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REVIEW

Starting points in plant-bacteria nitrogen-fixing symbioses: intercellular invasion of the roots

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Abstract

Agricultural practices contribute to climate change by releasing greenhouse gases such as nitrous oxide that are mainly derived from nitrogen fertilizers. Therefore, understanding biological nitrogen fixation in farming systems is beneficial to agriculture and environmental preservation. In this context, a better grasp of nitrogen-fixing systems and nitrogen-fixing bacteria-plant associations will contribute to the optimization of these biological processes. Legumes and actinorhizal plants can engage in a symbiotic interaction with nitrogen-fixing rhizobia or actinomycetes, resulting in the formation of specialized root nodules. The legume-rhizobia interaction is mediated by a complex molecular signal exchange, where recognition of different bacterial determinants activates the nodulation program in the plant. To invade plants roots, bacteria follow different routes, which are determined by the host plant. Entrance via root hairs is probably the best understood. Alternatively, entry via intercellular invasion has been observed in many legumes. Although there are common features shared by intercellular infection mechanisms, differences are observed in the site of root invasion and bacterial spread on the cortex reaching and infecting a susceptible cell to form a nodule. This review focuses on intercellular bacterial invasion of roots observed in the Fabaceae and considers, within an evolutionary context, the different variants, distribution and molecular determinants involved. Intercellular invasion of actinorhizal plants and *Parasponia* is also discussed.

Key words: Actinorhizal plants, intercellular invasion, legumes, molecular signaling, rhizobia, symbioses.

Introduction

The atmosphere is the major source of nitrogen in the biosphere. However, atmospheric nitrogen remains inaccessible to the majority of living organisms. Only a limited number of prokaryotes are able to convert atmospheric nitrogen into ammonia, making it available to be incorporated by the plants and, consequently, by the rest of the biota components. Throughout evolution, leguminous and actinorhizal plants (all belonging to the nitrogen-fixing clade within the Fabids or Eurosid I) acquired the ability to develop an endosymbiotic relationship with these bacteria. In this symbiosis, prokaryotes fix nitrogen that is supplied to the plants in exchange for carbon sources and a controlled environment.

The most extensively studied symbiotic association within the nitrogen-fixing clade is that established between legumes and rhizobia. This symbiotic association is quite common in the Fabaceae family (Soltis *et al.*, 1995). One of the features

associated with this interaction is the formation of specialized organs called nodules, which provide a suitable microenvironment for bacterial nitrogenase activity. Nodules are of two general kinds, indeterminate and determinate. Indeterminate nodules are elongated and originate by distal growth from a persistent meristem; while determinate nodules are spherical and develop from a non-persistent meristem (Hirsch, 1992). Only occasional reports have indicated that symbiotic nitrogen fixation in legumes belonging to the genera *Gleditsia* and *Peltophorum* does not involve nodule development (Allen and Allen, 1981; Bryan *et al.*, 1995). A great diversity of mechanisms allowing rhizobial invasion of legumes have been described. The likely most primitive and less studied involves intercellular penetration of the roots. This mechanism was assumed to be restricted to several odd and atypical legumes or plants adapted to flooded habitats (Goormachtig *et al.*, 2004*a*, *b*). However, emerging evidence indicates that it also occurs regularly in several legumes and can even occur simultaneously as a baseline phenotype in model species with the more sophisticated infection thread invasion (Madsen *et al.*, 2010).

Description of intercellular invasion in model legumes, together with widespread distribution in groups representing independent origins of nodulation (Werner *et al.*, 2014), emphasize the relevance of a deeper study of this infection mechanism. A comparative approach to study molecular determinants of this invasion might illustrate novel aspects of the plant genetic module involved and its evolution. In turn, this knowledge might enable the engineering of nitrogen-fixing symbiosis in non-host plants.

Rhizobia invade legume roots through different mechanisms

Root hair invasion by formation of infection threads is the best characterized mechanism of rhizobial invasion of legumes. This invasion mechanism takes place in legumes such as soybean, alfalfa, pea, bean and vetch (Sprent and James, 2007; Sprent, 2008) and has been extensively studied in the model legumes *Medicago truncatula* and *Lotus japonicus* (Oldroyd, 2001) (Fig. 1). Exudates from legume roots such as (iso)flavonoids and related compounds specifically induce the transcription of nodulation genes (*nod, nol, noe*) of compatible rhizobia by stimulating NodD transcriptional activation. The induced *nod* gene products are involved in the synthesis of nodulation factors (Nod factors, NFs) which are substituted N-acylated chitin oligomers. The chemical structure of NFs is critical for recognition between symbiotic partners, adding specificity beyond the composition of root exudates (Oldroyd, 2001). NFs are recognized by membrane LysM-receptor-like kinases (LysM-RLKs) of root epidermal cells. This triggers a signaling pathway (sym pathway) constituted by a signaling module called the common symbiotic pathway (CSP). This pathway was probably recruited from the more ancient mycorrhizal symbiosis (Parniske, 2008).

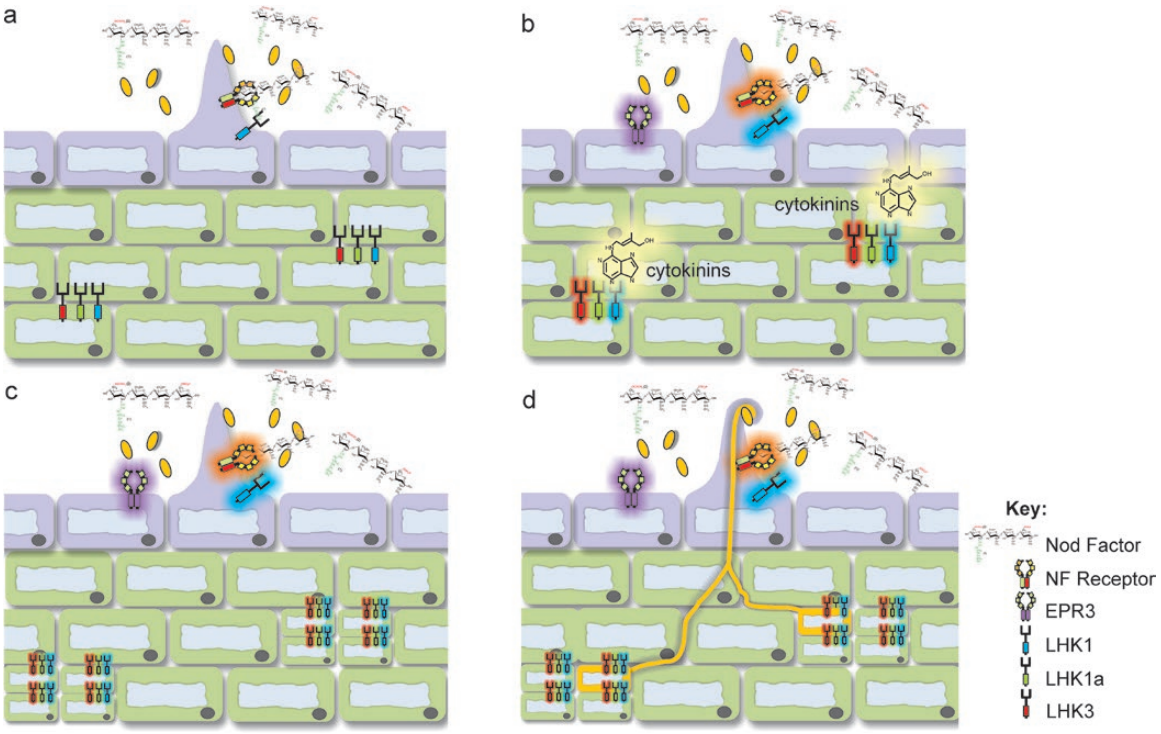

Fig. 1. Rhizobial infection of *Lotus japonicus* roots. (A) Rhizobia adhere to the roots and synthesize Nod factors that are perceived by LysM-RLKs Nod factor receptors LjNFR1 and LjNFR5. (B) NF perception triggers a complex signal transduction pathway at epidermal level resulting in synthesis of the phytohormone cytokinin. In addition, NF perception induces transcription of EPR3, a LysM-RLK that controls rhizobial exopolysaccharides (EPS). (C) Perception of cytokinins by LHK1, LHK1a and LHK3 receptors causes the reinitiation of meristematic activity of cortical cells to form the nodule primordia. (D) The hair root curves and a tubular structure called infection thread is formed, through which the rhizobia enters the root. Infection threads lead rhizobia to nodule primordia, where they infect plant cells and differentiate into nitrogen-fixing bacteroids.

In *L. japonicus*, CSP components include a leucine-rich repeat receptor kinase (SYMRK), nucleoporins (NUP133, NUP85 and NENA), ion channels (CASTOR and POLLUX), Ca^{2+}/calmodulin- dependent protein kinase (CCaMK), coiled-coil domain-containing protein (CYCLOPS) and GRAS family transcription factor NSP1 (Oldroyd, 2013). Ortholog genes were also identified in *M. truncatula* (Oldroyd, 2013). Interestingly, some genes of the CSP (CYCLOPS/IPD3 in advanced charophytes and POLLUX/Dmi1 and CCaMK in charophytes and even in chlorophytes) are present in green algae, suggesting that the ancestor of land plants was already pre-adapted for symbiosis (Delaux *et al.*, 2015*b*).

Two coordinated programs lead to root nodule formation, one occurring in the epidermis (bacterial infection) and the other in the cortex (nodule primordia inception). In the epidermis, activation of this signaling pathway leads to localized growth inhibition at the tip of root hairs, inducing their curling (Gage, 2004). Rhizobia entrapped in the curl enter the root hair by local hydrolysis of cell wall and invagination of the plasma membrane. Growing of the tip toward the base of the root hair results in an intracellular tube called the infection thread. NF-induced pectate lyase is required for the localized degradation of the plant cell wall at the site of infection thread initiation (Xie *et al.*, 2012). The infection thread then grows and branches through the cortex. These processes depend on plant cell wall degradation, synthesis of infection thread wall components and cytoskeletal rearrangements forming the preinfection structure (van Brussel *et al.*, 1992). In *L. japonicus*, infection thread development through the cortex also requires NF receptor NFR1 (Hayashi *et al.*, 2014). Simultaneously with the rhizobial infection, cortical cells reinitiate meristematic activity to form the nodule primordia. Activation of such a program is mediated by a mobile unidentified signal (possibly cytokinins or, less likely, NSP1 and NSP2) originating in the epidermis, which triggers a cortical signaling cascade including some components of the sym pathway (Frugier *et al.*, 2008; Hayashi *et al.*, 2014). A role in coordinating epidermal and cortical responses has also been attributed to NIN. This protein displays a complex and tissue-specific role during nodulation. It activates bacterial infection in the epidermis and nodule organogenesis in the cortex while negatively regulates the number of nodules that are formed. This is achieved through expression of CLE peptides that, in turn, promote the autoregulation of the nodulation (AON) system (Vernié *et al.* 2015). While CASTOR, POLLUX, NUP85 and NUP133 are exclusively required in the epidermis; CCaMK, CYCLOPS, NSP1 and NSP2 are required in both epidermis and cortex (Hayashi *et al.* 2014). Recent evidence suggests that SYMRK plays an important role in allowing infection threads to cross the epidermal-cortical interface, permitting rhizobia to reach nodule primordial (Saha *et al.*, 2016). Rhizobia are released from the infection thread into nodule primordia cells and then divide and differentiate into bacteroids. It has been proposed that clathrin-mediated endocytosis participates in the early stages of *L. japonicus* symbiosis (Wang *et al.*, 2015). In nodule primordia, bacteroids are separated from the cytoplasm of the host cell by a peribacteroid membrane forming an organelle-like structure called the symbiosome. Inside of these, bacteroids reduce atmospheric nitrogen into ammonia that is subsequently assimilated by the host plant.

A role for an appropriate bacterial cell surface in the symbiosis development has also been suggested, since mutants affected in production of surface exopolysaccharides (EPS) and lipopolysaccharides (LPS) have defective infection phenotypes. It was proposed that these molecules may suppress plant defense during infection (Mithöfer, 2002). The transmembrane receptor kinase EPR3 monitors the EPS status of bacteria during infection of *L. japonicus*. EPR3 binds EPS directly and distinguishes compatible and incompatible bacterial EPS. This protein is a LysM-RLK belonging to the NFR1 protein phylogenetic branch, and its expression in root hairs and epidermal cells is induced by NFs. Plant sensing of bacterial EPS by EPR3 occurs at the stage of colonization and infection of epidermal cells. Using bacterial mutants affected in EPS production, it was observed that the EPR3 receptor controls the infection, irrespective of the way the *L. japonicus* microsymbiont invades epidermal cells (Kawaharada *et al.*, 2015). Ortholog genes are present in other legumes and in non-legume species, suggesting that the mechanism for bacterial EPS recognition is widespread among plants. However, functionality of the receptor in non-legume species and its participation in EPS recognition still needs to be tested.

Although the majority of studies were conducted in legumes displaying root hair infection, evidence suggest that at least 25% of all legume genera display a non root hair infection pathway lacking infection threads (Sprent and James, 2007). This mode of rhizobial infection, via intercellular invasion, has also been described in the non-legume *Parasponia* (Trinick and Galbraith, 1980) and in actinorhizal symbiosis (Wall and Berry, 2008). In the intercellular rhizobial invasion, different target areas for bacteria penetrating the root have been described: (i) the middle lamellae between two adjacent root hair cells, (ii) wounds where lateral roots emerge (crack-entry), (iii) spaces at the base of root hairs on emerging lateral roots, or (iv) directly between epidermal cells in intact epidermis tissue. In some of the intercellularly infected legumes, the later intracellular penetration of nodule primordia cells occurs via an infection thread that develops after root tissue invasion, while in other legumes there is no infection thread formation at any step of the root bacterial entrance (Table 1). Very few of the intercellularly infected legumes have been subjected to an intense study of the molecular basis of the symbiotic interaction, even when they include some genera of economical importance.

Intercellular rhizobial invasion of the roots is widespread among the Fabaceae

Intercellular infection mechanism takes place in legumes belonging to the two major clades: the Mimosoideae-Caesalpineae-Cassieae (MCC) and the monophyletic subfamily Papilionoideae, in plants displaying both determinate and indeterminate nodules (Doyle, 2011). There are common features shared by most intercellular infection mechanisms,

Table 1. *Intercellular infection mechanisms within the Fabaceae*

	MCC Clade		**Papilionoids**						
	Mimosa scabrella	***Neptunia natans***	***Stylosanthes***	***Arachis hypogaea***	***Cytisus proliferus***	***Lupinus albus***	***Aeschynomene***		***Sesbania rostrata***
							A. indica	***A. afraspera***	
Actual infection site	Directly between intact epidermal cells	Natural wounds caused by emergence of lateral roots	Spaces between epidermal cells at the bases of the root hairs	Middle lamellae between two adjacent root hairs	At the junction between a root hair and an adjacent epidermal cell	Between cell walls of root hairs base and adjacent epidermal cells	Between two axillary root hairs	Cracks between an axillary root hair and an epidermal cell of the primary root	Via cracks formed by lateral or adventitious root protrusion
Spread through the cortex	Between cell walls and through intercellular air spaces	Intercellular. Bacteria accumulate in infection pockets at the periphery of the young nodule	Progressive collapse of invaded cells	Separating cortical cells at the middle lamellae	Intercellular	Cell wall swellings in the cortical cell underneath the root hair	Progressive collapse of invaded cells	Intercellularly over three or four layers of cortical cells. Ocasionally, cell collapse was observed	Cell death and formation of intercellular infection pockets
Uptake by susceptible cells	Through ill-defined infection threads. Alternatively, through breaches in cell wall caused by bacterial multiplication	Intercellular infection and structures resembling infection threads that invade host cells	Through structurally altered cell walls	Through structurally altered cell walls, possibly partial degradation by rhizobia	Through structurally altered cell walls	Through structurally altered cell walls	Local invagination of the cell wall	Local invagination of the cell wall	By intercellular and, subsequently, intracellular infection threads arising from infection pockets
References	(de Faria *et al.*, 1988)	(Subba-Rao *et al.*, 1995; James *et al.*, 1992)	(Chandler *et al.*, 1982)	(Chandler, 1978; Boogerd and Rossum, 1997; Uheda *et al.*, 2001)	(Vega-Hernández *et al.*, 2001)	(González-Sama *et al.*,2004)	(Bonaldi *et al.*, 2011)		(Goormachtig *et al.*, 2004*b*; Capoen *et al.*, 2010; D'Haeze *et al.*, 1998; D'Haeze *et al.*, 2003; Lievens *et al.*, 2005)

but also differences in the actual site of root invasion and in bacterial spread through the cortex to reach and infect a susceptible cell. Although the terms 'crack entry' and 'intercellular infection' are often used as synonyms, it is important to distinguish between them. The term 'intercellular invasion' describes all those infection mechanisms in which bacteria enter between epidermal cells without breaks. In contrast, 'crack entry' refers strictly to those mechanisms in which rhizobial entrance occurs via colonization of cracks or fissures naturally available in epidermic tissue. The junction of root hair cells or the wall between a root hair and an adjacent epidermal cell constitute common sites for bacterial entry in intercellular invasion. On the other hand, crack entry frequently occurs in wounds or cavities generated by lateral root protrusion. Crack entry is a type of intercellular invasion, but all intercellular invasions are not crack entry.

One of the simplest root invasion mechanisms in Fabaceae occurs in *Mimosa scabrella*, which belongs to the subfamily Mimosoideae in the MCC clade and forms indeterminate nodules. After attaching to the roots, rhizobia disrupt the mucigel and invade directly between epidermal cells through degradation of the primary cell wall. Further penetration into the cortex occurs between cell walls and through intercellular spaces. Once they reach the cortex, bacteria start dividing and, as a consequence, cell walls are pushed inwards, occasionally allowing intracellular infection. From these groups of bacteria, structures resembling ill-defined infection threads arise and cross cell limits, enabling the invasion of adjacent cells. Infection of cortical cells may also be achieved after a massive bacterial multiplication between cells and in intercellular air spaces. This breaches the cell walls and allows intracellular infection, without infection thread-like structures. This mode of intracellular invasion appears to cause the death of the cell. After intracellular infection of outer cortical cells, nodules are initiated through cell divisions in the inner cortex, and the progeny of the new meristematic cells are invaded by infection threads (de Faria *et al.*, 1988).

In the aquatic MCC clade legume *Neptunia natans*, which forms indeterminate nodules, bacteria enter the roots through natural wounds caused by emergence of lateral roots (crack entry) followed by colonization of the generated cavity. Afterwards, rhizobia induce formation of nodule primordia and accumulate in intercellular infection pockets formed by dead and sometimes collapsed cells at the periphery of the young nodule. Colonization of nodule primordia occurs through intercellular infection that narrows down to form structures resembling infection threads that penetrate host cells (Subba-Rao *et al.*, 1995). Similar intercellular invasion of the roots followed by penetration of individual host cells by infection thread-like structures was described in *N. plena* growing in vermiculite (James *et al.*, 1992).

Few detailed studies have been performed among the Caesalpinoid legumes of the MCC clade, with exception of *Chamaecrista*, which forms indeterminate nodules after interaction with compatible rhizobia. Symbiosis in *Chamaecrista* differs in the level of complexity of the structures developed. Some members of the genus (such as *C. ensiformis* var. *ensiformis*, *C. desvauxii* var. *glauca* and *C. glandulosa* var. *brasiliensis*) produce nodules with persistent infection threads (fixation threads) while in others (*C. absus*, *C. biensis*, *C. falcinella*, *C. fasciculata* among others) true symbiosomes are formed (Naisbitt *et al.*, 1992). In *C. fasciculata*, although infection threads are present in root hair cells, the threads abort and do not reach the nodules. However, infection threads are found within the nodule (Hirsch *et al.*, 2009). Therefore, it is proposed that rhizobia penetration occurs intercellularly and formation of infection threads occurs secondarily.

Among the Papilionoid legumes, *Stylosanthes* roots are also invaded intercellularly, and bacterial entrance takes place at the lateral root junctions through spaces found among epidermal cells at the base of root hairs (Chandler *et al.*, 1982). Root cortex cells are then penetrated through structurally altered cell walls. These cells collapse and are compressed by neighboring cortical cells. Further rhizobial penetration of the root cortex occurs by progressive collapse of invaded cells. Deeper into the cortex, rhizobia reach host cells, which do not collapse after invasion. Instead, they start dividing repeatedly to form a typical determinate aeschynomenoid nodule, with no uninfected interspersed cells (Chandler *et al.*, 1982). In addition, some abnormal associations were observed in *S. capitata* and *S. hamata* (Chandler *et al.*, 1982). In *S. capitata*, division of invaded cells was restricted and bacteria became enlarged and deformed. In *S. hamata,* after restricted cell division, host cells collapsed and no bacteroids were formed (Chandler *et al.*, 1982).

In *Arachis hypogaea* (peanut), which is also a member of the Papilionoid subfamily, another pattern of intercellular invasion takes place before development of determinate nodules (Chandler, 1978; Boogerd and Rossum, 1997; Uheda *et al.* 2001). Bradyrhizobia enter the root through the middle lamellae between two adjacent root hair cells, a place where the wall is loosely constructed (Fig. 2). After entry, bradyrhizobia spread intercellularly by separating cortical cells at the middle lamellae. Some of the axillary root hair basal cells are associated with 'large basal cells', which are the first to become infected intracellularly by bradyrhizobia, while other bacteria continue to spread intercellularly. These infected large basal cells divide repeatedly, giving rise to nodule tissue. It has been proposed that peanut infectivity is determined by the presence of an emerging root hair together with these enlarged basal cells, concomitant with the emergence of a lateral root (Boogerd and van Rossum, 1997).

Another particular invasion occurs in the roots of *Cytisus proliferus,* a member of the subfamily Papilionoideae (Vega-Hernández *et al.*, 2001), which forms indeterminate nodules after infection with compatible bradyrhizobia. This root invasion mode is singular since the infection threads are aborted before reaching a nodule primordium. Instead, bradyrhizobia penetrate the root intercellularly at the junction between a root hair and the adjacent epidermal cell. Afterwards, rhizobia invade the cortical cell immediately beneath the root hair through structurally altered cell walls and the infected cell divides to form the central infected zone of the nodule.

In another papilionoid, *Lupinus albus*, rhizobia colonize the root hair surface causing cell wall weakening and separation of neighboring epidermal cells. As in peanut, bacteria

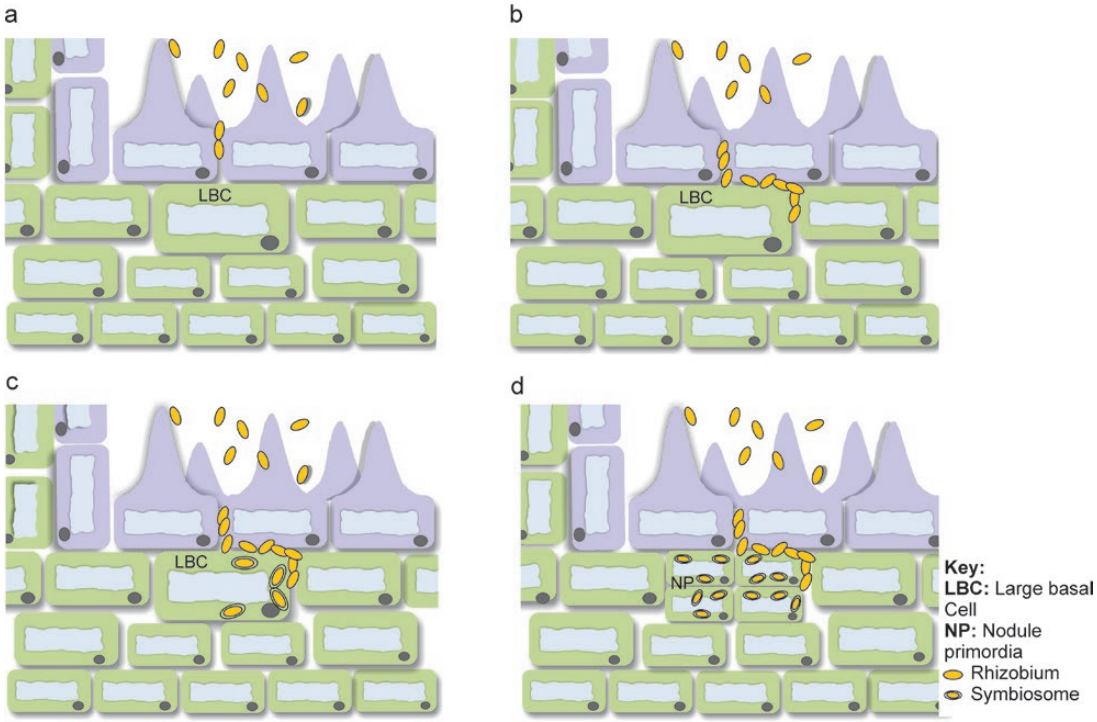

Fig. 2. Rhizobial infection of *Arachis hypogaea* roots. (A) Bradyrhizobia enter the root through the middle lamella between two adjacent hair cells, where the cell wall is loosely constructed. (B) After entry, bacteria spread intercellularly by separating cortical cells at the middle lamella. (C) Some axillary root hairs are associated with large basal cells, which are the first to become infected by bradyrhizobia. (D) These large invaded basal cells divide repeatedly, resulting in aeschynomenoid nodules with no interspersed uninfected cells.

penetrate between the cell walls of the root hair base and an adjacent epidermal cell, and then accumulate on the surface of an outer cortical cell. Rhizobial invasion occurs simultaneously with the induction of cell divisions in the outer cortex of the root, immediately below the root hair. Meanwhile, bacterial accumulations are observed inside cell wall swellings in the cortical cell underneath the root hair. Rhizobia are released from such structures into the cytoplasm of the cortical cell through structurally altered cell walls. The initially infected cell divides repeatedly. Development of primordia into indeterminate nodules results from the repeated division of both infected and non-infected cells (González-Sama *et al.*, 2004).

A legume genus that has captured great attention in the last decade is *Aeschynomene*, subfamily Papilionoideae. *Aeschynomene afraspera* and *A. indica* form determinate nodules after inoculation of rhizobia with or without genes for NF synthesis, respectively (Giraud *et al.*, 2007; Renier *et al.*, 2011). There are also some differences among the mechanisms of root infection by bradyrhizobia in both species (Bonaldi *et al.*, 2011). In *A. indica*, bacterial penetration occurs between two axillary root hairs. Further infection of the roots resembles the invasion mechanism in *Stylosanthes*. Bacteria penetrate cortical host cells through a local invagination of the cell wall that enclose the bacteria, causing cell disorganization and collapse. Other cortical cells in direct contact with these collapsed cells also become infected but, in contrast to the cells of the upper layer, they do not collapse and start dividing, giving rise to the aeschynomenoid nodule primordium (Bonaldi *et al.*, 2011). This type of nodule is characterized by having no interspersed uninfected cells in the infected region, and its infection processes do not involve transcellular infection threads (Sprent and James, 2007).

In *A. afraspera*, bacteria enter the plant through cracks generated by the emergence of lateral roots, between an axillary root hair and an epidermal cell of the primary root. After penetration of the epidermis, bacteria spread intercellularly over three or four deeper cortex layers and infect some cells along their way. In *A. afraspera*, these intracellular intrusions often penetrate deeper in the cell than in *A. indica*. Occasionally, some infected cells collapse. Subsequently, when bacteria reach a deeper zone in the cortex, the invaded cortical cells divide to form a primordium. Infected cells of the upper cortical layers give rise to an outgrowth in which bacteria remain enclosed in large tubular structures (Bonaldi *et al.*, 2011).

Sesbania rostrata, a papilionoid adapted to grow in frequently flooded habitats and forming determinate or indeterminate nodules depending on ethylene concentration, also shows a distinct infection mechanism. Roots can be invaded by rhizobia intercellularly or via root hair and infection thread development, depending on the environmental conditions (Goormachtig *et al.*, 2004*a*,*b*; Capoen *et al.*, 2010). When the plant is growing on well aerated conditions, roots are invaded via a typical root hair and infection thread mechanism, while an intercellular invasion – showing similar features to that described for *Neptunia* – is observed under hydroponic conditions. In the latter case, rhizobia penetrate the epidermis via cracks that are formed by lateral or adventitious root protrusion. They subsequently induce a localized cortical cell death

in a NF-dependent process involving hydrogen peroxide, ethylene and gibberellins. This local cell death results in the formation of intercellular infection pockets, where bacteria proliferate (D'Haeze *et al.*, 1998, 2003; Lievens *et al.*, 2005). Bacteria in the infection pocket act like a signaling center providing NFs for reinitiating meristematic activity of the cortical cells, forming a nodule primordium. From the infection pocket, bacteria are delivered into nodule primordia by intercellular and, subsequently, intracellular infection threads. Alternatively, intracellular infection threads can also begin to develop directly from the infection pockets.

A challenge for future research is to extend our current knowledge of nodulation ability and rhizobial invasion processes to more legume species. This is particularly necessary for the MCC clade, in which phylogenetic relationships among members are as yet unclear. Such studies might then provide us with a better understanding of the distribution of intercellular invasion among the Fabaceae.

Molecular bases of intercellular invasion in legumes

With exception of *Aeschynomene, Sesbania* and, to a lesser extent, *Arachis*, there are few studies dealing with the molecular aspects of intercellular invasion. This is likely associated with a historical bias towards the study of temperate legumes. However, it is now becoming clear that many temperate legumes also lack a root hair infection pathway (Sprent, 2008).

Considering that in legumes invaded via infection threads NFs are required for initial infection of the root hair cell at the epidermis (a process that is bypassed in the intercellular infection), an interesting question is whether these NFs also participate in intercellular root invasion and rhizobial entry.

In *A. indica* and *A. sensitiva* NFs are neither required for invasion of the roots nor for nodule primordia formation (Giraud *et al.*, 2007). The exact nature of bacterial signal triggering symbiotic signaling in these legumes is not fully understood, although purine derivatives (which may be cytokinin-like molecules) play an important role in nodule formation (Giraud *et al.*, 2007; Bonaldi *et al.*, 2010; Podlešáková *et al.*, 2013). Differences between the infection processes in *A. indica* (NF independent) and *A. afraspera* (NF dependent) (Bonaldi *et al.*, 2011) could be attributed to a dissimilar ability to perceive these bacterial molecular signals. Regarding the rhizobial 'entry key' required for initiating the nodulation program in the NF-independent species, Okazaki *et al.* (2015) indicated that bradyrhizobia can nodulate *Aeschynomene* plants in two ways: one that depends on a functional T3SS and other NFs, and T3SS independent, which relies on a still unknown mechanism.

In *Sesbania rostrata,* intercellular invasion of the roots depends on NFs to induce cell death for infection pocket formation (Capoen *et al.*, 2010). Intriguingly, this process does not depend on an active CCaMK, since gene knockdown abolished nodule development but not intercellular invasion and formation of infection pockets at lateral root bases (Capoen *et al.*, 2009). Therefore, colonization of the outer cortex depends on NFs but would be independent of calcium spiking. In addition, requirement of a particular NF chemical structure is less stringent for lateral root base nodulation (occurring after intercellular invasion) than for the root hair curling and infection thread process. Inoculation of mutants producing NFs lacking some substituents demonstrated that none of these decorations are strictly required for lateral root base nodulation, although the number of nodules synergistically increases with their presence (D'Haeze *et al.*, 2000). Therefore, it has been proposed that, after circumventing epidermal entry, there is a less stringent requirement for NF structures for inducing and infecting the nodule primordia.

NFs are not necessary for invasion of *Arachis hypogaea* roots (Ibañez and Fabra, 2011). Under laboratory conditions, peanut nodulation can also be achieved by rhizobia lacking *nod* genes (Guha *et al.*, 2016). However, this type of nodulation seems to be vestigial and non-significant in natural conditions, since *nod* gene-harboring rhizobia exclude *nod* gene-lacking strains in field nodules. Whether NF-independent nodulation of peanut depends on a functional rhizobial T3SS is still not known. Similarly, those determinants ('entry key') allowing NF-independent nodulation have not been identified. However, these results indicated that peanut, as other legumes, can harbor two different programs for nodulation: a NF-dependent and a NF-independent one. In the NF- dependent program, these bacterial molecules are required for initiating the cellular divisions for nodule primordia development (Ibáñez and Fabra, 2011). Considering that aeschynomenoid-type nodules are formed by divisions of a previously infected cell, and taking into account that peanut is intercellularly infected, at least two alternative models for infection and NF perception are possible. In the first model, NFs are required for intracellular infection of the basal cortical cell that forms the nodule primordia. Alternatively, the second model proposes that rhizobia can become intracellular without NFs but that these are required in order to reinitiate meristematic activity of the infected cell. Since the exact degree of colonization of the bradyrhizobial *nodC* mutant (whether it is inter- or intra-cellular) is still not known (Ibañez and Fabra, 2011), any of the two possibilities can be excluded. Putative peanut NFs receptors, homologs to LjNFR1 and LjNFR5, have been identified (Ibáñez *et al.*, 2015). However, their epidermic or cortical localization has not been determined. Moreover, even when the epidermis is crossed intercellularly, whether or not these epidermic cells perceive and respond to NFs is uncertain. CCaMK plays a crucial role in rhizobial dissemination during the development of peanut nodules but its contribution to intercellular invasion and cortical colonization has not yet been demonstrated (Sinharoy and DasGupta, 2009). Similar to legume infected via root hairs by formation of infection threads, an appropriate bacterial cell surface is important for peanut intercellular infection. Inoculation of *A. hypogaea* with a rhizobial mutant affected in the EPS production renders the formation of nodule-like structures but fewer nitrogen-fixing nodules (Morgante *et al*, 2007). Current vision on the origin of nitrogen-fixing symbiosis suggests that after a common precursor with a predisposition to nodulate about

eight independent origins of nodulation may have occurred, partially explaining the diversity found in the features of this interaction (Soltis *et al.*, 1995; Werner *et al.*, 2014). Existence of the single cryptic precursor implies deep homology in symbiosis evolution. Similarities among the different types of intercellular infection even among phylogenetically distant legumes (which probably represent different origins of nodulation), could illustrate the concept of deep homology underlying nitrogen-fixing symbiosis.

The identity of the molecular innovation and resulting traits conferring predisposition to nodulate is still unknown. Repeated whole genome duplications (WGDs) occurring during evolution of angiosperms may have provided the raw material for the emergence of this new trait. In this context, *de novo* assembly of transcriptional networks co-opted from arbuscular mycorrhizal symbiosis, nitrate responses and lateral root formation may have contributed to the emergence and development of this trait (Soyano and Hayashi, 2014). However, Cannon *et al.* (2015) suggest that whole genome duplications are not related to the origin of the 'precursor' (the state with the predisposition to nodulate) and that, within the papilionoids, a WGD event may account for the transition from the precursor to the single origin of nodulation. Taking into account that intercellular infection is considered to represent a ground state compared to the infection thread mechanism, insight into the nature of this molecular precursor should be provided in those legumes that display intercellular infections of the root.

Intercellular invasion of the roots in model legumes

Analysis of *Lotus japonicus* mutant lines lacking root hairs provided information about alternative infection mechanisms. Inoculation experiments in these lines revealed intercellular rhizobial infection through the formation of a wide intercellular infection pocket in the cortical surface of the nodule that narrows down to form an infection thread enabling cell colonization (Karas *et al.*, 2005). Even when the frequency of this event is low, it reveals a previously cryptic mechanism of infection. This intercellular infection mechanism was confirmed by using NFR5 and NFR1 mutants in a snf1 background (a line that forms spontaneous nodules in the absence of rhizobia as a consequence of an autoactivation of CCaMK kinase domain) (Madsen *et al.*, 2010). In these plants, infected nodules were formed, although at lower frequencies than in snf1 and wild-type plants. Further analysis indicated that rhizobia entered the roots through cracks formed at the junctions of spontaneous nodules and roots, followed by bacterial accumulation in intercellular pockets. Infection threads formed from these pockets allowed infection of the nodule cells (Madsen *et al.*, 2010). Moreover, infected nodules appeared in *L. japonicus* snf1 NFR5 and NFR1 plants inoculated with a rhizobial mutant not producing NFs, even at lower frequencies. Few cells, often only individual cells, were infected and no infection threads or trans-cellular infection threads were observed in these rare nodules. Instead, rhizobia in the infection pockets were delivered into nodules by intracellular infection pegs (Madsen *et al.*, 2010). These results indicated that intercellular invasion in *L. japonicus* occurs in absence of root hairs or NF signaling. Moreover, differences in rhizobial colonization when a wild-type or *nodA* and *nodC* mutant rhizobia are inoculated on snf1 NFR1 and NFR5 plants have been attributed to NFs perception by putative cortical receptors (Madsen *et al.*, 2010). In accordance with this, LjNFR1 (but not LjNFR5) is required for cortical infection thread formation, suggesting that NFR1 may form a heterodimer together with a LysM receptor homolog different to NFR5 (Hayashi *et al.*, 2014).

Intercellular infection mechanisms in other legume species of *Lotus* was described by Ranga Rao (1977), who reported that *L. hispidus* roots were intercellularly infected by rhizobia, mainly through direct epidermal invasion. Two simultaneous infection mechanisms in *L. uliginosus* plants growing under flooded conditions have been described. Under flooded conditions, two routes for rhizobial infection can be followed: the classical via root hairs and infection threads and a second route involving enlarged epidermal cells (James and Sprent, 1999). However, this could have been overlooked since it could represent another rare mechanism restricted to few legumes or plants growing in flooded habitats.

Similar to *L. japonicus*, NF-independent nodulation has also been described in *Glycine max* roots (Okazaki *et al.*, 2013). This mechanism relies on the presence of an intact T3SS in the microsymbiont, which plays crucial roles in infection of animal and plant hosts by pathogenic bacteria. In this particular case, T3SS is used by bradyrhizobia to deliver uncharacterized molecules that activate the symbiotic signaling cascade. Absence of root hair curling or infection thread formation in these soybean roots suggests that rhizobial invasion takes place intercellularly. Therefore, T3SS plays a role in intercellular infection occurring in soybean in the absence of NF signaling (Okazaki *et al.*, 2013).

Findings in *L. japonicus* and *G. max* indicate that alternative invasion modes were maintained during evolution and are not mutually exclusive (Madsen *et al.*, 2010, Okazaki *et al.*, 2013). Rhizobial intercellular invasion in plants that are normally infected through the more sophisticated root hair and infection thread mechanism come out as flashbacks, with the development of an apparently anachronistic infection mechanism in these legumes. Different variants in the mechanisms enabling the development of nitrogen fixing symbioses can be regarded as 'snapshots' of the same evolutionary sequence (Svistoonoff *et al.*, 2014). By putting these snapshots in order, observations of the intercellular invasion mechanism in model legumes allows for inference of symbiosis evolution. To this end, a detailed analysis of the molecular determinants required for the different modes of intercellular invasion and nodule morphogenesis is necessary. However, infection mechanisms that diversified after a common origin of nodulation must be compared. Only then will it be possible to organize the snapshots into a series of evolutionary steps that have contributed to transforming the interaction into a more specific, stable and reliable symbiotic association.

Intercellular invasion in non-legumes: actinorhizal plants and *Parasponia*

Actinorhizal symbioses refer to more than 220 plant species distributed across eight families and three orders – Fagales, Cucurbitales and Rosales – collectively referred to as actinorhizal plants. These form nitrogen-fixing root nodules with the actinobacteria *Frankia* (Wall, 2000; Pawlowski and Demchenko, 2012, Svistoonoff *et al.*, 2014). Actinorhizal plants are the second largest group of plants that, together with legumes and *Parasponia*, cover all known diversity of nitrogen-fixing plants within the so-called nitrogen-fixing clade (Fabids) (Doyle, 2011). The main difference between actinorhizal and legume root nodules is the anatomy, a consequence of a different ontogeny of nodule development. Root nodules in legumes are initiated in the dividing cells of the root cortex and pericycle after early interactions with the bacteria, even before infection has started. In actinorhizal root nodules the origin is located exclusively at the pericycle as happens with lateral roots (Pawlowski and Demchenko, 2012). As a consequence of this different ontogeny, the vascular bundles of the nodules show a different final location: lateral in legumes root nodules, and central in actinorhizal nodules. These anatomical features explain why actinorhizal nodules are considered to be modified lateral roots. Symbiotic specificity and recognition is characteristic of both, actinorhizal and legume symbioses (Wall 2000). It is believed that legumes are more specific for its microsymbiont than actinorhizal plants but this may be an artifact resulting from the different number of species studied in detail in both groups. Nevertheless, NFs purified from rhizobia do not show any effect on actinorhizal symbiosis, and *Frankia* DNA cannot complement *nod* mutants of rhizobia (Ceremonié *et al.*, 1999). And while different diffusible signals have been described, the nature of the putative *Frankia* signals is still not fully understood (Ceremonié *et al.*, 1999; Gabbarini and Wall, 2008; Beauchemin *et al.*, 2012; Svistoonoff *et al.*, 2014; Clavijo *et al.*, 2015). Many features of nodule development and infection are common between actinorhizal plants and legumes (Pawlowski and Demchemko, 2012). Some genes of the Sym pathway have been shown to be present and required in actinorhizal plants, supporting the existence of a common ancestor, as was described for SymRK (Gherbi *et al.*, 2008), CCaMK (Svistoonoff *et al.*, 2013) and NIN genes (Clavijo *et al.*, 2015).

Root infection leading to nodule development in actinorhizal plants can be classified in two main groups: (i) intracellular infection through the invasion of deformed root hairs and (ii) an intercellular infection pathway (also called intercellular root invasion) via dissolution of middle lamella between adjacent epidermal cells (Wall and Berry, 2008; Svistoonoff *et al.*, 2014). *Frankia* is able to form infection thread-like structures (Berg, 1999*a*) present in all intracellular infections via deformed root hairs in Fagales (i.e. *Casuarina*, *Alnus*), and also in Cucurbitales (i.e. *Datisca*) nodules. Infection thread growth in root tissue is preceded by cytoplasmic bridge formation in this infected plant tissue (Berg, 1999*b*). There is no infection thread formation in Rosales, as it was described in detail in *Ceanothus* (Liu and Berry, 1991) and in *Discaria trinervis* (Valverde and Wall, 1999*a*). In the cases of intercellular invasion in Rosales, there is no cortex cell division followed by cell infection by *Frankia*, as it happens in root hair-infected plants. Only a very faint cell cortex cell division that is not followed by infection has been described in *Ceanothus* (Liu and Berry, 1991). Intercellular infection in actinorhizal plants has been shown to modify the amount of material in the intercellular space involving pectic polysaccharides. Thus, the modification of the intercellular space seems to be a plant response to infection (Liu and Berry, 1991; Valverde and Wall, 1999*a*). In *D. trinervis* symbiosis it has been recently shown that intercellular infection is linked to the expression of a particular subtilase codified in the Dt12 gene. This Dt12 expression follows the intercellular infection pathway of *Frankia* in the root cortex (Imanishi, 2015). This subtilase of plant origin has been previously shown to be linked to actinorhizal intracellular infection and to be expressed in cells harboring *Frankia* infection threads from the starting point at the invasion of the deformed root hair (Svistoonoff *et al.*, 2003). The analysis of the expression of the Dt12 promoter fused to a GFP reporter shows subtilase expression along the interaction between *Frankia* and plant tissue, from the early adjacent epidermal cell invasion to the mature symbiotic cells in mature nodules full of *Frankia* differentiated into vesicle clusters for nitrogen fixation (Imanishi, 2015). Tracking of *Frankia* in the infection and nodule development of *D. trinervis* confirms the absence of infection thread-like structures at any stage of the symbiosis (Imanishi 2015). Thus, the intercellular infection pathway of *D. trinervis* acquires a particular relevance since it resembles the residual cases of infection and nodule development found in the interaction between mutants of *Lotus* and rhizobia that have been deprived for all the known signals involved in nodulation. This is just intercellular infection and single cell invasion without infection thread formation as an ancestral infection pathway (Madsen *et al.*, 2010). Another signaling step that appears to be different in the intercellular infection pathway in actinorhizal plants is the Ca^{2+} spiking as an early response to NFs (in legumes) or *Frankia* supernatants (in actinorhizal plants). While the Ca^{2+} spiking response appears to be common among legumes and actinorhizal plants infected via deformed root hairs (Granqvist *et al.*, 2015), it was not detected in *D. trinervis* epidermal root cells in response to *Frankia*, although the same cells were responsive for arbuscular mycorrhiza fungi signals (Mireille Chabaud and Joëlle Fournier, personal communication in Imanishi, 2015). Note that *D. trinervis* belongs to Rhamnaceae in the order Rosales, one of the most recent evolved groups of root nodulating plants (Doyle, 2011). Its simple intercellular infection pathway gives extra support to the hypothesis that intercellular infection is the ancestral infection condition in root nodule symbiosis (Sprent, 2007; Madsen *et al.*, 2010; Doyle, 2011).

Parasponia is a particular case in the diversity of root nodule symbiosis. While *Parasponia* is infected by *Bradyrhizobium* bacteria, its nodules resemble those that are anatomically actinorhizal with indeterminate growth and central vascular bundle development. The infection pathway in *Parasponia* is

a case of intercellular invasion that also resembles a crack entry mechanism (Bender *et al.*, 1987). Here, the susceptible region of the root for infection and nodule development is behind the root tip in the growing tap root in the area of root elongation, as has been described for legumes (Buhvaneswari *et al.*, 1980) and actinorhizal plants (Wall and Huss-Danell, 1997; Valverde and Wall, 1999*b*). This indicates the recognition events that take part in infection and early nodule development, since nodulation is not randomly distributed in the growing root (Wall and Huss-Danell, 1997; Valverde and Wall, 1999*b*). In the case of *Parasponia* the infection starts as an intercellular infection with degradation of the middle lamella of the epidermal cells. However, in contrast to events in actinorhizal plants such as *Discaria* and *Ceanothus*, large cell division is induced in the outer cortex. The expansion of the prenodule breaks the epidermis forming a crack opening for bacteria to continue colonization of the intercellular spaces of the expanding cortex. Subsequently, the bacteria infect the dividing cells via the formation of an infection thread with the particularity that bacteroids are not released in infected cells, rather they remain in a fixation thread. No root hair deformation or infection via root hairs is observed in *Parasponia* (Bender *et al.*, 1987). *Parasponia–Bradyrhizobium* recognition is mediated via NFs (Marvel *et al.*, 1987). The relation between nitrogen-fixing symbiosis and mycorrhizal root symbiosis is clearly expressed in *Parasponia*, giving extra support to the hypothesis that the NF receptor appears to be recruited from the ancient mycorrhizal symbiosis, being a common receptor factor for both symbioses in the case of *Parasponia* (Op den Camp *et al.*, 2011).

Potential application of studies on intercellular invasion

Commercial fertilizers are expensive and create a number of environmental problems because of nitrate accumulation in freshwater bodies. Biological nitrogen fixation is an alternative way to increase available nitrogen while reducing the dependency on commercial fertilizers. Consequently, there is a renewed interest in symbiotic nitrogen fixation in plants, an evolutionary innovation that is restricted to only four orders within the Eurosid I clade of angiosperms. One ambitious goal is to transfer symbiotic nitrogen fixation to non-legumes of economic significance (Rogers and Oldroyd, 2014, Delaux *et al.*, 2015*a*). This alternative relies on the fact that many processes related to nitrogen-fixing symbiosis in legumes depend on conserved genetic modules and seem to be based on the evolutionary recruitment concept (Soyano and Hayashi, 2014). Therefore, it is possible that some components of the two genetic programs involved in nodulation (infection and nodule primordia formation) are also present in non-legume plants (Charpentier and Oldroyd, 2010).

Developmental features of symbiosis, such as the infection mechanism, microsymbiont intracellular accommodation and nodule organogenesis, depend on the host genotype. Therefore, the prime target for engineering efforts that transfer nitrogen-fixing symbiosis to non-legumes seems to be the plant. This transfer should be performed by a stepwise improvement of the different stages implicated in legume-rhizobia symbiosis.

The first step in rhizobia-legume symbiosis involves the recognition between the partners, which is mediated through perception of rhizobial NFs by legume receptors belonging to the LysM-RLK family. Nod factor receptor NFP has been proposed to be required for all responses to lipochitooligosaccharides produced by mycorrhizal fungi in *Medicago truncatula* (Maillet *et al.*, 2011). Interestingly, LysM-RLKs that are closely related to NFs receptors were identified in the non-nodulating, non-mycorrhizal angiosperms Arabidopsis and rice, which are required for defense-related perception of fungal chitin oligomers (Kaku *et al.*, 2006; Wan *et al.*, 2008). Furthermore, non-legumes Arabidopsis, tomato and corn perceive and respond to rhizobial NF addition by reducing flg-22-induced reactive oxygen species production. In Arabidopsis, this response is mediated by LYK3, a member of LysM-RLKs (Liang *et al.*, 2013), suggesting that the mechanism for NF recognition and partial response is conserved in some non-legumes. Downstream of these receptors, a signaling pathway triggered by legumes is common to mycorrhizal symbiosis and was probably recruited from this more ancient interaction (Markmann and Parniske, 2009). Therefore, it is evident that some components of this pathway are also present in non-legumes.

Rhizobia can invade and interact with plants outside the Fabaceae. *Parasponia* species (family Cannabaceae, order Rosales) are the only non-legume plants known to be nodulated by rhizobia (Trinick, 1973), and like in most legume-rhizobia symbiosis, the process depends on NFs (Op den Camp *et al.*, 2011). Rhizobia can enter *Parasponia* root tissue intercellularly via the erosion of epidermal cells (Bender *et al.*, 1987; Rolfe and Gresshoff, 1988), this infection being concomitant with the formation of infection threads. Moreover, responses to rhizobial inoculation are also observed outside the Eurosid I clade. In the non-legume *Oryza sativa* (rice) various species and strains of rhizobia can gain access to the interior of roots by crack entry at lateral root emergence (Chi *et al.*, 2005), inducing structural changes resembling infection threads within the root hairs (Perrine-Walker *et al.*, 2007). Rice is a flood-adapted plant, similar to *Sesbania rostrata* and *Neptunia natans*, legumes in which rhizobial intercellular invasion also occurs upon flooding. Considering that rhizobial intercellular invasion actually occurs in non-legumes and that it is a ground state compared to infection thread formation, more studies on this infection mechanism would contribute to unraveling the genetic determinants of this step in the biological nitrogen fixing process. The cases of legumes infected and nodulated independently of NF, such as as *Aeschynomene* (Giraud *et al.*, 2007), open new possibilities of signaling pathways to be manipulated in the transfer of nitrogen-fixing traits to non-legume plant species of agricultural interest.

Related to the intracellular accommodation of rhizobia, symbiosome formation poses stringent demands on the genetic compatibility of symbiotic partners and plant control. Therefore ‘fixation threads’, the sites of bacterial nitrogen

fixation in some actinorhizal hosts and in some legumes of the families Caesalpiniaceae and Fabaceae and probably representing an ancient form of bacterial accommodation, might be more suited as model systems for transfer of nitrogen-fixing symbiosis to cereals (Markmann and Parniske, 2009).

It has been proposed that the most recent common ancestor of extant land plants and green algae was pre-adapted for symbiotic associations. Subsequent improvement of this precursor stage in early land plants (through rounds of gene duplication) led to the acquisition of additional pathways and the ability to establish a functional arbuscular mycorrhizal symbiosis (Delaux *et al.*, 2015*a*). A thorough understanding of the intermediate stages during the evolution of these symbiotic interactions could facilitate the engineering of nitrogen-fixing crops. In this sense, Delaux *et al.* (2015*a*) proposed that quantitative phylogenetics associated with comparative phylogenomics and phylogenetics will generate relevant lists of genomic features associated with nitrogen-fixing symbiosis.

However, components of the common signaling pathway may not be necessary for rhizobial colonization of non-legume plants. Recently, it was reported that rice knockout mutants for *DMI3* (*CCaMK*), *CASTOR* and *CYCLOPS,* which are defective in mycorrhizal symbioses, were all successfully colonized by GFP-tagged *Rhizobium leguminosarum* bv. *trifolii* strain R4. This suggests that common *Sym* genes are not required for infection and endophytic colonization of rice roots by nitrogen-fixing rhizobia. Nevertheless, it is not clear if independence of *Sym* genes for endophytic colonization is a general fact for rice-rhizobia associations, as only one bacterial strain was included in this study (Chen and Zhu, 2013).

It is interesting that another step of the nitrogen-fixing symbiosis, the nodule formation, involves features similar to lateral root development. It is believed that the former is evolutionarily derived from the latter by recruiting pathways already existing in plant development (Sprent, 1989; Mathesius *et al.*, 2000). Indeed, in white clover, cortical cells at sites of lateral root emergence exhibit similar responses to those of the tip after inoculation, and rhizobia can activate these mature cortical cells to form nodules (Mathesius *et al.*, 2000). This nodule morphogenetic process shares similarities with that of peanut. In this legume, nodules are formed at the site of emergence of lateral roots by division of a basal cortical cell, similar to that hijacked by rhizobia in mature zones of the roots in white clover. Therefore, molecular traits involved in lateral root formation in non-legumes should be analyzed in order to be engineered to transfer nitrogen-fixing symbiosis.

Taken together, these facts suggest that the programs required for symbiosis developments are, at least partially, present in non-legume plants. Hence, transfer of nitrogen-fixing symbiosis to non-legumes can make use of previously existing pathways, recruiting these processes for a new trait. Those non-fixing plants within the Eurosid I clade alive today, and identified by Werner *et al.* (2014) as still retaining the nodulation precursor, may represent suitable hosts for initiating gradual nitrogen-fixing symbiosis transfer experiments.

Taking into account that the knowledge now available indicates that molecular determinants related to the nitrogen-fixing process are shared by legumes and non-legumes, engineering of nitrogen-fixing crops is now more likely than a few years ago. Focusing studies on less specialized interactions, including those of *Aeschynomene* or *Arachis* with rhizobia or actinorhizal association with *Frankia*, rather than on legume model systems where the plant genetic programs involved seem to be strongly derived, will further enable this goal. Thus, studies on more primitive but efficient symbiotic processes involving a minimal set of bacterial and host genetic adaptations (such as those involving intercellular invasion) promise to be interesting for engineering artificial host systems.

Conclusion

As we have outlined in this work, much is known about the molecular determinants involved in the infection via the intracellular pathway and nodule formation in the interaction between rhizobia and the model legumes *Lotus japonicus* and *Medicago truncatula*. This review presented an overview of and recent advances in the understanding of bacterial intercellular root infection leading to nodule development in leguminous, *Parasponia* and actinorhizal plants. A better comprehension of the molecular mechanisms involved in biological nitrogen-fixing symbiosis outside the model legumes could provide new insights into ways of manipulating key steps in this process to eventually engineer this ability into major non-legume crops. A relevant and recent step forward has been the identification of a common genetic basis for plant root symbioses with arbuscular mycorrhizal fungi, rhizobia and *Frankia* bacteria in both legumes and non-legumes. This finding is congruent with the fact that genes involved in infection and nodule primordia formation are also present in non-legume crops. In addition, existence of the intercellular infection mechanism occurring in the absence of root hairs or NF signaling has been confirmed in mutants of the model legume *Lotus japonicus.* Advances in the understanding of intercellular rhizobial legume and non-legume root infection may represent original new avenues for designing non-legume nitrogen-fixing crops.

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