CURRENT REVIEW

Invasion by Invitation: Rhizobial Infection in Legumes

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Nodulation of legume roots typically begins with rhizobia attaching to the tip of a growing root-hair cell. The attached rhizobia secrete Nod factors (NF), which are perceived by the plant. This initiates a series of preinfection events that include cytoskeletal rearrangements, curling at the root-hair tip, and formation of radially aligned cytoplasmic bridges called preinfection threads (PIT) in outer cortical cells. Within the root-hair curl, an infection pocket filled with bacteria forms, from which originates a tubular invagination of cell wall and membrane called an infection thread (IT). IT formation is coordinated with nodule development in the underlying root cortex tissues. The IT extends from the infection pocket down through the root hair and into the root cortex, where it passes through PIT and eventually reaches the nascent nodule. As the IT grows, it is colonized by rhizobia that are eventually released into cells within the nodule, where they fix nitrogen. NF can also induce cortical root hairs that appear to originate from PIT and can become infected like normal root hairs. Several genes involved in NF signaling and some of the downstream transcription factors required for infection have been characterized. More recently, several genes with direct roles in infection have been identified, some with roles in actin rearrangement and others with possible roles in protein turnover and secretion. This article provides an overview of the infection process, including the roles of NF signaling, actin, and calcium and the influence of the hormones ethylene and cytokinin.

Rhizobia and legumes establish a mutualistic symbiosis, which leads to the morphogenesis of nodules where rhizobia eventually locate and fix N₂. The establishment of this symbiosis requires the successful infection of legume roots, which is a multifaceted developmental process that is driven by the bacteria but is ultimately under the control of the host. These events are reviewed in great detail in (Gage 2004) and more recently by Oldroyd and Downie (2008) and Kouchi and associates (2010), and a thorough look at infection from the microbial perspective has been provided by Downie (2010) and Jones and associates (2007). The following discussion is concerned with host factors required for infection prior to symbiosome formation and the roles of cytokinin and ethylene in infection thread (IT) formation. Recent genetic progress in the area of IT formation and progression and its relationship to the components of Nod factor (NF) signaling will be discussed.

PRE-ENTRY EVENTS

NF-signaling.

In the two model legumes *Lotus japonicus* and *Medicago truncatula*, rhizobial infection normally starts with attachment of the bacteria to the tips of growing root hairs and is followed by formation of a characteristic “shepherd’s crook” curl structure at the root-hair tip, within which a bacterial microcolony is established, forming a so-called infection pocket. At this point, the root hair ceases its outward growth and begins to grow inward, forming a tubular IT, in which bacteria reproduce behind the growing tip (Fournier et al. 2008). Eventually, the IT extends through the epidermis and into the growing nodule, where bacteria are taken up into cells and begin to fix N₂. Thus, the programs of IT development and the cortical programs of nodule organogenesis are coordinated (Guinel and Geil 2002; Oldroyd and Downie 2008). The programs of infection and nodule development are induced by the lipochito-oligosaccharides secreted by rhizobia called Nod factors (NF). This signal is perceived by the plant epidermal cells by the Nod factor receptors NOD FACTOR RECEPTOR 1/LYSM DOMAIN CONTAINING RECEPTOR-LIKE KINASE 3 (NFR1/LYK3) and NFR5/NOD FACTOR PERCEPTION (NFP), where it enables infection and is concomitantly relayed to the subtended cortical cells, where it initiates the formation of the nodule (Arrighi et al. 2006; Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003). Many of the developmental events that occur during nodulation can be induced by NF alone. These preinfection responses ready the root for infection by rhizobia. After exposure of growing root hairs to NF, actin rearrangements happen within 3 to 5 min (Allen et al. 1994; Weerasinghe et al. 2005), nuclear calcium spiking occurs within 15 min (Ehrhardt et al. 1996; Sieberer et al. 2009), and the first root-hair deformations occur within 1 to 3 h (Heidstra et al. 1994; Sieberer et al. 2009). Responses to NF in the inner root tissues are slower; microtubule rearrangements and nuclear activation are evidenced at 16 to 18 h in pericycle cells and similar events take place in the cortex at 18 to 24 h (Timmers et al. 1999). This results in the synchronization of primordium formation with the initiation of IT in epidermal cells. None of these events can occur without NF-signaling.

A branch in NF signaling: the common symbiosis and parallel pathways.

A prerequisite to infection and nodulation programs is the activation of signaling pathways by the NF receptors. The induction of nuclear Ca²⁺ oscillations by NF is one of the earliest responses in the root hairs of legumes. The mutants *nfp/nfr5* and *nfr1*, nucleoporin 85 (*nup85*), *nup133*, *castor*, *pollux/does not make infections 1* (*dmi1*), and *symbiosis receptor-like kinase*

(*symrk*)/*dmi2* lack the ability to produce NF-induced nuclear Ca^{2+} oscillations in growing root-hair tips and generally fail to initiate IT and cortical cell divisions (CCD) (Amor et al. 2003; Charpentier et al. 2008; Endre et al. 2002; Imaizumi-Anraku et al. 2005; Kanamori et al. 2006; Madsen et al. 2003; Miwa et al. 2006; Saito et al. 2007; Stracke et al. 2002). In *L. japonicus*, it was shown that, when the spontaneous nodulation allele of CCaMK (T265I) (spontaneous nodule formation [*snf1*] allele encoding autoactive CCaMK or CCaMK*) was introduced into these mutants, nodule infection by rhizobia still occurred via epidermal IT (Madsen et al. 2010). This illustrates that the primary purpose of these NF signaling elements in infection is to activate CCaMK, a calcium calmodulin kinase. CCaMK has been suggested to be responsible for interpretation of the Ca^{2+} signature, indicating a major role for Ca^{2+} signaling in the formation of NF-induced IT and nodules. CCaMK, NUP85, NUP133, CASTOR, POLLUX/DMI1, and SYMRK/DMI2 are also required for successful interactions with arbuscular mycorrhiza and comprise the so-called common symbiosis pathway (Kistner et al. 2005).

Several transcription factors act downstream of the common symbiosis pathway. Nodulation signaling pathway 1 (*nsp1*), *nsp2*, ERF required for nodulation 1 (*ern1*), and nodule inception (*nin*) can undergo symbiotic root-hair deformations but form infrequent microcolonies and lack epidermal IT and CCD, with some leakiness in *ern1* and *nsp1*, which form infrequent IT and nodule primordia (Catoira et al. 2000; Hayashi et al. 2010; Heckmann et al. 2006; Kalo et al. 2005; Levy et al. 2004; Middleton et al. 2007; Mitra et al. 2004; Miwa et al. 2006; Murakami et al. 2006; Oldroyd and Long 2003; Smit et al. 2005; Wais et al. 2000; Yano et al. 2008). Microcolonies have been reported on the weak *nin-2* allele but not on *nin-1* (Hayashi et al. 2010; Marsh et al. 2007). The aborted IT and small nodule primordia on *ern1* mutants may be a result of genetic redundancy because a second family member, ERN2, is NF inducible and expressed during nodulation, and ERN2

binds to and activates NF box-containing reporters (Andriankaja et al. 2007). Creation of an *ern1 ern2* double mutant would provide a clearer picture of the role of these transcription factors. In *L. japonicus*, the transcription factors NSP1, NSP2, and NIN were shown to be necessary for formation of IT and CCD in the *ccamk** background, showing they act downstream of CCaMK. CYCLOPS/IPD3 interacts with CCaMK (Messinese et al. 2007; Yano et al. 2008). However, *cyclops* mutants form microcolonies, indicating that they act downstream of CCaMK, NSP1, NSP2 and NIN during infection (Fig. 1) The *cyclops* roots transformed with a *ccamk** construct produced spontaneous nodules that did not become infected when inoculated with rhizobia, further indicating an important role for CYCLOPS in IT formation (Madsen et al. 2010; Yano et al. 2008).

The parallel pathway.

Earlier observations that the NF signaling pathway mutants *nup85*, *nup133*, *dmi1*, *dmi2*, and *ccamk* exhibit some NF-dependent root-hair responses that are absent in the NF receptor mutants suggested that a parallel signaling pathway required for initiation of infection is operating in concert with the common symbiotic genes downstream of NF perception (Esseling et al. 2004; Kanamori et al. 2006; Miwa et al. 2006; Saito et al. 2007). Recently, it was shown that, when provided with *ccamk**, *nfr1* and *nfr5* mutants did not form epidermal IT (Hayashi et al. 2010; Madsen et al. 2010). This shows that, although activation of CCaMK is necessary for bacterial entry, it is not sufficient. Thus, the NF receptors must mediate a CCaMK-independent function that is essential for the formation of IT (Fig. 1). In addition to rhizobial infection, the parallel pathway may also play a role in NF-mediated root-hair development (Oldroyd et al. 2001). It's not yet clear whether the NF-induced root-hair deformation and IT-initiation pathways are mechanistically related; however, pharmacological studies implicate lipid signaling in both processes. Inhibition of phosphatidylinositol

Fig. 1. Infection by rhizobia is genetically controlled by the host. Components of epidermal infection are shown using blue arrows and a proposed role for cytokinin during infection of the root cortex is shown with red arrows. The signaling pathway branches following the perception of Nod factors (NF). One signaling branch is the common symbiosis pathway which is also required for mycorrhization (*SYMRK*, *NUP85*, *NUP133*, *NENA*, *DMI1/POLLUX*, and *CASTOR*). This pathway serves to activate CCaMK and is required for NF-induced nuclear Ca^{2+} oscillations. Ethylene represses NF signaling, possibly through *LOT1*. The transcriptional regulators *NSP1*, *NSP2*, *ERN1*, and *NIN* are required for infection pocket formation and infection thread (IT) initiation. *NSP1* and *NSP2* form a heterocomplex in planta and *NSP1* binds to the *NIN* and *ERN1* promoters (Hirsch et al. 2009). *NSP2* is required for the induction of *NIN* (Murakami et al. 2006) and *NIN* is required for the formation normal microcolonies and is required for the initiation of IT. A second branch, the “parallel pathway”, plays an essential role and is required for root-hair deformations and the initiation and progression of IT. *RIT/NAP1* and *PIR1* act in this pathway and have roles in root-hair curling and IT growth and may, additionally, participate in basipetal nuclear migration and other tip-growth related processes, such as vesicle trafficking during IT progression. The role of *CYCLOPS* in infection differs from its role in organogenesis, where it is thought to act in concert with CCaMK. During infection, *CYCLOPS* may act downstream of CCaMK because it is not required for the formation of microcolonies, but it is needed for IT initiation. One possibility is that *CYCLOPS* acts in one of the alternate pathways upstream of either *VAPYRIN*, *PUB1*, or both. *FLOT2* and *FLOT4* act downstream of *NIN* and are required for IT initiation. *PUB1* expression is independent of *NIN* and *VAPYRIN* expression is independent of *NSP1*, *NSP2*, *ERN1*, and *NIN*, suggesting the existence of an alternative pathway for gene activation during infection. *LYK3* may deactivate *PUB1* to promote IT progression. It is not yet known which branch or branches of NF signaling control the expression of *SYMREM1*, *RPG*, and *CERBERUS/LIN*, which have roles in the progression of infection.

3-kinase or phosphatidylinositol-specific phospholipase C inhibited root-hair curling and initiation of IT (Peleg-Grossman et al. 2007). Phosphoinositide (PI) signaling has a well-established role in other tip growth processes. Thus, NF-mediated root-hair curling and IT development may, like root hair and pollen tube growth, be mediated by PI-regulated processes, which include endocytosis and generation of reactive oxygen species and changes in Ca^{2+} (Lee et al. 2008; Monteiro et al. 2005; Potocky et al. 2007; Samaj et al. 2006).

In conclusion, based on the above genetic and biochemical analysis, NF-induced IT initiation and growth involves two or more pathways: the common symbiotic pathway acting through *NSP1*, *NSP2*, *ERN1*, and *NIN*, which is required primarily for the activation of CcAMK; and a parallel CcAMK-independent pathways that controls root-hair responses and may involve PI signaling (Fig. 1).

Preinfection threads.

A critical insight into the mechanism of IT was made when van Brussel and associates (1992) showed that, upon application of NF to *Vicia sativa* (vetch) roots, radial rows of cytoplasmic bridges form across outer cortical cells. These cytoplasmic bridges are aligned with anticlinal cell divisions in underlying cortical cells, and these preinfection structures predict the path of IT progression (Timmers et al. 1999). Preinfection threads (PIT) were also seen resulting from NF addition to *L. japonicus* roots (Niwa et al. 2001). PIT are associated with a localized degradation of the cell wall at the poles of the cytosolic bridge, which allows for passage of the IT between cells layers (van Brussel et al. 1992). The preinfection responses in epidermal and outer cortical cells are partly independent from those in the pericycle or inner cortex because purified *Sinorhizobium meliloti* NF failed to induce PIT but could induce CCD on *M. sativa* (Timmers et al. 1999). In addition, the *S. meliloti* *nodF* and *nodL* double mutant can form microcolonies and induce CCD but can rarely initiate IT on *M. truncatula* roots, which, when they do form, abort in the root hair (Ardourel et al. 1994; Limpens et al. 2003). It was recently shown that, in the *snf1* mutant, NF-dependent infection in the cortex does not require the known NF receptors NFR1 and NFR5, leaving open the possibility that another set of NF receptors function in the cortex (Madsen et al. 2010). A separate set of cortical NF receptors with a different sensitivity or specificity to NF could explain the observed independent responses of the epidermis and cortex observed in these earlier studies.

The curious case of cortical root hairs.

It was observed that the PIT that form after addition of NF develop a locally weakened periclinal cell wall near the cytosolic bridge similar to the local cell wall deformation associated with advancing IT (van Brussel et al. 1992). Surprisingly, NF application also induced root-hair-like structures originating from cortical cells, so-called cortical root hairs (CRH) (van Brussel et al. 1992; van Spronsen et al. 1994). CRH are also induced by rhizobia but were observed less frequently than with NF application. It was suggested that this could be due to the absence of IT; in this case, the locally weakened cell wall can serve as either a gateway for IT or a site for the formation of CRH. CRH also form under certain conditions on the *root hairless 1* (*rhl1*) mutant, which is incapable of initiating epidermal root hairs, indicating the genetic independence of root-hair development in the cortex and epidermis. However, very surprisingly, it was found that CRH can functionally complement the root-hairless phenotype during infection by rhizobia by forming the classic shepherd's crook with enclosed infection pockets and normal IT (Karas et al. 2005). Judging from the efficacy of infection through these structures, it seems pos-

sible that this is a significant mode of infection for rhizobia in situations where epidermal root-hair growth is compromised or perhaps even in regions of the root containing mature root hairs (Karas et al. 2005).

What is a PIT? A role for components of the cell cycle.

A histone (*H4*) gene is highly expressed in the path preceding IT development in the outer cortical cell layers of infected pea roots, in advance of and coincident with the formation of PIT (Yang et al. 1994). This *H4* gene transcript is specifically associated with the S phase of the cell division cycle in *M. sativa* suspension culture, indicating that outer cortical cells with PIT have reentered the cell cycle and, presumably, have undergone DNA replication. However, transcription of *cyc2*, a gene that is specifically expressed in M phase of cell cycle, was not induced in these cells with PIT, indicating that these cells arrested at S phase without entering mitosis. However, inner cortical cells having PIT enter the cell division cycle and form nodule primordia (Yang et al. 1994). Indeed, the cytoplasmic strands traversing epidermal root-hair cells with actively growing IT described by Fournier and associates (2008) are not unlike cytoplasmic bridges formed in cortical cells during infection. Could epidermal cells also be forming PIT? In support of this notion is the observation that, during the transition from root-hair growth to IT growth, the epidermal cell nuclei nearly double in size, indicating entry into S phase (Dart 1974). Perhaps there are spatial restrictions delimited by hormone gradients that dictate whether a given cell can become mitotically engaged. In particular, it has been found that cytokinin and ethylene play significant roles in IT and nodule development.

A role for cytokinin.

Although epidermal and cortical events are clearly separable, there is a clear synchronicity of infections with underlying CCD. Cytokinin may play a role in coordinating these two programs. In *L. japonicus*, a cytokinin receptor mutant, *lotus histidine kinase 1* (*lhk1*), has supernumerary infections but lacks timely cell divisions, and the IT are initially blocked at the epidermal-cortical interface (Murray et al. 2007). In *M. truncatula*, RNAi downregulation of the *LHK1* ortholog *MtCre1* resulted in most infections being blocked in root hairs and a clear reduction in CCD (Gonzalez-Rizzo et al. 2006). The discrepancy between the *lhk1* mutant and *MtCRE1* knockdown phenotypes may represent real differences in the role of cytokinin between legume species but could also simply reflect differences in functional redundancy as they belong to a multigene family. A role for cytokinin in *M. truncatula* infection is further supported by the reduced infection phenotype in plants with reduced expression of *3-hydroxy-3-methylglutaryl CoA reductase 1* (*HMGR1*), an enzyme required for isoprenoid synthesis, including cytokinin (Kevei et al. 2007). Thus, cytokinin may have a role in IT formation in the outer cortex and epidermis in *Lotus* and *Medicago* spp., respectively. Cytokinin may interact with the infection and nodule development pathway through *NIN*. *NIN* expression is induced by cytokinin and, reciprocally, *NIN* is required for the induction of *MtARR4*, which acts downstream of *MtCRE1* during nodulation (Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Tirichine et al. 2007). This suggests a positive feedback mechanism exists (Fig. 1). However, nodules that form on *ccamk snf2* (*snf2* is an *LHK1* gain-of-function allele) double mutants fail to become colonized, indicating that *LHK1/MtCRE1*-mediated cytokinin signaling alone is insufficient to permit infection (Madsen et al. 2010).

It is not surprising to see the involvement of cytokinin in initiating CCD in response to NF or rhizobia because of its basic function in cell growth and division, but what role might this hormone play in IT progression? Gage and Margolin (2000)

noted that the PIT appears as an arrested form of cell divisions occurring in the cortex during nodule formation. If PIT are analogous to phragmoplasts, it is possible that their formation could require cytokinin. This could explain the block in IT progression in *Lotus lhk1* mutants at the cortical interface. In this scenario, the *Medicago MiCRE1* knockdown phenotype could be a secondary effect; for example, *Arabidopsis* cytokinin receptor mutants overproduce cytokinin, which, if it occurs in *lhk1* mutants, could negatively affect IT development through increased production of ethylene (Hansen et al. 2009; Riefler et al. 2006). Alternatively, it could suggest that cytokinin is directly required for root-hair infection. An answer to these questions will require a more detailed genetic analysis of the cytokinin receptor family and a better understanding of the events that occur downstream of cytokinin signaling during infection.

Ethylene as a major regulator.

Ethylene is able to block cell divisions in legume roots, including CCD in nodulation (Apelbaum and Burg 1972; Lee and Larue 1992), and has been shown to reduce the initiation of IT and the responses to NF, including the Ca^{2+} spiking associated with NF signaling (Oldroyd et al. 2001). Ethylene also inhibits PIT formation in vetch (van Spronsen et al. 1995). Increased infections are seen in the *Medicago sickle* (*ethylene-insensitive 2* [*ein2*]) mutant (Penmetsa et al. 2003, 2008) and transgenic plants having a dominant negative ethylene receptor gene, *ethylene response sensor 1* (*ERS1/H70A*) (Nukui et al. 2004) or *ethylene response 1* (*ETR1-1*), where ethylene sensitivity is affected (Lohar et al. 2009). Thus, ethylene plays a significant inhibitory role in rhizobial infection and nodule formation. The inhibition of infection by ethylene may be specifically mediated by *LOT1*. The *L. japonicus* mutant *low nodulation and trichome distortion 1* (*lot1*) has reduced IT and nodule formation and is insensitive to the suppression of nodule formation by ethylene but it retains a normal triple response (Oooki et al. 2005) (Fig. 1).

However, ethylene is not always a negative factor and plays a positive role during alternative modes of infection. Ethylene is required for rhizobial infection and nodule development in *Sesbania rostrata* flooded roots in which the crack entry mechanism is used (Capoen et al. 2009; Goormachtig et al. 2004), and crack entry infection in the *nena* mutant was also dependent on ethylene (Groth et al. 2010).

In contrast to its role in IT development, ethylene is a positive regulator of root-hair growth (Tanimoto et al. 1995). This represents a major difference between the hormonal regulation of these two tip-growth processes. Notably, whereas NF-induced Ca^{2+} oscillations are repressible by ethylene, NF-induced root hair–tip growth is not (Heidstra et al. 1997). This further demonstrates the existence of a parallel pathway independent of the common symbiotic pathway. It's possible that, during rhizobial infection, the plant locally decreases the production of ethylene in response to NF, and this marks the switch from root-hair growth to IT initiation and growth. The root hair itself switches from an ethylene-promoted process of polarized growth to a form of inverted polar growth that is not only ethylene independent but also strongly inhibited by this hormone. Understanding the orchestration of ethylene signaling in root-hair cells during infection will undoubtedly increase our understanding of this hormone's effect on cell growth processes in general.

IT PROGRESSION

More than one role for Ca^{2+} .

Ca^{2+} oscillations are induced near and within the nucleus of growing root hairs by NF earlier than the occurrence of root-hair cell deformations (Ehrhardt et al. 1996; Engstrom et al.

2002; Sieberer et al. 2009). Mutants that lack these oscillations fail to form IT and nodules; therefore, it is assumed that spiking is an essential component of NF-signaling, but proof of this will require identification of the Ca^{2+} channels involved (Oldroyd and Downie 2006). Independent roles for Ca^{2+} oscillations in root-hair growth and IT growth in response to NF are possible. Ca^{2+} oscillation patterns are associated with polar tip growth of plant root hairs and pollen tubes (Monshausen et al. 2008; Rincon-Zachary et al. 2010). Changing of Ca^{2+} gradients can alter the directional growth of root-hair tip and pollen tube, and Ca^{2+} gradients are also important for the growth of fungal hyphae (Bibikova et al. 1997; Malho and Trewavas 1996; Silverman-Gavrila and Lew 2003). If a Ca^{2+} gradient is required to direct tip growth processes, it is likely to be required for both NF-induced root-hair deformation and directional IT growth. Alternatively, it has been proposed that IT formation may be a modified version of cell plate formation based on a likeness that was noted between the transcellular bridges observed in dividing cells during the formation of the phragmoplast and PIT (Brewin 2004; Kijne 1992). Cell plate formation also requires Ca^{2+} gradients at the plane of division (Hepler 1994). In this scenario, a local PM-localized Ca^{2+} gradient would be required along the length of the IT where new cell wall is being formed. Whether IT growth is better modeled as tip-growth or as a modified cell plate, it seems likely that a localized Ca^{2+} gradient at the interface of the growing membrane will be required. The primary role of Ca^{2+} in this context may be in promoting secretory vesicle fusion at the plasma membrane (Schneggenburger and Neher 2005). Where might this Ca^{2+} come from? One possible source would be the IT matrix, which likely would have abundant Ca^{2+} bound both to pectin (Rae et al. 1992; Vandenbosch et al. 1989) and rhizobial exopolysaccharides (Aslam et al. 2008). Alternatively, an intracellular source such as the vacuole or nucleus might be used.

The actions of actin.

Several studies have shown that one of the most rapid responses to NF is the rearrangement of actin in growing root hairs, with some responses occurring within 3 to 5 min (Allen et al. 1994; Cardenas et al. 1998; de Ruijter et al. 1999; Vassileva et al. 2005; Weerasinghe et al. 2003, 2005). In the tips of NF-exposed root hairs, the longitudinal thick actin bundles were diminished and finer, more diffuse actin accumulated (Cardenas et al. 1998; de Ruijter et al. 1999; Weerasinghe et al. 2005; Yokota et al. 2009). The first genetic evidence implicating actin in infection growth was the *L. japonicus crinkle* mutant, which displayed defective growth of IT, pollen tubes, and root hairs (Tansengco et al. 2003). The mutant had swollen pollen tube tips that exhibited disorganized F-actin; however, the responsible gene has not yet been identified (Tansengco et al. 2004). The recently characterized mutants *nck-associated protein 1* (*nap1*) and *I21F-specific p53 inducible RNA* (*pir1*), which have defects in IT development, were shown to be mutated in genes that encode proteins involved in actin rearrangements (Miyahara et al. 2010; Yokota et al. 2009). The mutants also displayed defects in the polar growth processes of root hair and trichome development. Actin rearrangements observed in root hairs after NF application are absent in these mutants (Cardenas et al. 1998; Yokota et al. 2009). It has been shown previously that actin is required for NF-induced root-hair deformations (Miller et al. 1999). Consistent with this, the *nap1* and *pir1* mutants had impaired NF-induced root-hair deformation and defects in infection pocket formation. In addition, the mutants rarely formed IT and those that did form often disintegrated, demonstrating roles for actin in the formation and maintenance of IT. Finally, IT never progressed past the epidermal layer, suggesting an absolute requirement for actin in IT devel-

opment in the outer cortex. It is well established that actin is regulated by PI signaling acting through the activation of the Rho family of small GTPases (ROP) (Hilpela et al. 2004; Saarikangas et al. 2010). It's thought that ROP serve to generate tip-focused F-actin and Ca^{2+} gradients during polar cell growth (Yang 2002). PI signaling has also been shown to be required for both root-hair curling and IT initiation (Kelly-Skuepek and Irving 2006; Peleg-Grossman et al. 2007; Saarikangas et al. 2010). These points considered, it seems plausible that actin plays a central role in PI-driven tip growth during IT progression (Fig. 1). Interestingly, long-distance intracellular movement of the nucleus has been shown to require actin but not microtubules (Chytilova et al. 2000). In addition, it has been shown that the basipetal movement of the nucleus that occurs after root-hair growth requires actin (Ketelaar et al. 2002). It is possible that the nuclear movement that normally accompanies IT growth is also deficient in these mutants.

A role for lipid rafts in infection.

Lipid rafts are membrane microdomains that act as organizing centers for signal molecule assembly. Some lipid raft complex components have recently been implicated in infection. Silencing of a group of three flotillins in the roots of *M. truncatula* plants resulted in reduced numbers of IT (Haney and Long 2010). FLOT2 and FLOT4 were found to localize to membrane microdomains, and FLOT4 was uniquely found to mobilize to infected root-hair tips. *Flot4* and *Flot2* were induced by infection and this required *NIN* and *NSP2* but not *ERN1*. A nodule-specific remorin, *MEDICAGO TRUNCATULA SYMBIOTIC REMORIN 1* (*MtSYMREM1*), was shown to be involved in infection in *M. truncatula*. Remorins are also found in lipid rafts (Jarsch and Ott 2011). Mutations in this gene caused formation of enlarged and highly branched IT within the nodule; these abnormal IT failed to release bacteria into the symbiosomes while RNAi-mediated reduction of *MtSYMREM1* transcripts resulted in defective IT progression in root hairs (Lefebvre et al. 2010). Remorin also interacts with LYK3, NFP, and SYMRK in vivo, suggesting that there may be an NF-signaling complex that assembles within membrane microdomains and that this plays an essential role in the infection process.

New players with unknown roles.

Recently, several genes with roles in infection have been identified using genetics and biochemistry. In the *lin* and *cerberus* mutants, identified in *M. truncatula* and *L. japonicus*, respectively, most IT arrest in the epidermis and nodules form as small bumps but are not colonized (Kiss et al. 2009; Yano et al. 2009). Mutants *lin* and *cerberus* were found to be mutated in orthologous U-Box/WD40 genes. The U-box is found in E3 ubiquitin ligases that ubiquitinate proteins, which leads to their degradation by the 26S proteasome complex, suggesting a role for *LIN/CERBERUS* in protein turnover. Another mutant, *rpg*, exhibits deficient root-hair curling and slow-progressing IT (Arrighi et al. 2008). The underlying gene was identified as a long coiled-coil protein that localizes to the nucleus, hinting at a role in transcriptional regulation. The expression of *RPG* in *lin* and *hcl(lyk3)* mutant backgrounds was greatly reduced, suggesting that it acts downstream (Fig. 1). It has not been determined whether the transcriptional induction of *RPG* and *LIN/CERBERUS* is dependent on *NIN*, *NSP1*, *NSP2*, and *ERN1*.

VAPYRIN and PUB1, possible links to secretion.

It is clear that secretion will play a central role in infection. Potential roles include protein delivery to the PM for rhizobial attachment and NF signaling and secretion to the apoplast for

cell wall degradation at the site of IT initiation. In addition, the polarized growth of the IT will require vesicle-mediated delivery of cell wall components and specialized matrix proteins (Rae et al. 1992; Vandenbosch et al. 1989). Recently, a couple of genes with possible links to secretion during infection have been identified. *VAPYRIN* (*VPY*) is required for mycorrhizal infection and arbuscule development (Feddermann et al. 2010; Pumplin et al. 2010) as well as the development of IT (Murray et al. 2011). The *vpy* mutants have enlarged and misshapen IT that abort in root hairs. *VPY* is inducible by NF and this induction is dependent on *CCaMK* but not on *NIN*, revealing the existence of a *NIN*-independent pathway required for infection (Fig. 1). *VPY* encodes a protein containing a major-sperm (MSP) domain (also called a VAP domain) and an ankyrin repeat domain. A possible link of *VPY* with secretion comes from a well-studied MSP-containing protein in humans, VAP33, which interacts with a SNARE-complex member to regulate the exocyst-dependent secretion of glucose transporters in response to insulin. The exocyst is an octameric complex that tethers vesicles prior to SNARE complex fusion during exocytosis (Zhang et al. 2010). Another recently discovered protein, PUB1, may also have links to exocytosis. PUB1 is a demonstrated E3 ubiquitin ligase, which was detected using a two-hybrid screen for LYK3 interactors (Mbengue et al. 2010). PUB1 is phosphorylated by the kinase domain of LYK3, and RNAi knockdown of *PUB1* transcripts increased the frequency of infections on a weak allele of *lyk3*, providing evidence that PUB1 is a negative regulator of infection. Like *VPY*, induction of *PUB1* transcripts by NF required *CCaMK* but not *NIN* (Fig. 1). Mbengue and associates (2010) point out that an *Arabidopsis* homolog of PUB1, ARC1, which also interacts with a receptor kinase (SRK1), has been shown to interact with an exocyst complex member EXO70A1. One exciting possibility is that *VAPYRIN* or *PUB1* play a role in the regulation of NF-dependent secretion. The MtGEA database reveals that at least two *EXO70* genes are highly expressed during nodulation. In *Arabidopsis*, two EXO70 family members (*EXO70A1* and *EXO70C1*) are required for polarized growth processes (Li et al. 2010; Synek et al. 2006). Identification of the components of the plant secretion systems required for infection and the cargo they deliver is needed to better understand rhizobial attachment and entry and the formation and initiation of IT. What controls the change from secretion driving polar growth of root hairs to that of IT progression? Does LYK3 mediate NF-dependent secretion through PUB1? Is the exocyst required for IT initiation or growth? More genetic and biochemical studies are needed to identify the key players and to reveal how they are interlinked.

FUTURE DIRECTIONS

Sequencing the infectome.

A combination of deep sequencing technologies (RNA-seq) and microarrays has recently been used to characterize the gene expression of rhizobially infected root hairs in soybean (Libault et al. 2010). More than 1,900 differentially regulated genes were identified, the large number likely reflecting the increased resolution of this approach compared with harvesting the entire root. Many known symbiotic genes were found to be upregulated in root hairs from infected plants, including homologs of *NFR1*, *NFR5*, *NIN*, and *NSP1* and *NSP2*, supporting the proposed role of these genes in infection. This approach will prove especially powerful in model legumes, and similar root-hair-based expression analysis of infection mutants combined with techniques to isolate specific cell types such as laser-capture microdissection and fluorescence-activated cell sorting will help create a better understanding of gene expression changes during infection.

Forward and reverse genetics.

The identification of new infection mutants in *L. japonicus* promises rapid progress in this area in the upcoming years (Lombardo et al. 2006; Murray et al. 2006; Yano et al. 2006). The growing collection of publicly available gene expression data (Benedito et al. 2008; He et al. 2009) will serve to lay the ground for reverse genetic studies in both *Medicago* and *Lotus* spp. (Le Signor et al. 2009; Rogers et al. 2009; Till et al. 2003). In particular, *Medicago* spp. benefit from a large collection of *Tnt1*-transposon insertion lines (Tadege et al. 2008).

Imaging the IT.

The rhizobial IT is a unique structure that has captured the imagination of many researchers since it was first observed in 1887 (Ward 1887). As a superficial cell layer, root hairs present an attractive object of study. Recent efforts by (Fournier et al. 2008) have identified a fluorescent marker that can reveal the plasma membrane around the IT. In addition, they showed that a marker for the endoplasmic reticulum can effectively highlight the cytoplasmic bridge between the IT tip and the nucleus. These present powerful tools for the study of infection mutants but other markers are needed to highlight specific membrane trafficking processes such as exo and endocytosis that must play a central role. A combination of improved imaging techniques and genetics will help unravel the regulatory and physiological processes behind IT initiation and progression to shed light on the relationship in this special system of coordinated signaling and tip growth.

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