and a RAB7 homologue is required for symbiosome senescence in mature nodules (Limpens et al., 2009). These are few of what will certainly be many proteins required for endocytosis, trafficking and maintenance of symbiotic bacteria within their host cells.

**Bacterial invasion factors**

A number of bacterial genera in the α-proteobacteria can cause chronic infections of mammals (Brucella, Bartonella and Rickettsia sp.), plants (Sinorhizobium, Bradyrhizobium, and Agrobacterium sp.), and insects (Wolbachia sp.) (Sallstrom and Andersson, 2005). Although these closely-related bacteria infect and persist in different host environments, they share a number of genes required for infection including production of the correct types and amounts of cell surface polysaccharides (reviewed in Batut et al., 2004; Sallstrom and Andersson, 2005). Conserved virulence factors suggest the possibility of conserved host targets.
A reverse genetics approach to identify genes required for infection

Forward genetics may be limited in its ability to uncover plant genes required for the infection and invasion processes. This may occur if infection and invasion share common mechanisms with essential host functions. Detailed electron microscopy studies have described the cytology of rhizobia entry into legume root hairs (Newcomb, 1976). We observed that invagination of the root hair plasma membrane during infection thread initiation resembles a partial endocytosis event where the membrane begins to bud but instead of pinching off, elongates through the cell. When the infection thread reaches the end of the root hair cell, the bacteria release into the intercellular space and a new membrane invagination occurs in the underlying cell layers (reviewed in Gage, 2004). Like endocytosis and other membrane shaping events, infection thread growth in *Lotus* has been shown to depend on actin reorganization (Yokota *et al.*, 2009). Other genes involved in infection may not be revealed in forward genetic screens because they are essential, have pleiotropic phenotypes, or have genetic redundancy.

We hypothesized that symbiotic infection thread formation and bacterial endocytosis may have co-opted gene function normally involved in vesicle trafficking, endocytosis and membrane shaping, and that these might represent common host targets for intracellular bacteria. We employed a reverse genetics approach to identify plant endocytosis and membrane-shaping genes required for bacterial infection and invasion. We conducted a reverse genetics screen to identify genes that are (1) associated with infection or survival of *Brucella* in animal cells, (2) conserved in plants, (3) transcriptionally regulated during nodulation, and (4) expanded gene families in legumes. *M. truncatula* flotillins met all of these criteria, so their role in symbiosis was explored further (Chapters 2 and 4).

**Flotillins and membrane rafts in animals**

Flotillins were first identified in a search for membrane proteins that co-fractionate with detergent insoluble membrane fractions (Bickel *et al.*, 1997). At the same time, they were identified as proteins upregulated during axon regeneration in zebra fish
retina and as a result, named Reggies (Schulte et al., 1997). Animals have two flotillin/reggie-family proteins, FLOT1 and FLOT2, that are ~50% identical. Both proteins have patchy distributions associated with cellular plasma membranes and localize to several intracellular compartments (Solomon et al., 2002; Glebov et al., 2006; Langhorst et al., 2007). FLOT1 and FLOT2 lack trans-membrane domains and are membrane-associated by N-terminal fatty acid modifications (Morrow et al., 2002; Neumann-Giesen et al., 2004). The N-terminus of FLOT contains a stomatin/prohibitin/flotillin/HflK (SPFH) domain (Browman et al., 2007). Several SPFH domain-containing proteins can bind cholesterol (Huber et al., 2006), and it has been proposed that by binding cholesterol, they may play a role in structuring the plasma membrane (Browman et al., 2007). FLOT1 and FLOT2 co-localize (Langhorst et al., 2007) and form hetero- and homo-oligomers which are mediated by C-terminal coiled-coil domains (Neumann-Giesen et al., 2004; Solis et al., 2007).

FLOTs are frequently used as markers for the detergent resistant membrane (DRM) fraction, a technique which for many years was used to prove that a protein localized to “lipid rafts” (Lingwood and Simons, 2010). It was believed that proteins closely associated with cholesterol and sphingolipids would be shielded from detergents allowing them to be isolated from the remainder of the plasma membrane, and that these lipid-rich regions of plasma membrane corresponded to functionally active membrane rafts. However, it has been shown that detergent-based methods can introduce artifacts, and the DRM fraction may not correlate with protein distribution in a living cell (Munro, 2003; Lingwood and Simons, 2010). As a consequence, DRM localization does not necessarily have functional implications and two proteins that co-partition in the DRM fraction may not co-localize in a living cell. Membrane rafts (or lipid rafts) are defined as “small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes” (Pike, 2006). Despite the controversy and confusion surrounding the DRM/membrane raft debate, the domains marked by FLOTs meet the criteria for membrane rafts. FLOT puncta are small (50-100 nm; Stuermer et al., 2001) and likely cholesterol enriched (shown by indirect means; Kokubo et al., 2003). FLOT puncta co-distribute with
many of their interaction partners and as such, FLOT-containing membrane puncta can be said to compartmentalize cellular processes (see below).

Membrane puncta marked by FLOTs appear to be involved in compartmentalization of cellular processes. FLOTs co-distribute and can interact with cortical f-actin, Src kinases and the CAP adaptor protein (Liu et al., 2005). Via these interactions, FLOTs mediate membrane shaping events including membrane budding, actin-mediated neuronal differentiation, and filopodia formation (Baumann et al., 2000; Neumann-Giesen et al., 2004; Frick et al., 2007; Langhorst et al., 2008a). FLOTs co-localize with and are important for the function of several GPI-anchored proteins (Stuermer et al., 2001; Deininger et al., 2003; Reuter et al., 2004; Stuermer et al., 2004). FLOT1 and FLOT2 are both targets of the Fyn kinase which is required for GPI protein signaling (Neumann-Giesen et al., 2007; Riento et al., 2009).

The molecular function of FLOTs in signaling remains elusive. It has been suggested that FLOTs are required for receptor-mediated endocytosis of cholera toxin and epidermal growth factor (EGF) and may define a clathrin-independent endocytotic pathway (Glebov et al., 2006; Neumann-Giesen et al., 2007; Riento et al., 2009). However, while FLOTs are required for receptor-mediated signaling in response to many ligands, there are several examples where they are not required for endocytosis of the receptor (Katanaev et al., 2008; Langhorst et al., 2008b; Pust et al., 2010). There is evidence the role of FLOTs in receptor signaling may not be directly in receptor-mediated endocytosis (Schneider et al., 2008; Pust et al., 2010). There is also evidence that FLOTs are required for retrograde transport of bacterial toxins (Pust et al., 2010). It has been proposed that FLOTs are involved in recruitment and assembly of membrane proteins at plasma membrane cell-cell contacts or during tip growth (Langhorst et al., 2007; Stuermer, 2010). However, not all receptors that require FLOTs for their signaling also require FLOTs for their plasma membrane association (Glebov et al., 2006). More experiments are required to elucidate the exact function of FLOTs in signaling, and the existing models need to be refined to account for emerging flotillin behaviors.
FLOTs in animals have been implicated in bacterial pathogenesis and toxin-induced disease. Upon uptake into animal cells, *Brucella* is surrounded by a FLOT-positive host membrane (Watarai *et al.*, 2002; Arellano-Reynoso *et al.*, 2005). Silencing *FLOT1* has been shown to inhibit clathrin-independent, receptor-mediated endocytosis of cholera toxin (Glebov *et al.*, 2006). FLOTs are also required for the full toxicity of bacterial Shiga toxin and for the plant toxin ricin (Pust *et al.*, 2010). This suggests that pathogens may co-opt FLOT function, or the function of FLOT-associated proteins, to gain entry into host cells and to cause disease.

Flotillins are conserved across kingdoms, although their low sequence identity suggests FLOTs in diverse kingdoms may have adopted similar 3-dimensional structures via convergent evolution (Rivera-Milla *et al.*, 2006). The crystal structure of an archael SPFH domain-containing protein is highly similar to animal FLOT2 (Yokoyama *et al.*, 2008). Bacterial flotillins have been characterized and have a punctate, membrane-associated distribution (Donovan and Bramkamp, 2009; Lopez and Kolter, 2010); in *Bacillus*, flotillin mutants show a delay in sporulation (Donovan and Bramkamp, 2009) and defects in signaling via a membrane-associated histidine kinase (Lopez and Kolter, 2010). These results suggest that receptor signaling may be a conserved function of SPFH domain-containing proteins.

**Flotillins are conserved in plants**

Bioinformatic analysis predicts that plant flotillin-like proteins share some features with animal FLOT2 including two N-terminal hydrophobic stretches, a palmitoylation site at Cys-34, and a C-terminal coiled-coil domain (Rivera-Milla *et al.*, 2006). Plant flotillin-like genes are nearly all annotated as nodulins (a generic name for a gene that is expressed uniquely in nodules). This annotation is due to the early identification of a plant flotillin-like gene, GmNod53b, that is induced in soybean nodules (Winzer *et al.*, 1999). Proteomic analyses of the peribacteroid membranes in soybean and pea nodules identified peptide sequences identical to GmNod53b (Panter *et al.*, 2000; Saalbach *et al.*, 2002). An *Arabidopsis* flotillin is upregulated in roots in response to fungal chitin and bacterial flagellin, both of which elicit a defense response (Millet *et al.*...
These results indicate that flotillins are conserved in plants and likely have a role in plant-microbe interactions.

Contributions
In Chapter 2, I describe the characterization of *M. truncatula* flotillins (FLOTs) in symbiosis with *S. meliloti*. I found that *FLOT2* and *FLOT4* are required for symbiosis and that *FLOT4* has a unique requirement in infection thread elongation. This work is the first demonstration that flotillins are required for bacterial infection of any eukaryotic host. Additionally, these are the first functions ascribed to plant flotillin homologues. I showed that like their animal and bacterial counterparts, GFP-tagged *M. truncatula* FLOTs have a punctate, plasma membrane-associated distribution. This suggests that compartmentalization of plant membrane proteins may have a function during symbiosis.

I investigated the localization of receptor kinase proteins required for symbiosis and found that like GFP-tagged FLOTs, GFP-tagged symbiotic receptors have a patchy distribution associated with root hair plasma membranes. In Chapter 3, I describe localization of tagged functional symbiotic receptor kinases LYK3 and DMI2. I found that like FLOT4:GFP, LYK3:GFP localizes to infection threads in root hairs. Localization of LYK3:GFP and DMI2:GFP is discussed in the context of their proposed functions. These findings further implicate plant membrane compartmentalization in perception and transduction of signals.

I observed that FLOT4:GFP puncta have a change in distribution during the early events in symbiosis. Chapter 4 explores the localization of FLOT4:GFP in the context of known signaling events. I found that FLOT4:GFP localization is strongly affected in a *lyk3* kinase mutant. This suggests that membrane proteins themselves have a role in structuring protein distribution within plant membranes. I co-expressed tagged *FLOT4* and *LYK3* in *M. truncatula* roots and found that in the absence of bacteria, the two proteins have little co-distribution in space and time. Bacterial inoculation triggers redistribution of both FLOT4:GFP and LYK3:GFP and increases co-distribution of the two proteins. Based on the similarity in *flot4* and *lyk3*
phenotypes, the dependence of FLOT4:GFP localization on *LYK3*, and the observation that they co-localize after bacterial treatment, I propose that *FLOT4* is a component of the high stringency NF entry pathway previously defined by *LYK3* and *NIN*. These data demonstrate that changes in plant membrane protein distribution accompany root hair morphological changes in response to symbiotic bacteria.
CHAPTER 2: Plant flotillins are required for infection by nitrogen-fixing bacteria

ABSTRACT
To establish compatible rhizobial-legume symbioses, plant roots support bacterial infection via host-derived infection threads. Here we report the requirement of plant flotillin-like genes (FLOTs) in Sinorhizobium meliloti infection of its host legume Medicago truncatula. Flotillins in other organisms have roles in viral pathogenesis, endocytosis and membrane shaping. We identified seven FLOT genes in the M. truncatula genome and show that two, FLOT2 and FLOT4, are strongly upregulated during early symbiotic events. This upregulation depends on bacterial NF and the plant’s ability to perceive NF. Microscopy data show that M. truncatula FLOT2 and FLOT4 localize to membrane microdomains. Upon rhizobial inoculation, FLOT4 uniquely becomes localized to the tips of elongating root hairs. Silencing FLOT2 and FLOT4 gene expression reveals a non-redundant requirement for both genes in infection thread initiation and nodule formation. FLOT4 is uniquely required for infection thread elongation, and FLOT4 localizes to infection thread membranes. This work reveals a critical role for plant flotillins in symbiotic bacterial infection.
INTRODUCTION

Symbiotic nitrogen-fixing rhizobial bacteria live in association with legume roots inside developmentally unique structures called nodules. Bacteria penetrate nodules via plant-derived structures called infection threads. Invagination of the root hair plasma membrane during infection thread initiation resembles a partial endocytosis event where the membrane begins to bud, but instead of pinching off, elongates through the cell. As the infection thread finishes traversing the cell, the bacteria release into the intercellular space. New membrane invagination and infection thread formation take place in the underlying cell layers. Eventually, the bacteria are released into host cells via an endocytosis-like event where they remain surrounded by a host-derived membrane (Newcomb, 1976; Gage, 2004).

A bacterially-produced lipochitooligosaccharide called Nod Factor (NF) is a species-specific rhizobial signal that is recognized by the legume host. NF promotes nodule development (Debellé et al., 2001) and via signal transduction induces calcium spiking and transcriptional changes (Ehrhardt et al., 1996; Mitra et al., 2004a). Forward genetic studies in rhizobial-legume systems have revealed genes required for host NF signal perception, signal transduction, and transcriptional changes (see Oldroyd and Downie, 2008 and references therein). NF perception is mediated by LysM-family receptor-like kinases NFP and LYK3 (in Medicago truncatula; NFR1/NFR5 in Lotus japonicus). The NFP receptor induces calcium spiking via a pathway that includes a leucine-rich repeat receptor-like kinase and a putative ion channel. Calcium spiking appears to control activity of a calcium/calmodulin-dependent protein kinase and two downstream GRAS-family transcription factors NSP1 and NSP2 which interact in the plant nucleus and directly bind the promoters of nodulation genes (Hirsch et al., 2009). NSP1 and NSP2 are necessary for all known NF-dependent transcriptional changes (Mitra et al., 2004a).

A second “entry” pathway (controlling bacterial entry into root hair cells) has been proposed that includes the high stringency receptor encoded by LYK3. Loss of function lyk3 mutants undergo a small number of cortical cell divisions and form no infection threads. An additional transcriptional regulator NIN is required for nodule
organogenesis and infection events but not NF-dependent transcriptional changes. Current evidence suggests that NIN may coordinate signals through both the signaling and entry pathways (Marsh et al., 2007). Components of the proposed NF entry receptor pathway are currently limited to NIN and LYK3; how LYK3 signaling is perceived and translated into infection thread initiation is unknown.

Sinorhizobium meliloti, the nitrogen fixing symbiont of Medicago (the alfalfa genus), is a close relative of the animal pathogen Brucella. Sinorhizobium and Brucella have common genes required for infection and invasion of their hosts (Batut et al., 2004). Given these common features, we asked whether required eukaryotic host-cell factors might also be shared across kingdoms.

Upon uptake into host cells, Brucella is surrounded by a flotillin-positive membrane (Watarai et al., 2002; Arellano-Reynoso et al., 2005). Flotillins were initially used as markers for cholesterol-rich, detergent-resistant, membrane microdomains called “lipid rafts”, and are now believed to define a clathrin-independent, caveolin-independent endocytic pathway (Glebov et al., 2006). By interacting with effectors that can bind actin, flotillins mediate membrane shaping events including membrane budding, actin-mediated neuronal differentiation, and filopodia formation (Baumann et al., 2000; Frick et al., 2007; Neumann-Giesen et al., 2007; Langhorst et al., 2008a). Flotillins are also responsible for secretion and spread of long-range signaling forms of Wingless and Hedgehog in Drosophila, for insulin-stimulated glucose transport, and for epidermal growth factor signaling (Baumann et al., 2000; Neumann-Giesen et al., 2007; Katanaev et al., 2008).

Due to the importance of flotillins in pathogenesis, we investigated whether plant flotillin-like proteins might be candidates for symbiotic events from infection thread initiation and elongation to hormone transport to the final endocytosis of bacteria into their host cells. Seventeen flotillin-like proteins in 10 different plant species were identified by sequence similarity to mammalian flotillin-1 (Rivera-Milla et al., 2006). Plant flotillin-like proteins are predicted to have many features of animal flotillins including two N-terminal hydrophobic stretches, a palmitoylation site at Cys-34, and a C-terminal coiled-coil domain (Rivera-Milla et al., 2006). At the start of
this work, plant flotillin-like genes were annotated as nodulins (a generic name for a
gene that is expressed uniquely in nodules). The nodulin annotation was due to the
early identification of a plant flotillin-like gene, GmNod53b, which is induced in
soybean nodules (Winzer et al., 1999). Proteomic analyses of the peribacteroid
membranes in soybean and pea nodules identified peptide sequences identical to
GmNod53b (Panter et al., 2000; Saalbach et al., 2002). An Arabidopsis flotillin gene
was among the most strongly up-regulated genes in response to root application of
flagellin peptide Flg22 (Millet et al., 2010). A flotillin was identified in the detergent-
resistant microsomal fraction of Arabidopsis leaf cells (Borner et al., 2005). These
results imply that flotillins are conserved between plants and animals. The conserved
sub-cellular localization suggests the possibility of conserved function(s). In this work
we employ a reverse genetics approach to investigate the role of the Medicago
truncatula flotillin-like gene family (FLOTs) in symbiosis. We demonstrate that two
family members, FLOT2 and FLOT4 are required for early symbiotic events and have
non-redundant functions.
RESULTS

Identification of flotillin-like genes in *M. truncatula*

A search of the *M. truncatula* genome sequence yielded 7 genomic regions with high homology (E-value < 1e\(^{-151}\)) to the amino acid sequence of the soybean flotillin homologue GmNod53b, which we have designated *FLOT1*-7 (Table 2-1). All seven predicted ORFs have >85% identity on the nucleotide level (Figure 2-1A). *FLOT1*-5 are located within a 30 kb region of chromosome 3 (Figure 2-1B). While all other plant flotillin-like proteins described thus far have a conserved predicted palmitoylation site at Cys-35 (Rivera-Milla *et al.*, 2006), FLOT4 is predicted to have a Tyr substitution at this residue (Figure 2-1A). FLOT5 has no obvious translational start site and lacks a two-exon, one-intron structure. Only three *FLOT* genes are present in the Arabidopsis genome (Rivera-Milla *et al.*, 2006) implying an expansion of the *FLOT* family in *M. truncatula*.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>EST (TIGR)</th>
<th>Affymetrix probe set (expressed?)</th>
<th>BAC ID (imgag, genbank)</th>
<th>Genomic location Chromosome position (cM)</th>
<th>Number of exons</th>
<th>Estimated spliced mRNA size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>FLOT1</em></td>
<td>BF644444</td>
<td>Mtr.5691.1 (No)</td>
<td>Mth2-115c19, CT009553</td>
<td>3 (71.8)</td>
<td>2</td>
<td>1434 bp</td>
</tr>
<tr>
<td><em>FLOT2</em></td>
<td>EX527915</td>
<td></td>
<td>Mth2-115c19, CT009553</td>
<td>3 (71.8)</td>
<td>2</td>
<td>1440 bp</td>
</tr>
<tr>
<td><em>FLOT3</em></td>
<td>TC139669</td>
<td>Mtr.45231.1 (Yes)</td>
<td>Mth2-115c19, CT009553</td>
<td>3 (71.8)</td>
<td>2</td>
<td>1422 bp</td>
</tr>
<tr>
<td><em>FLOT4</em></td>
<td>TC133140, TC127236</td>
<td>Mtr.11786.1 (Yes), Mtr.42072.1 (Yes)</td>
<td>Mth2-115c19, CT009553</td>
<td>3 (71.8)</td>
<td>2</td>
<td>1425 bp</td>
</tr>
<tr>
<td><em>FLOT5</em></td>
<td>TC126348</td>
<td></td>
<td>Mth2-115c19, CT009553</td>
<td>3 (71.8)</td>
<td>5</td>
<td>?</td>
</tr>
<tr>
<td><em>FLOT6</em></td>
<td>AW574030</td>
<td>Mtr.3447.1 (No)</td>
<td>Mth2-193c3, AC161241</td>
<td>1 (49.4)</td>
<td>2</td>
<td>1416 bp</td>
</tr>
<tr>
<td><em>FLOT7</em></td>
<td>TC117648</td>
<td>Mtr.10214.1 (No)</td>
<td>Mth2-135j6, AC151528</td>
<td>1 (43.9)</td>
<td>2-3</td>
<td>?</td>
</tr>
</tbody>
</table>

Table 2-1. Summary of the *FLOT* gene family

**Figure 2-1. Alignment of predicted FLOT amino acid sequences and arrangement of FLOT1-5 within a single BAC**

(A) Sequences were aligned using CLUSTALW available from SDSC Biology Work Bench (http://workbench.sdsc.edu/). Conserved residues between all FLOTs are highlighted blue, residues conserved between four or more sequences are yellow, and similar residues are green. Note change in FLOT4 Cys35 to Tyr (residue 37 as numbered).

(B) Arrangement of FLOT1-5 BAC CT009553 (mth2-115c19) (IMGAG, http://www.medicago.org/genome/IMGAG/).

**FLOT2 and FLOT4 show NF-dependent upregulation during nodulation**

We assayed expression of *M. truncatula* FLOTS during nodulation by quantitative RT-PCR at times corresponding to key events in nodule development (Starker *et al.*, 2006). *FLOT2* and *FLOT4* expression increases early in nodule formation (Figure 2-2A). *FLOT2* is up-regulated for the entire 21-day developmental time course while *FLOT4* expression returns to baseline by 7 days post inoculation (dpi). In contrast, neither *FLOT1* nor *FLOT3* expression changes during nodule development. We explored expression of FLOTS in diverse plant organs and found that *FLOT4* expression is largely limited to roots and nodules, while *FLOT2* has highest expression in flowers and green pods, and *FLOT1* and *FLOT3* expression was detected in all plant organs (Figure 2-2B). We did not detect expression of *FLOT5* or *FLOT7* in any plant tissue including roots and nodules. *FLOT6* expression was occasionally detected in roots and nodules at levels so low that it could not be consistently detected even in the same sample. The roles of *FLOT5-7* in nodulation were therefore not explored further.
A small group of plant transcripts is up-regulated in the first 24 hours of symbiosis; NF is necessary and sufficient for the majority of these early transcriptional changes (Mitra et al., 2004a). We asked if NF was required for up-regulation of FLOT2 and FLOT4 at 24 hours post inoculation (hpi). An S. meliloti mutant unable to synthesize NF (ΔnodD1-nodABC) failed to up-regulate FLOT2 and FLOT4 at 24 hpi which implies NF is necessary, but purified NF was not sufficient for up-regulation (Figure 2-3A). We wondered if a second bacterial component such as a cell surface polysaccharide was also necessary for FLOT induction. Mutants deficient in synthesis of succinoglycan (exoA:Tn5), lipopolysaccharide (lpsB:Tn5), cyclic β-1,2-glucan (ndvB:Tn5) and a succinoglycan over-producer (exoX:Tn5) caused up-regulation of
FLOT2 and FLOT4 (Figure 2-3A) suggesting that known cell surface polysaccharides are not required.

Evidence suggests that NF effects are transduced via two pathways: a signaling pathway and a distinct entry pathway (Oldroyd and Downie, 2008). The most downstream component of the signaling pathway, transcription factor NSP2, is required for up-regulation of FLOT2 and FLOT4 at 24 hpi (Figure 2-3B). The parallel entry NF perception pathway, defined by LYK3, is also required for up-regulation of FLOT2 and FLOT4 at 24 hpi (Figure 2-3B) as is the transcriptional regulator NIN, which is common to both signaling and entry pathways (Figure 2-3B). However, ERN (BIT1), an ERF-like transcription factor required for upregulation of a subset of early nodulins but not for infection thread initiation (Middleton et al., 2007), is not required (Figure 2-3B). Rit-1 contains a mutation in an unknown gene, and has a block in early infection (Mitra and Long, 2004); FLOT2 and FLOT4 are not induced in the rit-1 mutant at 24 hpi. NF perception by both signaling and entry NF receptors and signal transduction via NSP2 and NIN are required for up-regulation of FLOT2 and FLOT4 at 24 hpi.

**Figure 2-3.** Up-regulation of FLOT2 and FLOT4 is dependent on NF and NF perception
(A) FLOT2 and FLOT4 expression was evaluated in response to bacterial mutants at 1 dpi using qRT-PCR. A17 plants were treated with NF, a ΔnodD1-nodABC (SL44) bacterial mutant, or with mutants altered in succinoglycan synthesis (exoA:Tn5 and exoX:Tn5), lipopolysaccharide biosynthesis (lpsB:Tn5), or cyclic β-1,2-glucan synthesis (ndvB:Tn5) (B) Plant mutants unable to perceive NF or unable to initiate subsets of NF-dependent gene transcription were treated with Rm1021 or buffer. Error bars show standard error of the ratio.
We asked whether \textit{FLOT2} and \textit{FLOT4} were up-regulated specifically in the plant cells that become infected by \textit{S. meliloti}. Using transcriptional fusions consisting of \textit{FLOT} promoters and the GUS reporter gene, we found that in uninoculated roots, \textit{FLOT1-4} are primarily expressed in vascular tissue (Figure 2-4A), and \textit{FLOT4} is also weakly expressed in elongating root hairs (Figure 2-4B). Upon inoculation with Rm1021, \textit{FLOT2} and \textit{FLOT4} show an expansion of expression in the root cortex in the region of elongating root hairs (Figure 2-4), which will eventually become colonized by bacteria (Gage, 2004). In nodules, \textit{FLOT2} and \textit{FLOT4} are expressed in the infection zone (Figure 2-4A, bottom panels). In contrast \textit{FLOT1} and \textit{FLOT3} show little change in their spatial expression or in intensity of expression in roots upon inoculation, and expression is limited to the nodule vascular tissue (Figure 2-4A).

\textbf{Figure 2-4.} \textit{FLOT2} and \textit{FLOT4} are expressed in the root elongation zone and in the infection zone of nodules  
(A) A17 plants were transformed to generate hairy roots expressing \textit{FLOT1-4} promoter-GUS fusions; GUS activity is shown for the indicated strains and times. Ten transgenic lines were observed for each construct at each time point (scale bars: 1 mm); representative roots are shown. (B) \textit{FLOT2} and \textit{FLOT4} are expressed in inoculated root hairs. A17 plants were transformed to generate hairy roots expressing \textit{FLOT2} and \textit{FLOT4} promoter-GUS fusions; GUS activity is shown for buffer- and Rm1021-inoculated roots at 24 hpi. Ten transgenic lines were observed for each construct at each time point (scale bars: 30 nm); a representative sample at the indicated time points is shown.
FLOT2 and FLOT4 localize to membrane microdomains and FLOT4 becomes polarly localized in response to bacterial signals

To study the localization and dynamics of FLOT2 and FLOT4 during nodulation, we constructed FLOT:GFP fusions using the *FLOT* genomic sequence (promoter and intron). FLOT2:GFP was not visible under control of its native promoter so we expressed FLOT2 using the CaMV 35S promoter. In root cells, signals from both FLOT2:GFP and FLOT4:GFP appear punctate and co-localize with FM4-64, a plasma membrane marker (Figure 2-5). In the absence of bacteria, FLOT4:GFP and FLOT2:GFP puncta are evenly distributed in the plasma membrane of root hair cells (Figure 2-6). Upon inoculation with *S. meliloti*, FLOT4:GFP accumulates in the tips of elongating root hairs while FLOT2:GFP puncta remain evenly distributed throughout root hair plasma membranes (Figure 2-6). FLOT2:GFP is polarly localized in uninoculated and inoculated epidermal cells while FLOT4:GFP is not (Figure 2-6).

Figure 2-5. FLOT2 and FLOT4 localize to membrane-associated puncta
We generated A17 hairy roots expressing 35S:FLOT2::GFP or FLOT4p:FLOT4:GFP. Transgenic roots were visualized using a spinning disk confocal microscope (scale bars: 15 μm). At least six transgenic lines were observed for each treatment. Representative images are shown. (A) 35S:FLOT2:GFP in root cells is punctate. (B) FM4-64 membrane-associated dye. (C) Co-localization of FLOT2:GFP puncta (green) and FM4-64 (red).
Figure 2-6. FLOTs localize to membrane-associated puncta and become polarly localized after inoculation

We generated A17 hairy roots expressing 35S:FLOT2:GFP or FLOT4:GFP driven by its native promoter. Transgenic roots were visualized using a spinning disk confocal microscope (scale bars: 15 μm). FLOT4:GFP and FLOT2:GFP are visibly punctate and evenly distributed in the membranes of uninoculated root hair cells. FLOT4:GFP puncta localize to root hair tips by 24 hpi while the localization of FLOT2:GFP does not change upon inoculation. FLOT2:GFP is weakly polar in uninoculated epidermal cells (arrows) and remains polar on inoculation. FLOT4:GFP is evenly distributed in epidermal cell membranes in inoculated and uninoculated roots. At least fifteen transgenic lines were observed for each treatment. Representative images are shown. Root hair images are maximum intensity projections of 100 sections, taken at 0.2 μm increments.

FLOTs are required for nodulation

We tested FLOT function using RNA interference (RNAi) or artificial micro RNAs (amiRNAs) to silence FLOT expression and found that FLOTs are required for symbiosis (Figures 2-7 and 2-8). We targeted FLOT2 and FLOT4, which are regulated during nodulation, and the constitutively expressed FLOT3 as a control.
(FLOT1 was also silenced and gave similar nodulation results as FLOT3). We confirmed efficacy of silencing by qRT-PCR at 24 dpi, a time point when no FLOTs are significantly up-regulated in inoculated hairy roots (Figure 2-8C). Constructs are designated by their primary target gene (greater than 50 percent reduction in expression); numbers in parentheses show genes that have partial reduction in expression due to cross silencing. The most dramatic phenotype of the FLOT-silenced lines was observed when FLOT2,3, and 4 are silenced as a group (FLOT2,3,4 RNAi, Figure 2-7B; this construct also silences FLOT1 (Figure 2-8A,B)). Roots transformed with the empty vector formed nodules 87 percent of the time while only half of the FLOT2,3,4 RNAi roots formed nodules. Pink nodules were observed on control plants more than 25 percent of the time compared to only 2.5 percent for FLOT2,3,4 RNAi roots. On average control roots formed around 6 nodules while FLOT2,3,4 RNAi root formed an average of only 2 nodules. FLOT2,3,4 RNAi roots also had altered morphology including a decrease in primary root length, an increase in the number of primary and secondary lateral roots (Figure 2-8A) and reduction in root weight (Figure 2-8D). These results indicate that FLOTS are required for symbiosis and normal root development.
Figure 2-7. Silencing FLOTs results in a decrease in nodule number, a decrease in infection events and nodules that do form are non-functional

(A) Expression of FLOT2,3, and 4 in individual hairy roots expressing the indicated RNAi or amiRNA construct was assessed using qRT-PCR, normalized to an internal actin control, and then to expression in plants transformed with the empty vector (average of at least 10 roots). Constructs are designated by their primary target gene(s) (*>50 percent reduction in expression); numbers in parentheses show genes that have partial but significant (#P< 0.05) reduction in expression due to cross silencing.

(B) Nodulation phenotypes were assayed from a minimum of three biological replicates representing 50-100 plants per construct.

(C) Acetylene reduction assays were performed at 21 dpi to determine the efficacy of nodules that form (14-20 plants were assayed per silenced line).

(D) Hairy roots were stained for Rm1021 expressing phemA: lacZ and stained for lacZ activity at 7 dpi to visualize infection events. Infection events and nodules were scored on ten transformed plants per amiRNA or RNAi and normalized to root weight (Figure 2-8D). Between 104 (FLOT2,3,4 RNAi) and 371 (empty vector) total infection events were observed per line.
(E) Progression of infection threads on silenced roots (from Figure 2-4C). Infection threads were scored as aborting in the root hair, penetrating the hypodermal/cortical cell layers but not releasing bacteria into cortical cells, or releasing bacteria into cortical cells. Release was inferred by diffuse blue staining in the nodule cortex.

(A-E) Error bars represent ±SEM; *#P < 0.05

(F-I) Abortive infection threads in plants transformed with the FLOT4 amiRNA construct (F and G) compared to control infection threads (H and I) (scale bars: 15 μm).

(J) Linear regressions were conducted on plants described in (A) to determine if a correlation exists between expression of FLOTs and nodule number. FLOT2 expression level has a strong linear relationship with nodule number (y = 5.1x + 2.7; P<sub>intercept</sub> = 0.005; P<sub>slope</sub> = 3x10⁻⁵); FLOT4 expression level has a weaker but statistically significant correlation (y = 2.0x + 5.8; P<sub>intercept</sub> = 3x10⁻⁸; P<sub>slope</sub> = 0.02). FLOT3 expression does not correlate with nodule number (P<sub>slope</sub> = 0.6). FLOT2 and FLOT4 expression have additive effects (y = 7.1x + 0.9; P<sub>intercept</sub> = 0.003; P<sub>slope</sub> = 3x10⁻¹⁰).

Silencing different combinations of FLOTs results in varying degrees of nodulation and root morphological defects, although none as severe as FLOT2,3,4 RNAi roots (Figures 2-7 and 2-8). Silencing FLOT2 results in fewer nodules per plant, an increase in Nod- plants, and a decrease in plants that form pink nodules. Silencing FLOT2 resulted in a decrease in primary root length and long primary lateral roots but no significant change in root weight. Plants silenced for FLOT4 expression are significantly less likely to form pink nodules than roots transformed with the vector control; FLOT4-silenced roots had an increase in numbers of secondary lateral roots but no decrease in primary root length or root weight. The FLOT3(4) amiRNA construct silences FLOT3 as completely as FLOT2,3,4 RNAi but these roots show no significant nodulation defects compared to controls (this construct also targets FLOT1—Figure 2-8B). We observed shorter roots and reduced root weight in lines with the greatest reduction in FLOT3 expression. These data suggest that FLOT2 and FLOT4 play the largest role in symbiosis and the symbiotic defects are separable from the small root phenotype observed upon silencing FLOT2,3, and 4.
Figure 2-8. Root and nodule phenotypes of FLOT-silenced roots

(A) A representative plant for amiRNA and RNAi constructs described in this study is shown. Nodules that formed in silenced lines were small and white (with the exception of FLOT1+3(4) amiRNA line). Note smaller overall roots in FLOT3-silenced lines, shorter primary roots in FLOT2-silenced lines and increase in short secondary lateral roots in FLOT4-silenced lines.

(B) Silencing data including the data for FLOT1 and one additional construct that primarily targets FLOT1 (FLOT1(2) amiRNA). Gene expression of FLOTs in individual hairy roots expressing the indicated RNAi or amiRNA construct was assessed using qRT-PCR, normalized to an internal actin control and then to expression in control plants (average of at least 10 roots). Constructs are designated by their primary target gene(s); numbers in parentheses show genes that have partial but significant ($P < 0.05$) reduction in expression due to cross silencing.

(C) Hairy root time course. Jemalong seedlings were transformed using A. rhizogenes with the amiRNA empty vector to generate hairy roots. Plants were
inoculated with *S. meliloti* Rm1021 or 1/2X BNM and harvested at the indicated time. QRT-PCR was performed to analyze expression of *FLOT2* and *FLOT4* at all time points; *FLOT1* and *FLOT3* expression were monitored at 21 dpi only. Expression of each gene is normalized to an actin internal control; the ratio of inoculated to uninoculated plants is shown. Error bars represent standard error of the ratio.

**D** Average root weight of silenced lines. The ten plants per construct used to count infection events (Figure 2-7A to G) were weighed. Error bars represent ±SEM; *P* <0.05.

**E** Linear regressions were conducted on plants described in Figure 2-7B to determine if a correlation exists between expression of *FLOTs* and nodule number. *FLOT1* expression does correlates with nodule number (*P*< 0.01, Figure 2-7C).

**FLOT2- and FLOT4-silenced plants are defective in both nodule form and function**

Nodules that form in *FLOT*-silenced lines are predominantly small and white suggesting that they are unable to fix nitrogen. We found that all silenced lines (with the exception of *FLOT3(4)* amiRNA) had a significant decrease in their ability to reduce acetylene, a reporter for nitrogen fixation (*P* < 0.01, Figure 2-7C).

To explore the individual contribution of each *FLOT* to nodule number, we used the data set from Figure 2-7A to determine whether a correlation exists between nodule number and the expression of a particular *FLOT* gene (each hairy root transformation event results in a unique genomic insertion of the RNAi or amiRNA construct and a different degree of gene silencing). We found that that *FLOT2* expression has a strong linear correlation with nodule number while *FLOT4* expression has a weaker but significant linear correlation with nodule number (Figure 2-7J). In contrast, *FLOT1* and *FLOT3* expression levels have no significant correlation with nodule number (Figures 7J and 8E). To assess if *FLOTs* have additive effects, we compared the pooled expression of different combinations of *FLOTs* and found that the combination of *FLOT2* and *FLOT4* expression levels was the only grouping that increased the strength of the correlation (Figure 2-7J). This is consistent with a model in which *FLOT2* and *FLOT4* contribute independently to nodule number.
**FLOT silencing results in infection thread initiation and elongation defects**

We used lacZ-staining of *S. meliloti* to visualize infection threads in silenced roots. *FLOT*-silencing results in reductions in both the number of nodules and the number of infection events (with the exception of *FLOT3(4)* amiRNA) (Figure 2-7D). The decrease in the total number of infection events in *FLOT2* and *FLOT4*-silenced roots indicates that the decrease in nodule number observed may in part be due to an infection thread initiation defect.

We wondered if the infection defects (Figure 2-7D) are limited to infection thread initiation or if *FLOT2*- and *FLOT4*-silenced roots also have infection thread elongation defects. We evaluated the progression of infection threads on silenced roots and found that infection threads on *FLOT4*-silenced roots were significantly more likely to abort in the root hair than control plants (Figure 2-7E). Infection threads on roots silenced for *FLOT4* and all *FLOTs* were also significantly less likely to have bacterial release into cortical cells. *FLOT2*- and *FLOT3*-silenced roots showed normal infection thread development (Figure 2-7E). Representative images of the abortive infection threads observed on *FLOT4*-silenced roots are shown in Figure 2-7F and G (compare to controls in Figure 2-7H,I). This suggests that *FLOT4*, but not other *FLOTs*, is required for normal infection thread elongation. It may suggest a defect in bacterial release in *FLOT4*-silenced plants, or that infection threads fail to reach the cell layers where release normally occurs.

**FLOT4 localizes to infection thread membranes**

Because silencing *FLOT4* results in infection thread elongation defects (Figure 2-7E to I), we explored whether FLOT4 localizes to infection threads. In infected root hair cells, FLOT4:GFP localizes to infection thread membranes (Figure 2-8A). A section through the infection zone of a 7 day old nodule revealed that FLOT4 is present in infection thread membranes in the nodule (Figure 2-8B). In contrast, FLOT2 is not present on infection thread membranes in root hairs (Figure 2-8C).
Figure 2-9. FLOT4 localizes to infection thread membranes
(A-C) Transgenic roots expressing FLOT4p:FLOT4:GFP or 35S:FLOT2:GFP (green) are inoculated with S. meliloti Rm1021 expressing ptrp:mCherry (red) (Scale bars: 30 μm). (A) FLOT4:GFP localizes to infection thread membranes in root hair cells (stack of 50 images taken at 0.2 μm increments, arrows mark infection threads). (B) FLOT4:GFP localizes to infection thread membranes in the infection zones of maturing nodules (arrows). (C) FLOT2:GFP does not localize to infection thread membranes in root hair cells.
DISCUSSION

A requirement for \textit{FLOT2} and \textit{FLOT4} for early nodulation events

Data presented in this study indicate that \textit{FLOT2} and \textit{FLOT4} have roles in early nodulation. \textit{FLOT2} and \textit{FLOT4} are among a select group of plant genes up-regulated at 24 hpi; this up-regulation is dependent on both the NF signaling and entry receptor pathways, and on \textit{NIN} (Figure 2-3B). The unexpected result that NF is insufficient for induction of \textit{FLOT2} and \textit{FLOT4} suggests that a second bacterial factor may also be required, although known surface polysaccharides are not apparently required (Figure 2-3A).

\textit{FLOT4} shows a prominent accumulation in the tips of elongating root hairs upon inoculation with \textit{S. meliloti} (Figure 2-6). Plant proteins with similar localizations have been implicated in polar growth of root hairs and pollen tubes (Yang, 2008). Upon silencing \textit{FLOT4}, we did not observe an obvious defect in normal root hair elongation or in curling around bacterial colonies, so it seems unlikely that \textit{FLOT4} is required for root hair elongation or directional growth. It seems more likely that this protein is involved in polar growth of the infection thread.

We observed that silencing \textit{FLOT2} and \textit{FLOT4} results in fewer infection threads (Figure 2-7D) and silencing \textit{FLOT4} causes infection thread elongation defects (Figure 2-7E). By analogy to flotillins in other organisms, one might predict that \textit{FLOT2} and \textit{FLOT4} have a role in the initial invagination of infection threads into the root hair and that \textit{FLOT4} additionally functions in elongation of the infection thread. At is also possible that \textit{FLOTs} coordinate infection thread initiation and nodule organogenesis (as has been proposed for \textit{NIN}; Marsh \textit{et al.}, 2007) or that \textit{FLOTs} are involved in endocytosis or trafficking of a signal involved in nodule organogenesis. Further studies are needed to determine if, like their animal counterparts, plant flotillins interact with the actin cytoskeleton to facilitate membrane rearrangements.

\textit{FLOTs} were previously isolated from soybean and pea peribacteroid membranes (Panter \textit{et al.}, 2000; Saalbach \textit{et al.}, 2002); however, we were unable to detect either \textit{FLOT2:GFP} or \textit{FLOT4:GFP} on symbiosome membranes in \textit{M. truncatula}. This may be due to limitations in the sensitivity of our microscope or to an
absence of the proteins on the symbiosome membranes in this system. We found
evidence that FLOTs are required for later stages of nodulation: FLOT2-silenced
nodules were populated with infection threads that looked similar to wild type
infection threads (Figure 2-7E); however, these nodules were unable to fix nitrogen
(Figure 2-7C) indicating a defect post-infection. Future studies are needed to
determine if FLOTs have a role in endocytosis and trafficking of bacteria.

**Flotillin-like genes are ubiquitous in plants**

Possible roles for flotillin-like genes in general plant development may be suggested
by the root phenotypes of FLOT-silenced plants (Figure 2-8). We found that FLOT3-
silenced plants had a reduction in root weight, FLOT2-silenced plants had a decrease
in primary root length and FLOT4-silenced plants had an increase in secondary lateral
roots (Figure 3-8). These silencing phenotypes suggest that FLOTs may have a
normal role in plant growth and development and that legumes may have co-opted the
ancestral FLOT function for symbiosis.

Because plant membranes have different compositions than animal membranes
(reviewed in Zappel and Panstruga, 2008), it is likely that plant membrane
microdomains will have different properties than animal membrane microdomains.
Plant membranes contain little cholesterol but rather a mix of sterols that vary across
plant taxa. Most plants including the model Arabidopsis contain primarily sitosterol,
campesterol, and stigmasterol. *Medicago* has a less common membrane composition
consisting largely of spinasterol (Lefebvre *et al.*, 2007). Determining how the
membrane microdomains defined by FLOTs are similar to and different from the
membrane microdomains defined by flotillins in other plants and other organisms will
give valuable insight into plant membrane microdomains and plant membrane
organization.
MATERIALS AND METHODS

Plant growth and bacterial treatments

*Medicago truncatula* Gaertner cv Jemalong, cv Jemalong A17 (an inbred line of Jemalong), *nin-1* (Marsh *et al.*, 2007) and *bit1-1* (Middleton *et al.*, 2007) were grown, inoculated and harvested as described (Mitra and Long, 2004). *S. meliloti* strain Rm1021 is a streptomycin-resistant derivative of WT field isolate SU47 (Meade *et al.*, 1982). SL44 has a deletion of the *nodD1-nodABC* region (Fisher *et al.*, 1988). The *exoA* strain (Rm7031) is described in Leigh *et al.* (1985). The *exoX* (MB801), *lpsB* (R1A6) and *ndvB* (B587) mutants are described in Griffitts *et al.* (2008) and Griffitts and Long (2008). Bacteria were grown in liquid TY-medium or Luria-Bertani (LB) medium supplemented with appropriate antibiotics. For inoculations, bacteria were grown to an OD$_{600}$ between 0.5 and 1. They were then pelleted and resuspended in 1/2X Buffered Nodulation Medium (BNM; Ehrhardt *et al.*, 1992) at an OD$_{600}$ of 0.05.

RNA sample preparation

RNA used for 24 hour time points (A17 inoculated with NF, or with SL44, or *exoA* bacterial mutants and uninoculated and inoculated *nps2-2* and *hcl-1* plant mutants) were the same RNA samples that were used in Mitra *et al.* (2004a). The RNA samples from the A17 time course were the same samples used by Starker *et al.* (2006) with the exception of the 21 dpi RNA samples which were prepared by Adriana Parra-Rightmyer using methods described in Starker *et al.* (2006). Remaining RNA samples were isolated using the TRIzol (Invitrogen) method previously described by Mitra and Long (2004).

Quantitative RT-PCR

To ensure primer specificity, primers were designed to amplify the most divergent region of each *FLOT* and to flank intron splice sites (Table 2-2). Primer pairs were tested against a cloned genomic region containing *FLOT1,2,3* or *4* to ensure that each primer pair would only amplify its intended target. Primer pairs were also tested for specificity using cDNA from uninoculated and inoculated *M. truncatula* cv Jemalong.
and cv Jemalong A17. The resulting products were sequenced to ensure they were from a single gene. Quantitative RT-PCR, data quantification and analysis were performed as described in Mitra et al. (2004a).

To prepare template for qRT-PCR, RNA was DNase treated using DNase-free turbo (Ambion). 35 PCR using actin-specific primers (Table 2-2) was used to check for DNA contamination post DNase treatment. The DNase-treated RNA was used in single stranded cDNA synthesis using Superscript III (Invitrogen) and oligo(dT) primer (Invitrogen). 25 PCR cycles were used to check for successful cDNA synthesis. Template concentration per reaction was determined empirically based on relative abundance of the transcript of each FLOT; each template was run at two different concentrations. Template quantification was done at the level of total RNA; an internal actin control in each PCR controlled for differences in efficiency of cDNA synthesis. Actin was amplified from cDNA made from 2.5 ng and 7.5 ng of RNA; FLOT2, FLOT3, and FLOT4 were amplified from cDNA made from 7.5 and 25 ng RNA; FLOT1 was amplified using cDNA made from 25 and 75 ng total RNA. qPCR was performed using DyNAmo Flash SYBR Green qPCR kit (Finnzymes, Helsinki).

A. rhizogenes-mediated hairy root transformations
Plasmids were transformed into A. rhizogenes Arquai (Quandt, 1993) and selected using the appropriate antibiotic. A. rhizogenes-mediated hairy root transformations were done according to Boisson-Dernier et al. (2001) with modifications. We found that transformation efficiency was increased if the plants were first placed on modified Fahraeus medium containing 1 mM α-aminoisobutyric acid (AIB) without selection for 7 days. All newly formed roots were removed, and plants were transferred to selective media with no ethylene inhibitor and either 25 mg/L kanamycin (remained on selective media for 17-21 days) or 10 mg/L hygromycin (for 10 days). Plants remained on selective media until roots reached approximately 2.5 cm (we found that less time was often needed for Jemalang A17 than for Jemalong). Plants were then transferred to 1/2X Gamborg’s B5 Basal Salt medium (Sigma) with 1% agar to
recover from antibiotic selection. For confocal microscopy studies, 300 mg/mL Augmentin (Research Products International) and 500 mg/L Cefotaxime (Sigma) were added to the B5 medium to reduce the amount of *A. rhizogenes* carryover. Plants remained on B5 for 1 week then were transferred to Buffered Nodulation Medium (BNM; Ehrhardt *et al.*, 1992) containing 0.1 μM aminoethoxyvinylglycine (AVG). Plants were flood inoculated one week later with 10 mLs of the appropriate *S. meliloti* strain diluted to OD_{600} = 0.05 in 1/2X BNM.

**β-Glucuronidase assays**

The upstream region of each open reading frame (3 kb at most, less if another ORF is < 3 kb upstream) was amplified from BAC mth2-115c19 and cloned into pENTR D/TOPO (Invitrogen) (Table 2-5). LR recombination was performed with the Gateway-compatible vector pMDC163 (Curtis and Grossniklaus, 2003). Plasmids were used to transform *A. rhizogenes* Arqua1. *A. rhizogenes*-mediated hairy root transformation was performed as described above with hygromycin selection. GUS assays were conducted as described in Oldroyd *et al.* (2001a).

**RNAi and amiRNA construct design and screen**

For RNAi constructs, 100-200 bp of either the conserved region of *FLOTs* or the 3’UTR were amplified from DNA prepared from BAC mth2-115c19 (Table 2-3). The PCR products were first cloned into pENTR/D TOPO (Invitrogen). LR recombination was performed with the binary vector pHELLSGATE8 (Helliwell and Waterhouse, 2003).

AmiRNA constructs were designed as described in Ossowski *et al.* (2008) using the web designer described in Schwab *et al.* (2006) ([http://wmd2.weigelworld.org](http://wmd2.weigelworld.org)) which cross-references with available *Medicago* EST databases (Table 2-4). The full length sequence for the desired *FLOT* target was entered as the “target gene” and available ESTs for that target were entered as “accepted off-targets”. The suggested amiRNAs were BLASTed against the available *M. truncatula* genome to ensure there were no off target sequences that are absent
from the available EST library. The pRS300 plasmid (Ossowski et al., 2008) was used as a template to create the amiRNA hairpin with an intron.

The final amiRNA PCR product was digested at the XhoI and XbaI sites flanking the sequence encoding the amiRNA hairpin. The resulting product was ligated into the pHELLSGATE8 vector XhoI and XbaI sites (thus removing the Gateway cassette). The result was an amiRNA construct driven by the CaMV 35S promoter with the same vector backbone as the RNAi constructs.

Using A. rhizogenes to generate transgenic hairy roots, we conducted a preliminary screen of eight RNAi and twelve amiRNA constructs to identify those that effectively silenced expression of one or more FLOT. ANOVA was used to identify constructs that caused significant reduction in gene expression. Two-tailed t-tests were used to determine if nodule number, percent Nod⁻ and percent pink nodules were different in silenced lines compared to the empty vector control. Linear regressions were done using Microsoft Excel data analysis feature. To determine if second and third order correlations existed, second and third order transformations were performed on the data and linear regressions were conducted. Gene expression was pooled by averaging the expression level of each gene in the same plant.

**Plant Assays**

Acetylene reduction (Turner, 1980) was performed as described (Oke and Long, 1999). Plants were initially grown on BNM plates as described above; at 21 dpi the entire plant was moved to test tubes for the assays. At least 3 uninoculated plants were assayed per construct to ensure that silencing FLOTs did not intrinsically cause an increase in ethylene production.

To visualize infection events, plants were inoculated with Rm1021 expressing lacZ from the plasmid pXLGD4 and stained for β-galactosidase activity at 7 dpi as described (Boivin et al., 1990). Due to the altered lateral branching observed in some FLOT-silenced lines, we used root weight rather than root length as a measure of root size.
Localization of fusion proteins

The genomic regions of *FLOT2* and *FLOT4* were amplified from BAC Mth2-115c19 DNA and inserted into pCH010 (Table 2-5). pCH010 was constructed in two steps by first inserting eGFP (with added 5’ EcoRI and XmaI sites) into the BamHI/XbaI sites of pJG159; then the NPTII ORF was amplified from the pHELLSGATE8 vector and inserted into the XhoI site in pJG159. pJG159 is a small (7.8 kb) binary vector that was constructed by J. Griffitts (unpublished) by a three way ligation of inserts A and B from pEGAD (Cutler *et al.*, 2000); Table 2-5) and the Sphl/XhoI fragment from pCAMBIA1300 (www.cambia.org). To create insert A (Sph-RB-P35S-RI), pEGAD was amplified with primers oJG346/347 and 348/349 followed by overlap extension-PCR with oJG346/349. Insert B (RI-nosT-P35S-XhoI) was amplified from pEGAD with oJG350/351.

For 24 hpi localization studies, plants were inoculated with Rm1021 over-expressing NodD3 from the plasmid pRmE65 (Fisher *et al.*, 1988). For infection thread co-localization studies, plants were inoculated with Rm1021 constitutively expressing mCherry from the plasmid pQDN03. pQDN03 was constructed by replacing GFP in pDG71 (Gage, 2002) with mCherry (Table 2-5).

Root segments and nodule hand sections (for imaging the infection zone) were excised and mounted in 0.1 M potassium phosphate buffer pH 7. Spinning disk confocal microscopy was performed on a system described previously in Gutierrez *et al.* (2009) using a 63X/1.3 NA glycerol immersion objective. GFP and RFP were excited at 491 nm and 561 nm, respectively, by solid-state lasers. Z-projections of root hairs are from 100-200 images taken at increments of 0.2 μms (MCL NanoDrive). Stacks were processed using ImageJ software (http://rsbweb.nih.gov/ij/). Typical exposure times were 1000 ms for GFP, 500 ms for mCherry.

FM4-64 was dissolved in 0.1 M phosphate buffer pH 7.0 to a final concentration of 20 μM and kept on ice until use (Bolte *et al.*, 2004). The GFP/FM4-64 experiment was imaged on a system described in Paredez *et al.* (2006) with the same excitation settings listed above for GFP/RFP and 1000 ms exposures. Typical exposure times were 1000 ms for GFP, 500 ms for mCherry and 1000 ms for FM4-64.
Hairy root time course

To determine if regulation of FLOTs was altered in hairy roots, we assayed FLOT expression levels in uninoculated and inoculated *M. truncatula* cv Jemalong seedlings transformed with the amiRNA empty vector construct EX117 (Table 2-4) at 1, 4, 7, 14 and 21 dpi. Plants were grown as described above and inoculated with 1/2X BNM or Rm1021 in 1/2X BNM. Plants were harvested just below the callus at the appropriate time point and flash frozen in liquid nitrogen. Three independent replicates of the entire time course were performed; each time point sample was a pool of the ten plants from a single plate. RNA was isolated with a yield of 50-100 μg per ten plants.

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Table 2-2. PCR and qPCR primers to monitor FLOT expression
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<th>Primer Name</th>
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<td>FLOT1-4</td>
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<td></td>
<td></td>
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<td>GTTTCCAAGTCAGTATTGAGAA</td>
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<td>Q: FLOT4(3) RNAi</td>
<td>FLOT1-4</td>
<td>chh228</td>
<td>CACCACATAGTTGATGACATATTAG</td>
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<td>chh229</td>
<td>ACCTGAAACTGAGACAGTGCAG</td>
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<tr>
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<td>FLOT1 UTR</td>
<td>chh277</td>
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<td>chh278</td>
<td>GACTGAGAAACTGAGACAGTGCAG</td>
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</tr>
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<td>chh285</td>
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Table 2-3. RNAi Construct Primers
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<td>chh319</td>
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<td>chh327</td>
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<tr>
<td>EX117: empty vector</td>
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Table 2-4. amiRNA Construct Primers
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<td>pEGAD</td>
<td>pCAMBIA 1300</td>
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<td>pHELLS-GATE8</td>
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Table 2-5. Vector Construction
CHAPTER 3: Localization of the symbiotic receptor kinases LYK3 and DMI2

ABSTRACT

Legume-rhizobia symbiosis initiates with plant recognition of the bacterial molecule Nod Factor (NF). In *Medicago truncatula*, two receptor kinases, LYK3 and DMI2, are known to be required for perception of the bacterial signal. *LYK3* encodes a LysM-family receptor required for bacterial entry into plant root hair cells. *DMI2* encodes a leucine-rich repeat receptor kinase that is required for early signaling events. We report localization of GFP-tagged LYK3 and DMI2 prior to bacterial inoculation and during symbiosis. In uninoculated root hairs, we found that both proteins have punctate plasma membrane-associated distributions. Within minutes of bacterial treatment, we observed an increase in DMI2:GFP-containing vesicles followed by a rapid loss of DMI2:GFP signal. We did not observe DMI2:GFP on infection threads or in nodule cells. We also observed an increase in LYK3:GFP-positive vesicles following bacterial treatment but not until 6 hours post inoculation, and vesicles persisted at 24 hours. We observed LYK3:GFP along infection threads in root hairs but not associated with infection threads in mature nodules. Our data support genetic evidence that DMI2 plays a role in signaling while LYK3 is involved in infection. Our observation that LYK3:GFP infection thread association is restricted to root hair cells suggest that infection thread growth in root hairs may involve different cellular processes or receptors than infection of cortical cells.
INTRODUCTION

Symbiosis between nitrogen-fixing bacteria and legume plants initiates with a signal exchange between plant host and bacterium. Plants secrete compounds called flavonoids into the surrounding soil. Flavonoids activate a *Sinorhizobium meliloti* transcription factor NodD1 which upregulates expression of genes involved in biosynthesis of a bacterial lipochitooligosaccharide called Nod Factor (NF) (Peck et al., 2006). *Medicago truncatula* NF perception requires LysM-family receptors LYK3 and NFP. Other plant LysM receptors bind chitin (Iizasa et al., 2010) so LYK3 and NFP are good candidates for receptors that directly bind the chitin backbone of bacterial NF. NFP mutants have no measurable response to bacterial NF, while LYK3 mutants can perceive NF and initiate a number of preliminary events including calcium spiking (Wais et al., 2000) and root hair tip growth, but form no infection threads (Catoira et al., 2001; Smit et al., 2007). A leucine-rich repeat receptor kinase DMI2 is genetically downstream of NFP (Catoira et al., 2000). These phenotypes support a model where the NFP and LYK3 receptors bind NF and signal through parallel pathways to initiate signaling events and bacterial entry into root hairs respectively (Geurts et al., 2005; Smit et al., 2007).

Little is known about the cell biology of receptors during bacterial recognition events or during infection. It is also unknown whether the receptors interact with one another or if cross-talk is mediated downstream of the receptors. There is one report that DMI2 is present on infection thread and cellular plasma membranes in nodules (Limpens et al., 2005). When LYK3 was transiently expressed in *Nicotiana benthamiana*, the protein appeared homogenous along the plasma membrane (Lefebvre et al., 2010). We expressed GFP-tagged LYK3 and DMI2 by their native promoters in the roots of *M. truncatula*. In uninoculated roots we observed that both receptors had patchy distributions associated with the plasma membrane. After bacterial treatment, DMI2:GFP and LYK3:GFP displayed distinct behaviors. Our observations add to understanding of the function of these receptors during perception of symbiotic bacteria and the role of bacterial perception during later stages of symbiosis.
RESULTS

Localization of symbiotic receptor kinases LYK3 and DMI2

To localize the LYK3 and DMI2 proteins, plants were stably transformed with a full genomic copy of LYK3 (pLYK3:gLYK3) or DMI2 (pDMI2:gDMI2) translationally fused to either GFP or an HAS tag dual-affinity tag (B. Riely and D. Cook, personal communication). Root hair walls and other structures are known to display auto-fluorescence; the HAS-tagged lines were used as controls for auto-fluorescence. The hcl-1 mutant, which contains a point mutation in the predicted kinase domain of LYK3 (Smit et al., 2007), was transformed with the pLYK3:gLYK3:HASt and pLYK3:gLYK3:GFP transgenes; two lines were recovered with each transgene. The dmi2-1 mutant was transformed with the pDMI2:DMI2:GFP and pDMI2:gDMI2:HASt transgenes; one line was recovered with each transgene. The dmi2-1 mutant has a point mutation resulting in an early stop codon in the DMI2 open reading frame and is a predicted null allele of DMI2 (Endre et al., 2002). In the absence of the complementing transgenes, hcl-1 and dmi2-1 mutants form no nodules or infection threads (Catoira et al., 2000; Catoira et al., 2001).

We confirmed that the LYK3 and DMI2 transgenes were able to complement the hcl-1 and dmi2-1 mutant phenotypes by comparing nodulation and nitrogen fixation to wildtype M. truncatula cv Jemalong A17 plants. All lines with the exception of one pLYK3:gLYK3:HASt line (163.8) formed significantly fewer nodules than wildtype plants (P < 0.01 by two-tailed t-test) (Figure 3-1A). We assessed nitrogen fixation by indirect means by measuring the plants’ ability convert acetylene to ethylene (Turner, 1980). We found that both pLYK3:gLYK3:HAS lines (163.5 and 46.10) and the pDMI2:gDMI2:GFP (63.8) had significantly reduced rates of acetylene reduction per plant and per nodule (Figure 3-1B,C). The other lines had no significant difference from wildtype plants in their ability to reduce acetylene. All lines tested had some nitrogen fixation ability indicating that the DMI2 and LYK3 C-terminal fusions are functional.
In concert with our attempt to localize GFP-tagged \textit{DMI2} and \textit{LYK3} in stable transgenic lines, we used \textit{Agrobacterium rhizogenes}-generated hairy roots transformed with the g\textit{LYK3:GFP} and g\textit{DMI2:GFP} fusion proteins. We confirmed that when introduced by hairy root transformation, the transgenes could complement the nodulation defects in \textit{hcl} and \textit{dmi2} mutants. We found that introduction of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3-1.png}
\caption{\textbf{LYK3 and DMI2 fusion proteins rescue mutant phenotypes}}
\end{figure}

Approximately 30 plants per genotype or transgenic line type were assayed for nodulation and acetylene reduction phenotypes at 21 dpi; error bars represent SEM. *$P < 0.05$ by two-tailed t-tests; n.d. not detected. In some cases, multiple transgenic lines were recovered with each construct; line numbers are in parentheses. (A) Average nodule number per plant. (B) Acetylene reduction assays showed that nodules on transgenic lines were able to fix nitrogen. (C) Adjusted acetylene reduction level per plant per nodule.
pLYK3:gLYK3:GFP into hairy roots restored nodulation in the hcl-1 allele as well as hcl-2 and hcl-3 alleles (See Table 3-1 for a description of each mutant allele and results of complementation assays). We found that introduction of pDMI2:gDMI2:GFP into hairy roots restored nodulation to dmi2-1 as well as the dmi2-3 and dmi2-4 mutants (Table 3-1).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Genetic Background</th>
<th>Nature of mutation</th>
<th>Predicted effect on gene function</th>
<th>Hairy roots or Stable line</th>
<th>Nod+/total plants</th>
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<td>No transgene</td>
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<td>Point mutation</td>
<td>Non-functional kinase domain</td>
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<td>Point mutation</td>
<td>Non-functional kinase domain</td>
<td>Lines 22.3 and 26.1</td>
<td>28/30; 29/29</td>
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<td>Hairy roots</td>
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<td>Point mutation</td>
<td>Non-functional kinase domain</td>
<td>Hairy roots</td>
<td>3/5</td>
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<tr>
<td>pLYK3:gLYK3:GFP</td>
<td>hcl-2 (W1)</td>
<td>Point mutation in splice site</td>
<td>Loss of transcript; predicted null</td>
<td>Hairy roots</td>
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Table 3-1. Transgenes complement LYK3 and DMI2 mutants
LYK3 and DMI2 transgenes were stably integrated or inserted via A. rhizogenes hairy roots into wildtype and mutant plants. Roots were scored for their ability to form nodules.
Both stable and hairy-root transgenics were used for LYK3:GFP localization and yielded equivalent results. In uninoculated root hairs, we observed LYK3:GFP in a punctate distribution at the extreme cell periphery in a gradient that was denser near the growing tip (Figures 3-2 and 3-3). There was little co-localization of LYK3:GFP with a cytoplasmic marker and few labeled cytoplasmic vesicles were visible (Figure 3-3). The LYK3:GFP signal followed the membrane after plasmolysis (Figure 3-4) confirming that the labeled protein was not localized to the cell wall. These observations suggest that majority of the LYK3:GFP signal is associated with the plasma membrane. No signal was detectible in the LYK3:HASt transgenic lines. As the signal was somewhat brighter in the LYK3:GFP (22.3) line (Table 3-1) and in hairy roots in the hcl-2 background, these were used for subsequent localization studies.

Figure 3-2. LYK3 has a punctate distribution in root hairs
In uninoculated root hairs of the stable transgenic line 22.3 (hcl-1 mutant transformed with pLYK3:LYK3:GFP). The GFP signal was most evident in emerging (A) and elongating (B) root hairs. The signal was punctate and densest near the growing root hair tips. Images are maximum intensity projections of ~50 z-sections taken at 0.3µm increments. Scale: 10 µm.
Figure 3-3. LYK3:GFP has little overlap with a cytoplasmic marker in root hairs
Hairy roots were generated in the hcl-2 mutant background with a plasmid containing the genomic LYK3 fused to GFP (pLYK3:gLYK3:GFP) and dsRed which marks the cytoplasm and nucleus (Curtis and Grossniklaus, 2003). The top half of each panel shows single z-slices through root hairs; the bottom halves show maximum intensity projections of ~50 z-sections taken at 0.3µm increments. Scale: 10 µm. LYK3:GFP has a punctate distribution (A) emerging, (B) elongating and (C) fully elongated root hairs.
Figure 3-4. LYK3:GFP signal stays with the membrane upon plasmolysis
Roots in the transgenic line 22.3 were treated with 0.5 M sodium chloride for 10 minutes until the plasma membrane (arrow) had visibly retracted from the cell wall (arrowhead). (A) Single optical section of plasmolysed root hair. (B) Maximum intensity projection of 100 z-sections taken at 0.3µm increments showing reconstruction of the root hair cell. (C) DIC image of plasmolysed root hair. Scale: 10 µm.

DMI2:GFP signal was visible in hairy roots but not in the single available stable transgenic line. In hairy roots in all dmi2 mutant backgrounds tested (Table 3-1), DMI2:GFP had a punctate distribution at the extreme cell periphery similar to LYK3:GFP (Figure 3-5). However, the signal gradient observed with LYK3:GFP, with the densest signal at the root hair tip, was not observed with DMI2:GFP. There was little co-localization of DMI2:GFP with a cytoplasmic marker and few intracellular vesicles were evident (Figure 3-5).
Figure 3-5. DMI2 has a punctate distribution associated with root hair plasma membranes

Hairy roots were generated in the dmi2-1 mutant background with a plasmid containing the genomic DMI2 fused to GFP (pDMI2:gDMI2:GFP). The top half of each panel shows single z-slices through root hairs; the bottom halves show maximum intensity projections of ~50 z-sections taken at 0.3µm increments. Scale: 10 µm. Plasmids for hairy root transformation contain a dsRed marker that marks the plant cytoplasm and nucleus enabling screening for transgenic roots (Curtis and Grossniklaus, 2003). DMI2:GFP has a patchy appearance in (A) emerging, (B) elongating and (C) fully elongated root hairs.
LYK3:GFP localizes to intracellular vesicles and infection threads post inoculation

Few LYK3:GFP vesicles were visible in the cytoplasm in uninoculated root hairs (Figure 3-4 and Figure 3-6A). Following treatment of roots with an S. meliloti strain producing constitutive high levels of NF (Fisher et al., 1988), an increase in LYK3:GFP intracellular vesicles was evident by 6 hours post inoculation (hpi) (Figure 3-6B) and persisted at 24 hpi (Figure 3-6C). An increase in vesicles was also observed with 1 nM purified S. meliloti NF at 24 hpi, although the response was less robust (Figure 3-6D). No consistent increase in vesicles was evident at 0.5 or 3 hpi (not shown).

After bacterial treatment, LYK3:GFP signal was visible on the membrane surrounding initiating and elongating infection threads in root hairs (Figure 3-6E to N). By analogy to the membrane localization of LYK3:GFP in plasmolysed root hairs (Figure 3-3), we inferred that LYK3 localized to infection thread membranes and not the infection thread wall. No signal was associated with infection threads of control plants expressing LYK3:HASt (Figure 3-6F). No signal was visible along infection thread membranes in cortical cell layers at 4, 7, 10 and 14 dpi (Figure 3-7). We observed pronounced auto-fluorescence associated with older infection threads in the infection zone in control LYK3:HASt nodules (Figure 3-7).
Figure 3-6. LYK3 localizes to intracellular vesicles and infection threads post inoculation

(A-F) *M. truncatula* stable lines transformed with pLYK3:LYK3:GFP (A-E) or pLYK3:LYK3:HAS* (F). (A) In uninoculated roots, LYK3:GFP localized to the cell periphery and few intracellular vesicles were visible. (B) By 6 hours post inoculation with rhizobia, intracellular LYK3:GFP vesicles were visible. (C) LYK3:GFP vesicles persisted at 24 hours post inoculation. (D) Treatment with NF also increased appearance of vesicles, although less robustly than bacterial treatment. (A-D) are single optical sections. (E) After inoculation LYK3:GFP (green) was visible on growing infection thread membranes at 4 dpi (bacteria are expressing *ptrp:mCherry* and shown in red). (F) No GFP signal was visible on infection threads of plants expressing *LYK3:HAS* (E-F) are maximum intensity projections of ~50 optical sections taken at 0.3 µm increments. Arrows mark infection thread membranes; arrowheads point to bacterial colonies in curled root hairs.

(G-N) Introduction of pLYK3:gLYK3:GFP into hairy roots complemented the infection defect in the *LYK3* mutant hcl-2. Arrows mark infection thread membranes; arrowheads point to bacterial colonies in curled root hairs. (G) LYK3:GFP was visible at the sites of initiating and (K) elongating infection threads. (H, L) The inserted plasmid contains *pUbq:dsRed* which marked the cytoplasm and nucleus (*). (I,M) Plants were inoculated with *S. meliloti* expressing *ptrp:CFP*. (J) is an overlay of (G-I) and (N) is an overlay of (K-M). (G-J) are single optical sections; (K-N) are maximum intensity z-projections of ~50 optical sections taken at 0.3 µm increments. Scale bars: 10 µm.
Figure 3-7. LYK3:GFP is not visible on infection threads in interior nodule cells
Transgenic roots expressing either LYK3:GFP (line 22.3; bottom panels) or LYK3:HASl (line 46.10; top panels) were inoculated with S. meliloti expressing ptrp:mCherry. Images show infection threads (arrows) in the infection zone of a 10 day old nodule where bacterial release was evident (*) but little differentiation had occurred. No signal that was consistently different than auto-fluorescence was observed on infection threads in mature nodules in the LYK3:GFP-expressing plants. Images are maximum intensity z-projections of ~50 optical sections taken at 0.3 µm increments. Scale bars: 10 µm.

DMI2:GFP localizes to intracellular vesicles post-inoculation
Like uninoculated pLYK3:gLYK3:GFP-expressing root hairs, the majority of the DMI2:GFP signal localized to the extreme cell periphery and not to intracellular vesicles (Figures 3-5 and 3-8A). As early as 30 minutes post inoculation with either S. meliloti or purified NF, a DMI2:GFP signal was evident on numerous intracellular structures (Figure 3-8B). By 1 dpi, little signal was associated with either the plasma membrane or cytosolic vesicles (Figure 3-8C). A faint cytoplasmic GFP signal was visible at this time. No DMI2:GFP signal was detectible on infection threads in root hairs (Figure 3-8D) or infection threads in nodules (Figure 3-9).
**Figure 3-8. After bacterial treatment, DMI2:GFP localizes to intracellular vesicles**

Hairy roots were generated with pDMI2:gDMI2:GFP on the dmi2-1 mutant background; dsRed marks the cell cytoplasm and nucleus (*). (A) In uninoculated root hairs, the majority of the DMI2:GFP signal localizes to the extreme cell periphery and little DMI2:GFP signal co-localizes with a cytoplasmic marker (red). (B) By 30 minutes post treatment with NF, there is an increase in DMI2:GFP cytoplasmic vesicles. (C) By 1 day post treatment with NF, very little DMI2:GFP signal is visible along the plasma membrane or cytosolic vesicles. (D) 4 days post inoculation with S. meliloti expressing ptrp:CFP, no DMI2:GFP signal is visible along infection thread membranes (arrow). (A and B) are single optical sections; (C-D) are maximum intensity z-projections of ~50 optical sections taken at 0.3 µm increments. Scale bars: 10 µm.

**Figure 3-9. DMI2:GFP is not visible on infection threads in interior nodule cells**

Transgenic hairy roots generated on dmi2-1 mutants expressing either DMI2:HAS (top panels) or DMI2:GFP (bottom panels) were inoculated with S. meliloti expressing ptrp:CFP. Images show infection threads (arrows) in the infection zone of a 10 day old nodule where bacterial release was evident (asterisk) but little differentiation had occurred. No signal that was consistently different than auto-fluorescence was observed in the DMI2:GFP-expressing plants. Images are maximum intensity z-projections of ~50 optical sections taken at 0.3 µm increments. Scale bars: 10 µm.
DISCUSSION

We demonstrated that \textit{LYK3} and \textit{DMI2} N-terminal fusions can complement infection, nodulation and nitrogen fixation defects in \textit{lyk3} and \textit{dmi2} mutants. We show that the nodules on stable transgenic lines can fix nitrogen, but that some lines have reduced fixation compared to wild type nodules (Figure 3-1). This indicates that the fusion proteins were functional; the most likely explanation for partial complementation is that the transgenes did not reflect endogenous expression levels due to positional effects. The formation of nodules with reduced fixation suggests that \textit{LYK3} and \textit{DMI2} are required at multiple stages or continually during symbiosis, and not just during initiation of symbiosis. This is consistent with previous characterizations of partial loss of function mutants and RNAi lines showing that both \textit{LYK3} and \textit{DMI2} are required for infection to progress (Limpens \textit{et al.}, 2005; Smit \textit{et al.}, 2007).

\textit{LYK3} is required for infection thread initiation and continued infection thread growth (Catoira \textit{et al.}, 2001; Smit \textit{et al.}, 2007). The presence of \textit{LYK3}:GFP at the plasma membrane prior to interaction with bacteria, and the persistence of \textit{LYK3} on infection threads, are consistent with these genetic data (Figures 3-2, 3-3 and 3-6). We observed that \textit{LYK3}:GFP is associated with intracellular vesicles post inoculation. The origin of these vesicles is unknown; it is possible that they are the result of endocytosis of \textit{LYK3}:GFP from the membrane or that they are the result of new protein synthesis and packaging into secretory compartments. \textit{LYK3} eventually localizes to infection threads in root hairs; determining the origin of \textit{LYK3}-containing vesicles will help elucidate both how proteins are trafficked to infection threads during symbiosis, and the mechanism of \textit{LYK3}-mediated infection.

While \textit{LYK3}:GFP was present on infection threads in root hairs, we could not detect \textit{LYK3}:GFP along infection thread membranes in interior nodule cells (Figure 3-7). This lack of signal is consistent with previous reports that \textit{LYK3} expression is weak to absent in the infection zone of mature nodules (Limpens \textit{et al.}, 2005; Mbengue \textit{et al.}, 2010). The apparent absence of the \textit{LYK3} protein associated with interior nodule cells is consistent with several interpretations: (a) low levels of \textit{LYK3}
are sufficient for NF perception in nodules, (b) LYK3 may not be required for later stages of infection, and/or (c) a different receptor is involved.

The observed behavior of DMI2:GFP was distinct from that of LYK3:GFP. While LYK3 vesicles were evident no earlier than 6 hpi and persisted at 24 hpi, DMI2 vesicles were evident within 30 minutes of bacterial or NF treatment (Figure 3-8). These vesicles did not persist and little to no DMI2:GFP signal was visible in root hairs or in infected cells beyond 1 dpi. The rapid appearance of DMI2:GFP vesicles upon NF application followed by signal loss is reminiscent of the ligand-dependent endocytosis observed with the plant flagellin receptor FLS2 (Robatzek et al., 2006).

DMI2 is not predicted to bind NF; if the observed vesicles are in fact the result of endocytosis, the most likely explanation is that NFP receptor (which itself lacks a predicted kinase domain) binds NF and then interacts with DMI2, and NFP and DMI2 are internalized as a complex. This sort of receptor cooperation has been shown for the BAK1 receptor like kinase and several receptor partners including the brassinosteroid receptor BRI1 (Li et al., 2002; Nam and Li, 2002) and flagellin receptor FLS2 (Chinchilla et al., 2007). Future studies are needed to determine if the increase in DMI2:GFP vesicles are in fact the result of endocytosis of the membrane signal, and whether this depends on NFP. Exploring the origin of DMI2 vesicles will help elucidate the mechanism by which plants perceive and transduce the NF signal.

Our DMI2:GFP localization data conflict with a previous report that DMI2:GFP is visible along infection threads and plasma membranes in infected nodule cells (Limpens et al., 2005). Our experimental design closely mirrored the Limpens et al. (2005) study; in both experiments, DMI2:GFP driven by its native promoter was introduced into the dmi2-1 mutant background via A. rhizogenes-mediated hairy roots. The prior study reported using a laser scanning confocal for their localization while we used a more sensitive spinning-disk confocal; it is unlikely that sensitivity of the instrument can account for the absence of signal in our experiment. Additionally, the previous study did not report a DMI2:GFP signal in root hairs prior to inoculation, again suggesting that our methods are equally or more sensitive. A few subtle differences in the experimental designs exist. First, Limpens
et al. (2005) used a 2.2 kb promoter instead of our 2.0 kb promoter; it is possible that there is an enhancer element in the missing 200 bps that is important for DMI2 expression. Second, Limpens et al. (2005) used a slightly different hairy root transformation method; it is feasible that their method allowed for recovery of roots with stronger expression of the DMI2:GFP transgene. A final possibility is that what Limpens et al. (2005) reported as signal was actually auto-fluorescence. No controls for auto-fluorescence were used in their study, and we observed substantial auto-fluorescence on older infection threads (Figure 3-9). Resolving these issues will help establish whether DMI2 functions locally to regulate infection thread growth and bacterial release (as proposed by Limpens et al., 2005) or more globally to help establish and monitor the progress of symbiosis (Endre et al., 2002; Bersoult et al., 2005).

We demonstrated that symbiotic receptor-like kinases have patchy distributions associated with plant plasma membranes that are reminiscent of membrane raft signaling domains in animals (Lingwood and Simons, 2010). Our findings suggest that compartmentalization of membrane proteins may be important for signaling in plants. Prior to inoculation, the similarity in appearance and distribution of LYK3:GFP and DMI2:GFP signals raises the possibility that the proteins are co-distributed and possibly form a symbiotic receptor complex. The differences in observed behaviors after inoculation support genetic data suggesting that DMI2 functions as a component of the NF perception signaling pathway (Catoira et al., 2000; Bersoult et al., 2005) while LYK3 is involved in infection (Smit et al., 2007). Our studies are the first steps in elucidating the localization and regulation of symbiotic receptor kinases before and during symbiosis. These studies complement a decade of genetics that established the roles of receptor kinase genes in early symbiotic events.
MATERIALS AND METHODS

Plant growth and bacterial treatments.
*Medicago truncatula* Gaertner cv Jemalong, cv Jemalong A17 (an inbred line of Jemalong), and mutant lines *hcl-1, hcl-2,* and *hcl-3* (Catoira *et al.*, 2001), *DMI2-1* (Catoira *et al.*, 2000), *DMI2-3* and *DMI2-4* (Endre *et al.*, 2002) and transgenic lines were grown, inoculated and harvested as described (Mitra and Long, 2004). Bacterial growth and inoculations were done as described in Chapter 2.

Generation of LYK3 and DMI2 translational fusion constructs and transgenic plants

*pLYK3:gLYK3:GFP* and *HASt* and *pDMI2:gDMI2:GFP* and *HASt* constructs and transgenic lines were obtained from Brendan Riely in Doug Cook’s Lab (University of California, Davis; personal communication).

Fluorescent bacterial plasmid construct design

*pQDN01* was constructed by replacing GFP in pDG71 (Gage, 2002) with CFP. *pQDN03* was constructed by replacing GFP in pDG71 with mCherry (Haney and Long, 2010). Both mCherry and CFP were amplified using forward primer TTTGGATCCACCATGGTGAGCAAGGGC and reverse primer TTTTCTAGATTTTGTACAGCTCGTCCATGC. PCR products and were digested with BamHI and XbaI and ligated into the corresponding sites in pDG71.

Acetylene reductions and hairy root transformation

were conducted as described in Chapter 2 materials and methods.

Confocal Microscopy

For 24 hpi localization studies, plants were inoculated with Rm1021 over-expressing NodD3 from the plasmid pRmE65 (Fisher *et al.*, 1988). For infection thread localization studies, plants were inoculated with Rm1021 constitutively expressing mCherry or CFP from the plasmids pQDN03 or pQDN01 pretreated for ~2 hours with
3 μM luteolin. The signal was brighter in the 22.3 LYK3:GFP than in the 46.10 line possible likely due to positional effects of the transgene. The 22.3 line was used for localization studies.

Root segments and nodule hand sections (for imaging the infection zone) were excised and mounted in BNM buffer pH 6.5. Spinning disk confocal microscopy was performed on a system described previously in Gutierrez et al. (2009) using a 100X/1.4 NA oil immersion objective for root hair membrane imaging or a 63X/1.3 NA glycerol immersion objective for infection thread and nodule imaging. CFP, GFP and dsRed/mCherry were excited at 442, 491 nm and 561 nm, respectively, by solid-state lasers; emission filtering was accomplished with band pass filters (470/40 for CFP, 530/50 nm for GFP, and 640/50 nm for mCherry/dsRed; Chroma Technology). Maximum intensity z-projections of root hairs are from ~100 images taken at increments of 0.3 μms (MCL NanoDrive). Time-lapse images were taken at 2s increments. Stacks were processed using ImageJ software (http://rsbweb.nih.gov/ij/). Typical exposure times were 1000 ms for GFP, 300 ms for dsRed, 500 ms for mCherry, and 500 ms for CFP.
CHAPTER 4: Co-localization of flotillin protein FLOT4 with the symbiotic receptor LYK3

ABSTRACT

In response to signals, proteins with patchy distributions in animal membranes redistribute laterally, a previously unreported phenomenon in plants. A *Medicago truncatula* flotillin protein, FLOT4, has a punctate plasma membrane distribution and is required for infection by the nitrogen-fixing symbiotic bacterium *Sinorhizobium meliloti*. We show that FLOT4 puncta density is altered in a predicted dead kinase mutant of the symbiotic receptor *LYK3*. Like FLOT4, LYK3 has a patchy plasma membrane distribution and localizes to infection thread membranes. In buffer-treated control roots expressing tagged *LYK3* and *FLOT4*, FLOT4 puncta are relatively stable while LYK3 puncta are dynamic; the two labeled proteins show relatively little co-localization. Bacterial treatment causes an increase in FLOT4 and LYK3 co-localization, and both proteins occupy positionally stable plasma membrane domains. Our work indicates that plant membrane protein arrangement and dynamics are altered in response to symbiotic bacteria.
INTRODUCTION

Proteins, sterols and lipids are often heterogeneously distributed in cellular plasma membranes, defining domains of varying size. Studies of animals and fungal cells show that extracellular stimuli and changes in membrane potential elicit changes in microdomain structure and composition (Giri et al., 2007; Grossmann et al., 2007). Emerging evidence suggests that some plant membrane components also have patchy distributions (Homann et al., 2007; Gutierrez et al., 2009; Raffaele et al., 2009; Haney and Long, 2010). How plant membrane proteins are organized and the significance of lateral compartmentalization has yet to be established except by analogy to animals and fungi. Legume-rhizobia symbioses provide a model for exploring changes in membrane protein distribution during signal perception. In this system, membrane protein distribution and dynamics can be monitored in an intact live host, abundant molecular and genetic tools are available, and there is an extensive body of work establishing a clear sequence of distinct cellular behaviors for early plant responses to bacteria.

Recent studies implicated the flotillin proteins (FLOT) and a remorin protein (SymREM) in symbiotic plant-microbe interactions (Haney and Long, 2010; Lefebvre et al., 2010). FLOT2 and FLOT4 function non-redundantly during symbiosis between Medicago truncatula and its bacterial symbiont Sinorhizobium meliloti (Haney and Long, 2010). Fluorescent protein fusions to FLOT2 and FLOT4 have a punctate plasma membrane distribution. Both FLOT4:GFP and SymREM localize to host-derived infection structures called infection threads and are required for normal infection thread growth. FLOT4:GFP redistributes after bacterial inoculation to form a bright cap at the root hair cell tip (Haney and Long, 2010) suggesting that perception of symbiotic bacteria alters membrane protein distribution.

Legumes are able to recognize rhizobial signaling molecules known as Nod Factors (NFs). NFs consist of a chitin backbone with several species-specific modifications (Roche et al., 1991a). NF perception and signaling in M. truncatula is dependent on the receptors NFP, LYK3 and DMI2 (Catoira et al., 2000). NFP and LYK3 encode LysM-type receptors that are hypothesized to bind bacterially secreted
NFs (Amor et al., 2003; Smit et al., 2007; Lefebvre et al., 2010); LysM-family receptors bind chitin in Arabidopsis (Iizasa et al., 2010). DMI2 is a leucine-rich repeat receptor kinase predicted to act downstream of NFP (Catoira et al., 2000). NFP and DMI2 mutants are impaired in nearly all responses to symbiotic bacteria (Catoira et al., 2000) whereas LYK3 mutants (also known as hcl) are impaired in infection (Catoira et al., 2001; Smit et al., 2007).

Using the patchy distribution of FLOT4:GFP (Chapter 2), we explored the role of signal perception (via NFP, LYK3 and DMI2) in regulating organization of membrane proteins. While the function of FLOT4 distribution is not known, it can serve as a scorable marker for membrane organization and can be used as a phenotype to assess the effect of genetic changes on membrane structure. We found that the density of FLOT4 puncta is decreased in a predicted dead kinase mutant of LYK3. Like FLOT4, tagged LYK3 has a patchy distribution in plant membranes, forming puncta at or below the limit of optical resolution. LYK3 puncta undergo a shift in dynamics from mobile to immobile after treatment with symbiotic bacteria. Immobile LYK3 puncta co-localize with FLOT4 after bacterial treatment. These results show that plant membrane proteins undergo complex lateral reorganization in response to a signal.
RESULTS

A change in FLOT4 distribution is associated with rhizobia-triggered re-initiation of root hair growth

Prior to inoculation, FLOT4:GFP localizes to puncta that are evenly distributed along root hair plasma membranes; by 24 hours post inoculation FLOT4 is distributed in a cap at the tip of some elongating root hairs (Haney and Long, 2010). To explore the timing of these events in more detail, we repeated these experiments at 1, 6, 12, 18, 24, and 36 hours post inoculation (hpi). We found that the tip localization was observed in root hairs that had re-initiated directional growth but had not formed a full curl (Figure 4-1). Root hair responses to bacteria are asynchronous; nonetheless we were able to assign these events primarily to the interval between 12 and 24 hpi. We also found that in responding root hairs, FLOT4:GFP puncta appear dimmer and more diffuse (Figure 4-1). By 36 hours, many root hairs had formed full curls and tip localization was less apparent, possibly due to auto-fluorescence in the inner portion of the curl (Figure 4-1, right panel).

Figure 4-1. Redistribution of FLOT4 during re-initiation of root hair tip growth

Hairy roots expressing pFLOT4:gFLOT4:GFP were observed at 1, 4, 6, 12, 24, and 36 hours post inoculation. Tip localization of FLOT4:GFP was observed in root hairs that had re-initiated directional growth but not formed a full curl; root hairs in this stage were primarily observed between 12 and 24 hours post inoculation with S. meliloti Rm1021 over-expressing NodD3. At least 10 root hairs on 5 roots were observed at each time point. Images are maximum intensity projections of ~50 optical sections taken at 0.3 µm increments. Scale bar: 10 µm.
Over-expression of FLOT2 results in increased FLOT4 polarity

Since GFP-tagged FLOT2 and FLOT4 have similar localizations in root hairs but differ in epidermal cells (Chapter 2), we explored the degree of co-distribution of tagged FLOT2 and FLOT4 proteins. We used Agrobacterium rhizogenes to generate A17 hairy roots co-transformed with pFLOT4:gFLOT4:GFP and 35S:gFLOT2:mCherry (FLOT2 was not visible under its native promoter). In roots with visible green and red puncta, FLOT4:GFP had an increase in polarity compared to roots transformed with just FLOT4 alone (Figure 4-2A and B). In root hair cells, FLOT2:mCherry and FLOT4:GFP puncta had a high degree of overlap. We quantified the overlap in three dimensional space and found a Pearson’s correlation coefficient ($r$) of 0.73±0.06 (n=12 root hairs from 4 plants; Figure 4-2C). Post inoculation, we observed a decrease in overlap of FLOT2 and FLOT4 puncta in root hairs that had reinitiated directional growth and now $r=0.45±0.12$ (n=15 root hairs from 3 plants; Figure 4-2D; P=0.0004). We occasionally observed tip localization of the FLOT2:mCherry signal in root hairs that have reinitiated growth, which was not observed in plants transformed with FLOT2:GFP (Chapter 2) or FLOT2:mCherry alone.
Figure 4-2. Co-expression of FLOT2:GFP and FLOT4:mCherry

(A) In uninoculated epidermal cells, FLOT4:GFP was evenly distributed along the plasma membrane and FLOT2:mCherry was polarly localized (arrows). Images are single optical sections; scale bar: 10 µm. (B-D) Hairy roots co-expressing pFLOT4:gFLOT4:GFP and p35S:gFLOT2:mCherry. Images in (B) are single optical sections of epidermal cells; (C-D) are maximum intensity projections of ~50 optical sections taken at 0.3 µm increments. Scale bars: 10 µm. (B) In uninoculated epidermal cells, an increase in polar distribution of FLOT4:mCherry was observed (arrows). (C) FLOT4:GFP and FLOT2:mCherry puncta co-localize in uninoculated root hairs. (D) Upon inoculation, there is a lower degree of co-localization in root hairs that have re-initiated directional growth and there is an increase in signal from both proteins at root hair tips (arrows).

FLOT4 mis-localizes in a LYK3 mutant

Early plant responses to bacteria and Nod Factor, including re-initiation of root hair growth, depend on NFP and LYK3 pathways. To determine if these pathways are also required for FLOT4 redistribution in response to S. meliloti, we expressed FLOT4:GFP in mutant backgrounds of the symbiotic receptors DMI2, NFP, and LYK3. We found that in the absence of bacteria, the density of FLOT4 puncta decreased in epidermal cells of the LYK3 mutant hcl-1 (predicted dead kinase allele) but was unaffected in nfp (predicted null allele) or dmi2-4 (predicted dead kinase) genetic backgrounds (Figure 4-3A through D, I). In wildtype plants, FLOT4 puncta are relatively evenly dispersed along cell membranes of epidermal cells and root hairs (Haney and Long, 2010). There was a slight increase in polar localization of FLOT4:GFP puncta in all three mutant backgrounds (Figure 4-3J; P<0.01). By contrast, FLOT2:GFP, which normally shows marked polarity in epidermal cells but not root hairs, had no decrease in spot density or increase in polar localization when expressed in the hcl-1 background (Figure 4-3G and H). Thus, increased polar localization is specific to FLOT4, and mis-localization of FLOT4 is not due to a general perturbation of membrane proteins in the hcl-1 mutant. We conclude that prior to bacterial inoculation, FLOT4 distribution is partially dependent on symbiotic receptors while FLOT2 is not.

The hcl-1 mutant allele harbors a single-base substitution within the highly conserved ATP binding pocket of the LYK3 kinase domain, a change that is predicted
to abolish kinase activity (Smit et al., 2007). By contrast, the hcl-2 and hcl-4 alleles contain mutations in first intron splice sites and display reduced (hcl-4) or no (hcl-2) LYK3 transcript (Smit et al., 2007). We tested if decreased FLOT4 puncta density was specific to the hcl-1 allele or if it also occurred in the LYK3 splice mutants. The density of FLOT4:GFP puncta in hcl-2 and hcl-4 was indistinguishable from wild type roots (Figure 4-3E,F,I and J). We also assessed FLOT4 distribution and found FLOT4:GFP was significantly more polar in hcl-2 while localization in hcl-4 was indistinguishable from wild type roots (Figure 4-3J). The degree of increase in FLOT4 polar localization correlates with the strength of the symbiotic defect suggesting that FLOT4 localization depends on the activity of LYK3 (Smit et al., 2007).
Figure 4-3. FLOT4 mis-localizes in roots carrying mutations in a symbiotic receptor

(A-F) Hairy roots transformed with pFLOT4:gFLOT4:GFP; images are maximum intensity z-projections of ~45 optical sections taken at 0.3 μm increments. (A) FLOT4:GFP puncta in epidermal cells of wild type (A17), (B) hcl-1, (C) dmi2-4, (D) nfp-1 (E) hcl-2 or (F) hcl-4 roots. Arrows point to cells with polar protein localization. (G-H) Epidermal cells of hairy roots transformed with 35S:gFLOT2:GFP (images are single optical sections) in (G) wild type (A17) or (H) hcl-1 roots. (I) Decreased spot density was specific to FLOT4:GFP in the hcl-1 mutant background. (J) Polar localization was approximated by the ratio of apical cell membrane signal density to abaxial membrane signal density (see methods). Increased polar localization was found for FLOT4:GFP when expressed in hcl-1, hcl-2, dmi2-4 and nfp mutants. (I-J) Maximum intensity z-projections of ~45 optical sections taken at 0.3 μm increments were used for analyses. Error bars represent ± SEM; *P<0.01. Scale bars: 10 μm.

FLOT4 and LYK3 have increased co-localization after bacterial inoculation

FLOT4 and LYK3 have similar RNAi phenotypes, gene expression patterns, and protein localization, suggesting they may function cooperatively to mediate symbiotic infection and that FLOT4 and LYK3 membrane puncta may be co-distributed (Smit et al., 2007; Haney and Long, 2010; Mbengue et al., 2010). We generated hairy roots expressing pFLOT4:gFLOT4:mCherry in wild type plants, the hcl-1 mutant, or the hcl-1 mutant stably transformed with pLYK3:gLYK3:GFP. The hcl-1 mutant displayed both a decrease in density of FLOT4:mCherry puncta and a polarity defect; the pLYK3:gLYK3:GFP transgene complemented only the density phenotype (Figure 4-4).
Figure 4-4. LYK3:GFP complements the decrease in FLOT4 puncta density in the hcl-1 mutant

(A-C) Hairy roots expressing pFLOT4:gFLOT4:mCherry were generated on wild type A17 plants, hcl-1 mutant plants or the hcl-1 mutant stably transformed with pLYK3:gLKY3:GFP. Maximum intensity z-projections of ~45 optical sections taken at 0.3 µm increments were used for analyses. Scale bar: 10 µm. (A) FLOT4:mCherry localizes to membrane puncta in A17 plants. (B) In the hcl-1 mutant, FLOT4:mCherry has a lower density of puncta and has increased polarity. (C) The LYK3:GFP transgene complements the decrease in density of FLOT4 puncta in an hcl-1 mutant background. (D) Quantification of localization shows that LYK3:GFP rescues the decrease in FLOT4 puncta but not polarity. (a vs. b P < 0.05 by two-tailed t-test).

In buffer-treated root hairs, FLOT4:mCherry and LYK3:GFP showed a limited overlap in distribution but had distinct dynamic behaviors (Figures 4-5 and 4-6, left panels). Using 3-D image volumes acquired at the root hair cell tip, we calculated Pearson’s correlation coefficients (r) in voxels (Costes et al., 2004). In buffer-treated root hairs, r=0.15±0.03, indicating that FLOT4 and LYK3 co-localize only slightly more often than is predicted by an uncorrelated distribution of the proteins. Time-lapse imaging revealed that bright FLOT4 puncta were relatively stable while the majority of LYK3 puncta were dynamic (Figure 4-6A,B, and C). Intensities from FLOT4 and LYK3 signals along linear transects of the time-averaged images show that in the absence of bacteria there is little correlation of average LYK3 and FLOT4 position and intensity (Figure 4-6D,E).

Bacterial treatment of root hair cells had a pronounced effect on FLOT4 and LYK3 co-localization at 24 hours (Figure 4-5, right panels). In cells that reinitiated tip growth FLOT4 and LYK3 co-localization rose sharply (Figure 4-5C; r=0.66±0.03; P=5x10⁻¹⁵). In inoculated root hairs that had not reinitiated tip growth (swollen or no
visible response), we observed an increase in co-localization compared to buffer treated roots, although to a lesser degree ($r=0.40\pm0.05; P=1x10^{-4}$). As observed with FLOT4:GFP (Figure 4-1; Haney and Long, 2010), FLOT4:mCherry became distributed in more spots by 24 hpi and redistributed to form a cap at the cell tip (Figure 4-5A).

After bacterial treatment, the dynamics of LYK3:GFP also changed significantly in root hairs that had re-initiated tip growth. The LYK3:GFP signal shifted from motile to relatively stable puncta, and both the positions and relative intensities of stable LYK3:GFP puncta were well correlated with FLOT4:mCherry puncta (Figure 4-6, compare left and right panels). LYK3:GFP vesicles were evident in the cytoplasm of these root hairs, but these vesicles were not marked by FLOT4:mCherry (Figure 4-7).
Figure 4-5. LYK3 and FLOT4 co-localize in inoculated root hairs in 3-D space

(A) Co-distributions of FLOT4:mCherry and LYK3:GFP signals in 3-D space; z-stacks were obtained at 0.3 µm increments from the top 15 µms of root hairs.

(B) Higher magnification of images in (A).

(C) Pearson’s correlation coefficients ($r$) for 3-D voxel intensities were calculated from 2-D correlation plots (Costes et al., 2004). Left panels: buffer-treated root hairs ($r=0.15±0.03$; n=23 root hairs on 4 plants). Right panels: 24 hours after bacterial treatment a significant increase in co-distribution was observed ($r=0.66±0.03$; n=22 root hairs on 3 plants; $P=5x10^{-15}$). Maximum intensity projections, correlation plots, and $r$-values of representative root hairs are shown.
Figure 4-6. After inoculation, LYK3 puncta shift from dynamic to stable
Time-series images of FLOT4 and LYK3 after buffer or bacterial treatment acquired at 2s intervals for 180s.
(A) Single frame shows that the puncta distribution of LYK3 and FLOT4 is maintained in the absence or presence of bacteria
(B) 90-frame averaged images are shown; diffuse signals are indicative of mobile puncta while discrete spots designate stable puncta.
(C) Kymographs of intensities in 90-frame time-series images (dashed blue line in B). In the absence of bacteria, LYK3 was dynamic and individual puncta were unstable, which produced an unstructured pattern in the kymograph. FLOT4 puncta were relatively stable and yielded vertical streaks. In the presence of bacteria, both LYK3 and FLOT4 puncta were stable and created vertical streaks in the kymographs. (A-C) Scale bars: 5 µm.

(D) Signal intensities from FLOT4 (red) and LYK3 (green) signals along linear transects of the time-averaged images (dashed blue lines) are shown graphically. In the absence of bacteria there is little correlation of average LYK3 and FLOT4 position and intensity ($R^2=0.27\pm0.06$; n=12 transects on 3 plants). With bacterial treatment, both the position and the relative intensity of the puncta are correlated ($R^2=0.85\pm0.02$; n=16 transects on 4 plants; $P=1\times10^{-10}$).

(E) Slope vs. intensity plots for charts in (B) are shown. These show that the rate of change in signal intensity as a function of position is well correlated between FLOT4:mCherry and LYK3:GFP only in the presence of bacteria. In buffer-treated root hairs $R^2(slope)=0.10\pm0.03$; in bacteria-treated root hairs $R^2(slope)=0.74\pm0.02$; $P=4\times10^{-13}$ by two-tailed t-test.

Figure 4-7. FLOT4:mCherry does not label LYK3:GFP intracellular vesicles
Hairy roots expressing $pFLOT4:gFLOT4:mCherry$ were generated on plants transformed with $pLYK3:gLYK3:GFP$. One day post inoculation with $S. meliloti$, LYK3 and FLOT4 showed an increase in co-distribution at the plasma membrane (arrowheads) but not LYK3:GFP intracellular vesicles (arrow). Images are single optical sections. Scale bar: 5 µm.
DISCUSSION

FLOT2 affects FLOT4:GFP distribution
We found that FLOT4:GFP localization in epidermal cells was affected by FLOT2. When FLOT4:GFP was co-expressed with FLOT2:mCherry driven by the constitutive 35S promoter, FLOT4:GFP showed increased polar localization in epidermal cells. By analogy to animal FLOTs, this result suggests that FLOT2 and FLOT4 might form hetero-oligomers (Solis et al., 2007; Babuke et al., 2009).

The observation that FLOT4:GFP expressed under its native promoter affects FLOT2:mCherry localization raises questions of copy number and expression level in hairy roots. FLOT2:GFP accumulation at root hair tips was only observed in hairy roots co-transformed with FLOT4:mCherry. When A. rhizogenes strains for the two constructs were mixed in a 1:1 ratio we obtained a frequency of co-transformation of about 1 in 4 recovered roots (data not shown). If transformation primarily occurred as a single event per root, we would not expect nearly this high a rate of co-transformation. This implies that transgenic hairy roots contain multiple copies of the transgenes, and even when expressed under native promoters, expression levels may not be reflective of endogenous levels.

Plant membrane protein compartmentalization
We have found strong evidence that FLOT4 and LYK3 are components of the same pathway. We show that the phenotype of FLOT4-silenced roots (Chapter 2) closely resembles the phenotype of LYK3-silenced roots (Smit et al., 2007). A point mutation in LYK3 caused decreased FLOT4:GFP spot density and increased polarity (Figure 4-3), and after bacterial treatment, tagged FLOT4 and LYK3 co-localize in space and time (Figures 4-5 and 4-6). The most parsimonious explanation for these observations is that FLOT4 and LYK3 are components of a shared complex. An alternative is that FLOT4 and LYK3 co-localization arises because they occupy distinct (sub-microscopic resolution) protein complexes within stable microdomains. Further studies are required to determine the functional significance of co-localization and regulation of co-distribution of membrane microdomain proteins during symbiosis.
We found evidence for root hair membrane puncta with three different properties: (1) pre-inoculation stable puncta marked by tagged FLOT2/FLOT4, (2) pre-inoculation mobile puncta marked by tagged LYK3, and (3) post-inoculation stable puncta marked by tagged LYK3/FLOT4. Our results show that plant membranes have complex heterogeneity and that proteins exhibit distinct behaviors that can be altered in response to signals. These data also demonstrate that changes in membrane-protein architecture accompany altered root hair morphology during early symbiotic events.

From these observations, we suggest that regulation of protein distribution and dynamics within plant membranes may be a means of regulating protein function. While LYK3 is required for infection thread initiation, the underlying mechanism is unknown. Our data show that after bacterial treatment, LYK3:GFP is not restricted to infection sites but instead shifts from dynamic to stable puncta that persist on plasma membranes. One interpretation of this observation is that LYK3 functions within immobile puncta to send a global signal that reprograms the root hair cell for infection. An alternative is that sequestering LYK3 in immobile puncta negatively regulates LYK3 function and serves to limit infection to one site per cell or to only certain cells. Differentiation between the functional states of mobile and immobile populations of LYK3 will provide insight into the mechanism by which LYK3 regulates infection thread initiation.

More generally, our observations suggest two possible models of how regulation of protein localization within plant membranes may be achieved. One possibility is that multiple types of microdomains always exist in plant membranes and that after perception of a signal, proteins travel from one domain to another. A second possibility is that some proteins (such as LYK3) move freely in and out of stable microdomains (such as those marked by FLOT4); upon signal perception, movement of mobile proteins is restricted to stable domains. Both models allow for the possibility that regulation of plasma membrane protein localization may be a means by which plants direct responses to external signals.
MATERIALS AND METHODS

Plant growth, bacterial treatments and hairy root transformations were performed as described in the materials and methods of Chapter 2. FLOT2:mCherry and FLOT4:GFP co-transformations were done by mixing equal ratios of *A. rhizogenes* carrying each plasmid.

**Construct design**

*pLYK3:gLYK3:GFP* and :HASt constructs and transgenic lines were obtained from Brendan Riely in Doug Cook’s Lab (U.C. Davis, unpublished). Plasmids pCH153 containing *pFLOT4:gFLOT4:mCherry* and pCH046 containing *35S:FLOT2:mCherry* were generated by replacing GFP in plasmids pCH118 and pCH035 (Haney and Long, 2010) with mCherry. MCherry was amplified with primers AAAACCGGGATGGTGAGCAAGGGCGAGGAG and AAAACTAGATCTTTGTACAGCTCGTCCATG and inserted into the XmaI and Xbal sites in pCH118 and pCH035 (Haney and Long, 2010).

**Confocal microscopy**

Microscope setup, optics and filters are described in Chapter 3 materials and methods. Brightest point z-projections of stacks of epidermal cells or root hairs were used for quantification of signal or density of spots. Final images included about 45 z-sections taken at 0.3 μm increments to cover just the top half (~15 μm) of epidermal cells. As a proxy for polarity, the ratio of signal density on the apical cell pole to the epidermal surface was determined as follows: (1) the average apical signal was measured by drawing a 1-pixel wide line along the apical membrane and using ImageJ’s Measure function to determine the average intensity per pixel (or signal density), (2) the epidermal signal was measured by a similar method but with a rectangular selection, (3) the apical signal density was divided by the epidermal signal density to determine ratio of signal density. To measure density of flotillin puncta, images were automatically thresholded based on statistical distribution of signal so that just the puncta on the epidermal surface were defined. Using the “analyze
particles” feature in Image J, the total number of spots was determined and divided by the total area measured to give a spot density. No constraints were placed on spot size or dimensions. At least 5 cells were measured from at least 3 independent plants for a total of at least 15 measurements per treatment.

Co-localization experiments were done in three dimensional space using Imaris’s (Bitplane) co-localization tool as described in Costes et al. (2004). Images were cropped in three dimensions to include approximately 20 μm of the growing root hair tip. The background thresholds were set at 1300 and 1080 for the green and red channels respectively and determined based on auto-fluorescence and background signals measured in hairy roots on LYK3:HAS plants co-transformed with an empty vector pCH040 (same as pCH010 described in Chapter 2 except the plasmid lacks GFP). The Pearson’s correlation co-efficient (r) was determined for co-localization in 3-D space. Twenty-three buffer-treated root hairs were analyzed from 4 individual plants. Forty-three root hairs were analyzed on 3 independent inoculated plants, 22 of which had reinitiated direction growth and 21 of which had not. Two-tailed t-tests with equal variance were used to determine significance of the differences in r.

Time series data was acquired at 2 second intervals over a three minute period. Analysis was done in ImageJ. Linear manual selections (1 pixel width) were made on average intensity projections and stacks to generate average pixel intensity and kymographs. Average pixel intensities were exported to Microsoft Excel to generate graphical representations and coefficients of determination (R²). Slope at pixel position x was approximated by the 3-point estimation: (f(x-h) - f(x+h))/2h where the step h was a single pixel. Two time series data sets were analyzed from each of two independent inoculated or uninoculated roots (four images per treatment). Three selections were analyzed per root hair. Two-tailed t-tests with equal variance were used to determine significance of the differences in R² values.
Plant membrane protein compartmentalization

Some plasma membrane-associated plant proteins have patchy distributions (Sutter et al., 2006; Homann et al., 2007; Krugel et al., 2008; Gutierrez et al., 2009; Raffaele et al., 2009). We found several plant proteins required for legume-rhizobia symbiosis, including two receptor kinases (LYK3 and DMI2) and two flotillin proteins (FLOT2 and FLOT4), that also have patchy distributions associated with plasma membranes. We showed that the distribution of FLOT4:GFP and LYK3:GFP is altered during response to a symbiotic bacterium. The finding that signal perception affects membrane protein distribution is not novel for animal cells (e.g. Stuermer et al., 2004; Giri et al., 2007); yet, these are the first demonstrations of the phenomenon in plant cells. In animal cells, evidence suggests that cellular processes are compartmentalized in punctate plasma membrane domains called “rafts”. Currently, the animal field defines “membrane rafts” as “small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes” (Pike, 2006). The function and even existence of these domains has been the subject of much controversy and attention (discussed below and in Lingwood and Simons, 2010).

Are the membrane puncta marked by LYK3 and FLOT4 analogous to animal membrane rafts? LYK3:GFP and FLOT4:GFP puncta are sub-resolution by light microscopy, so they are likely on the order of 10-200 nm. Animal membrane rafts are nearly always defined by their cholesterol-association (Pike, 2006) and plants have little to no cholesterol (Zappel and Panstruga, 2008). Our observations are consistent with the possibility that LYK3 and FLOT4 co-localization serves to compartmentalize a response to symbiotic bacteria. Yet the field of plant membrane biology is too early in its development to say how similar these domains are to their animal counterparts in terms of size, lipid and sterol composition, and dynamics.

To date, plant cell biology has been affected by misconceptions about how to define membrane rafts (discussed in Opekarovà et al., 2010). Detergent resistance has been used synonymously with membrane raft with little regard for size, composition,
or function (Peskan et al., 2000; Mongrand et al., 2004; Borner et al., 2005; Morel et al., 2006; Lefebvre et al., 2007; Zappel and Panstruga, 2008; Lefebvre et al., 2010; Mongrand et al., 2010). Detergent-based methods can introduce artifacts, and the DRM fraction may not correlate with protein distribution in a living cell (Munro, 2003; Lingwood and Simons, 2010). As a consequence, DRM localization does not necessarily have functional implications, and two proteins that co-partition in the DRM fraction may not co-localize in a living cell. Despite clear evidence that “rafts” are not equivalent to DRMs, detergent resistance is still used in many fields to claim “raft” localization (e.g. Lefebvre et al., 2010; Lopez and Kolter, 2010; Ludwig et al., 2010). Despite these concerns, is difficult to imagine how proteins could have such striking, punctate distributions without functional consequence.

The environment of the plant membrane likely results in domains with distinct properties from animal membranes. Protein diffusion in animal membranes is constrained by a labyrinth of sterols, proteins, and cytoskeletal elements (Kusumi et al., 2010). Because plant membrane composition differs from animal membranes, it is likely that protein diffusion in plant membranes is under distinct constraints and may differ in diffusion rates. Additionally, plant cells are walled, resulting in forces on the plasma membrane from both the cell contents and the cell wall itself. Both stable and dynamic plasma membrane-cell wall contact sites (Kohorn, 2000) could be sites of membrane protein stabilization. Consequently, plant membranes may have unique domains that are constrained by the cell wall. Plant membrane puncta size and dynamics have not been extensively characterized, but evidence suggests that in walled yeast cells, some punctate domains may be larger than 200 nm (Malinska et al., 2003) and may be stable for hours (Malinska et al., 2004). Further work will define the size range of plant membrane puncta, the sterol and lipid environment, and the behaviors of these domains.

**Plasma membrane protein compartmentalization during symbiosis**

Nearly concurrent with the discovery of a requirement for FLOTs in symbiosis was the discovery of a symbiotic remorin (REM) (Lefebvre et al., 2010). REMs, like FLOTs,
have punctate plasma membrane distributions (Raffaele et al., 2009) and localize to infection threads during symbiosis (Lefebvre et al., 2010). Both REMs and FLOTs are peripheral membrane proteins, predicted to be anchored by palmitoylation, and have coiled-coil domains. That FLOTs and REMs have such similar predicted topologies and have nearly indistinguishable but non-redundant requirements in symbiosis begs the question: why both FLOTs and REMs?

One possibility is that FLOTs and REMs function similarly to animal FLOT1 and FLOT2. The two animal flotillins function non-redundantly and cooperatively in several processes (Solis et al., 2007; Babuke et al., 2009; Riento et al., 2009). As plants appear to have only one family of flotillins (Rivera-Milla et al., 2006), REMs may perform a similar function to the second family of animal flotillins. A second possibility is that FLOTs and REMs occupy distinct membrane compartments and that a receptor, such as LYK3, may undergo a sort of “handoff” from one protein to the other. In this model, one might predict that LYK3 and SymREM would co-localize before inoculation and then a “handoff” of LYK3 to FLOT4 would occur after inoculation.

We observed that GFP-tagged DMI2 and LYK3 have similar punctate distributions. DMI2 is required for infection and appears to act before LYK3 (Catoira et al., 2000; Limpens et al., 2005). Genetic data support a model where LYK3 could be phosphorylated by DMI2, resulting in LYK3 redistribution. Determining: 1) if LYK3 and DMI2 co-distribute prior to bacterial treatment, 2) if DMI2 is necessary and sufficient for LYK3 re-distribution and immobilization, and 3) if LYK3 is a target of the DMI2 kinase domain, will provide insight into the mechanisms by which symbiotic receptors regulate infection.

What is the function of FLOTs during symbiosis?

In animals, flotillins associate with effectors that interact with the actin cytoskeleton (Langhorst et al., 2007; Neumann-Giesen et al., 2007; Langhorst et al., 2008a). Cytoskeletal rearrangements are associated with symbiotic infection (Yokota et al., 2009); by analogy, perhaps *M. truncatula* FLOT4 forms a complex with LYK3 and
with effectors that bind actin. In this way, flotillins could link NF perception and the cytoskeletal rearrangements associated with infection. However, if this were the case, one would expect FLOT4 localization, or FLOT4 and LYK3 co-localization, to be limited to the site of initiating and growing infection threads. Instead, we observed that FLOT4 and LYK3 co-localization and LYK3 immobilization is not restricted to the infection site. This observation suggests that the function of FLOTs in symbiosis may not be directly in infection thread initiation and growth.

Membrane depolarization is associated with NF perception in legume root hairs and with signaling in animal neurons and T cells. Animal flotillins function during T cell activation (Stuermer et al., 2004; Langhorst et al., 2006), a process which depends on membrane depolarization. Animal flotillins also associate with ion channels (Morrow and Parton, 2005; Suzuki et al., 2008; Robinson et al., 2010). This begs the question, is there a link between flotillins and membrane depolarization during symbiosis? Within minutes of NF application, root hairs undergo a rapid change in membrane potential followed by calcium spiking (Ehrhardt et al., 1992; Ehrhardt et al., 1996); both membrane depolarization and calcium spiking are required for root hair curling (Wais et al., 2000). However, flotillin-silenced roots do not exhibit root hair curling defects, suggesting that flotillins are not required for membrane depolarization or calcium spiking during symbiosis (Chapter 2). Drug-induced membrane depolarization in yeast causes a change in membrane protein distribution (Grossmann et al., 2007). However, within the first 30 minutes post NF application, when membrane depolarization and calcium spiking occur, we did not detect a redistribution of FLOT4:GFP or a change in LYK3:GFP dynamics (Chapter 4 and not shown, respectively). While these observations do not rule out all possible connections between flotillins and calcium signaling during symbiosis, we did not find evidence for a role of flotillins in calcium spiking or membrane depolarization.

Our data are most consistent with a role for flotillins downstream of calcium signaling, prior to and during infection. In animal cells, flotillins have been implicated in membrane traffic associated with signaling (Langhorst et al., 2008b; Riento et al., 2009; Pust et al., 2010; Stuermer, 2010). We did not observe a significant pool of
intracellular flotillins in any plant cell type under any conditions tested, so it seems unlikely that plant flotillins are involved in endocytosis or exocytosis during symbiosis. Rather, a role for structuring clusters of LYK3 receptors, or bringing LYK3 into contact with other proteins, is most consistent with our observations (Stuermer, 2010).

**Possible functions for plant membrane protein compartmentalization during host-microbe interactions**

We demonstrated that several proteins required for perception of and infection by symbiotic bacteria have patchy distributions associated with the plasma membrane. By analogy to animal membrane rafts, changes in membrane protein compartmentalization may be a general response during plant-microbe interactions. This is supported by the observation that an *Arabidopsis* flotillin is upregulated in roots in response to the fungal elicitor chitin (Millet *et al.*, 2010). As the backbone of bacterial NF is composed of chitin, it is tempting to speculate that FLOTs may have a generalized function in organizing membrane receptors in response to microbial perception. As most plants species have multiple flotillin homologues (Rivera-Milla *et al.*, 2006), individual flotillins may have different receptor specificities, or specificity may be regulated on the level of cell-type-specific expression.

A second possible role for membrane protein compartmentalization is in exocytosis of secondary metabolites. In response to perception of potential pathogens, plants roots deposit a waxy substance called callose in a patchy distribution on the plasma membrane (Millet *et al.*, 2010). Patchy callose deposition is reminiscent of patchy membrane protein distribution and may require targeted exocytosis to specific plant plasma membrane microdomains. Similarly, plant roots secrete secondary metabolites including antimicrobial compounds during defense (Bais *et al.*, 2005) and flavonoids, which act as signaling molecules during legume-rhizobia symbiosis (Peters *et al.*, 1986). It is unknown how these compounds are secreted from plant roots into the surrounding environment, but exocytosis is a likely mechanism. Secondary metabolites are of increasing interest to studies of both plant- and animal-
microbe interactions. Understanding how secondary metabolites are secreted, and determining what role membrane physiology plays in exocytosis in plants, is of enormous importance in answering the question: how do eukaryotic hosts communicate with and defend against microbial communities?
REFERENCES


and association with the microdomain/lipid raft reggie proteins in regenerating CNS axons. Mol Cell Neurosci 22, 544-554.


Madsen, E.B., Madsen, L.H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., and


