Running head: Rhizobial infection determines nodule development

Jeremy Dale Murray
Cell and Developmental Biology,
John Innes Centre,
Norwich NR4 7UH, United Kingdom
Tel: 01603-450130
jeremy.murray@jic.ac.uk

Plants Interacting with Other Organisms
Rhizobial infection is associated with the development of peripheral vasculature in nodules of *Medicago truncatula*

Dian Guan¹, Nicola Stacey¹, Chengwu Liu¹, Jiangqi Wen², Kirankumar S. Mysore², Ivone Torres-Jerez², Tatiana Vernié¹, Million Tadege³, Chuanen Zhou², Zeng-yu Wang², Michael K. Udvardi², Giles E. D. Oldroyd¹, and Jeremy D. Murray¹.

¹ Department of Cell and Developmental Biology, John Innes Centre, Norwich NR4 7UH, United Kingdom

² Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA

³ Oklahoma State University, Department of Plant and Soil Sciences, 368 Agricultural Hall, Stillwater, OK 74078-6028
Financial sources: This work was supported by BBSRC grants CA403A13B (David Phillips Fellowship) and BB/J004553/1, the Samuel Roberts Noble Foundation, the National Science Foundation, the European Foundation as Marie Curie IEF 255467 to T. Vernié, and support for D. Guan from the John Innes Foundation.

Email address of corresponding author: jeremy.murray@jic.ac.uk
Nodulation in legumes involves the coordination of epidermal infection by rhizobia with cell divisions in the underlying cortex. During nodulation rhizobia are entrapped within curled root hairs to form an infection pocket. Trans-cellular tubes called infection threads then develop from the pocket and become colonized by rhizobia. The infection thread grows towards the developing nodule primordia and rhizobia are taken up into the nodule cells where they eventually fix nitrogen. The epidermal and cortical developmental programmes are synchronized by a yet-to-be identified signal that is transmitted from the outer to the inner cell layers of the root. Using a new allele of the *Medicago truncatula* mutant *Lumpy Infections, lin-4*, that forms normal infection pockets but cannot initiate infection threads; we show that infection thread initiation is required for normal nodule development. *lin-4* forms nodules with centrally-located vascular bundles similar to that found in lateral roots rather than the peripheral vasculature characteristic of legume nodules. The same phenomenon was observed in *M. truncatula* plants inoculated with the *Sinorhizobium meliloti exoY* mutant, and the *M. truncatula vapyrin-2* mutant, all cases where infections arrest. Nodules on *lin-4* have reduced expression of the nodule meristem marker *MtCRE1* and do not express root-tip markers. In addition, these mutant nodules have altered patterns of gene expression for cytokinin and auxin markers, *CRE1* and *DR5*. Our work highlights the coordinating role that bacterial infection exerts on the developing nodule and allows us to draw comparisons to primitive actinorhizal nodules and rhizobia-induced nodules on the non-legume *Parasponia andersonii*. 
Legumes form an endosymbiosis with nitrogen fixing bacteria called rhizobia in a carefully orchestrated developmental process called nodulation which involves the infection of the epidermal cell layer and divisions of underlying cortical cells to form a new organ called a nodule. During nodulation the bacteria enter the plant roots through special structures called infection threads which provide access to the inner cell layers, a process that is tightly controlled by the host. In most legumes, including *Medicago truncatula*, infection threads originate from a so-called ‘infection pocket’, a structure formed by the curling of root hairs at their tip which serves to entrap the rhizobia. Infection threads then form as invaginations of the cell membrane and cell wall from the infection pocket and extend down through the root hair cell and eventually into the cortical layers where the bacteria enter re-differentiated cells of the nodule (Murray, 2011). Here they are taken up into organelle-like structures called symbiosomes where they differentiate and eventually fix nitrogen. As epidermal infection takes place, the inner cell divisions of the nodule have already started. Legume nodules can be either indeterminate as those found in *M. truncatula*, having a persistent apical meristem, or the determinate type in which no focal meristem is evident, such as those found in *Lotus japonicus*. In *M. truncatula* the nodule meristem takes several days to form (Timmers et al., 1999) and is subtended by a zone containing infection threads where bacteria are released into still-developing nodule cells, followed by the nitrogen-fixing zone which is a core of enlarged cortical cells containing the symbiosomes (Oldroyd and Downie, 2008; Oldroyd et al., 2011). The nodule also has two or more peripheral vascular bundles that converge towards the nodule apex and serve in the exchange of nutrients between roots and nodules.

The events in nodulation are initiated by lipochitooligosaccharides called Nod(ulation) factors (NF) that are produced by the rhizobia. NFs are perceived by the plant, probably by members of the LysM receptor-like kinase family LYK3 and *Nod Factor Perception* (NFP) (Amor et al., 2003; Limpens et al., 2003; Arrighi et al., 2006), and the signal is transduced by the Common Symbiosis Pathway which is shared with the arbuscular mycorrhizal symbiosis (Oldroyd and Downie, 2008; Oldroyd et al., 2011). The common symbiosis pathway includes, CCaMK, a calcium calmodulin dependent protein kinase (Gleason et al., 2006; Tirichine et al., 2006) whose activation leads to the induction of transcription of several transcription factors including *Nodule Inception* (*NIN*) (Schauser et al., 1999; Marsh et al., 2007). *NIN* is required for both the initiation of infection threads and the initial cell divisions that lead to nodule formation, and expression of *NIN* can be induced by cytokinin (Murray et al., 2007; Plet et al., 2011). The downstream cytokinin response regulator *MtRR4* is also strongly induced during nodulation in a *Cytokinin*
Response1 (MtCRE1) dependent manner, and localizes to the early cell division centre in nodule primordia and to the meristem in mature nodules (Plet et al., 2011).

The mechanism that links infection and nodule development is not known. Loss of function mutants such as nin (Schauser et al., 1999; Marsh et al., 2007) and ccamk (Gleason et al., 2006), that don’t form infection threads invariably do not form any nodules, while mutants in which infections abort in the root hairs such as vapyrin (Murray et al., 2011), lin (Kuppusamy et al., 2004), and rpg (Arrighi et al., 2008), lead to delayed nodule development. Plant mutants such as ipd3 (Horvath et al., 2011; Ovchinnikova et al., 2011) and the rhizobial mutant bacA (Oldroyd et al., 2011) which support normal infection thread growth but fail to form symbiosomes, have normal nodule architecture. Remarkably, constitutively active forms of the cytokinin receptor LHK1/MtCRE1 (Tirichine et al., 2007) or CCaMK (Tirichine et al., 2006; Gleason et al., 2006) in the absence of rhizobia, infection by a non NF-producing rhizobia mutant constitutively expressing a cytokinin biosynthesis gene (Cooper and Long, 1994), and exogenous application of either NF (Truchet et al., 1991; Grosjean and Huguet, 1997) or cytokinin (Heckmann et al., 2011), can result in formation of nodule-like structures, showing that the initiation of nodule organogenesis does not require infection. However, previous studies show that rhizobial infection progression is necessary to maintain the nodule apical meristem (Voroshilova et al., 2009).

Despite the fact that mutations in several genes result in defects in both infection and organogenesis, several lines of evidence point to the genetic independence of these two programmes. One line of evidence is that cortex and outer cell layers have differential responses to various species of NFs. Double nodF/nodL mutants that produce NFs with missing or substituted decorations are unable to induce infection threads but were still able to induce cortical cell divisions (Ardourel et al., 1994). More recently, work in L. japonicus showed that in the nfr1 nfr5 snf1 background, no epidermal infection threads formed but trans-cellular infection threads in nodules occurred at a low rate with WT but not with nodA or nodC rhizobia (Madsen et al., 2010). Moreover, expression of gain-of-function alleles of CCaMK and LHK1 in mutants that do not support epidermal infection, such as nfr1 or nfr5, can induce so-called ‘spontaneous nodules’ in the absence of rhizobia (Madsen et al., 2010). Conversely, in the hyperinfected 1 (hit1) mutant a delay in nodule cortical cell divisions is associated with hyperinfection of the epidermis (Murray et al., 2007). While it’s clear the two programmes are genetically separable infection and organogenesis appear to be interdependent once the nodule is established as rhizobial infection is needed for nodule apical meristem formation (Yang et al., 1992; Voroshilova et al., 2009).

We show using plant and rhizobial mutants that blockage of infection leads to the
development of nodule-like structures that have centralized vasculature and that this phenomenon is associated with changes in auxin and cytokinin signalling. This work reveals that nodule development is influenced by the progression of bacterial infection and highlights the coordination that occurs between the cells accommodating rhizobia in the epidermis and outer root cortex and those cells in the inner cortex that are acquiring a nodule identity.

RESULTS

A mutant that aborts rhizobial infection in the root hair curl

A *M. truncatula* mutant defective for infection by *S. meliloti* was identified as a nodulation mutant from a population of *Tnt1* transposon-tagged (ecotype R108) plants (Pislariu et al., 2012). Rhizobial infections that formed on the mutant aborted in the root hair curl, forming a normal looking infection pocket but no infection threads (Supplemental Fig. S1). Rarely, what appear to be very short intracellular infections formed but they aborted almost immediately and never progressed across the root epidermis. We named the locus *Knocks but Can’t Enter* (*KCE*). Genetic analysis showed that the *kce* mutant phenotype is conditioned by a monogenic, recessive mutation ($\chi^2=1.102$, $p>0.31$, $n=86$) which maps to the lower arm of chromosome 1 in the same region as the symbiotic gene *LIN*. We sequenced *LIN* in the *kce* mutant and found a G to A transition at position 1740 in cDNA which introduced a TGA stop codon. We therefore concluded that *kce* is an allele of *LIN* and we reassigned it as *lin-4*. When quantified at 6 and 14 dpi, *lin-4* mutant had more infection events than wild type plants (Fig. 1C). Despite the early abortion of infections *lin-4* developed nodules in a very slow manner; at 60 dpi some white bumps began to emerge, by 90 dpi *lin-4* nodules were short white outgrowths, compared with wild type nodules that were long and pink (Fig. 1A, B). These nodules only formed on *S. meliloti* infected plants and were associated with aborted infections. Nodule numbers on *lin-4* were reduced at all time points examined (Fig. 1D). An acetylene reduction assay on *lin-4* nodules indicated they were deficient in nitrogen fixation (data not shown), and the plants showed signs of nitrogen deprivation (yellowing of leaves and anthocyanin accumulation in the shoot) and started to die after 3 months of growth.
The infection-induced nodules in \textit{lin-4} are centrally vascularised

Sectioning of WT nodule primordium revealed that the initial cell divisions were an ordered series of periclinal and anticlinal divisions in the cortical, endodermal and pericycle cell layers accompanied by cell expansion that resulted in a bulge in the epidermis (Supplemental Fig. S2A). In \textit{lin-4} primordia early cell divisions also occurred in all three cell layers but were significantly delayed, and cell expansion was not seen at this stage (Supplemental Fig. S2B). This is identical to \textit{lin-1} as described by Kappusamy et al. (2004). Also, the area where the cortical cell divisions occurred was limited to a shorter interval and the divisions were less coordinated such that the layered organization of cell layers seen in WT was lost (Supplemental Fig. S2). Surprisingly, as the \textit{lin-4} nodules grew over time, they developed a central vascular bundle (Fig. 2B, D), similar to lateral roots (Fig. 2E, F). This contrasted markedly from the WT nodules where multiple vascular bundles developed at the nodule periphery (Fig. 2A, C). However, \textit{lin-4} nodule vascular strand organization was often different from that of the lateral roots; in particular, the \textit{lin-4} nodules sometimes had a centrally fused xylem strand (Fig. 2D). In addition, examination of longitudinal sections revealed that the apical region of the \textit{lin-4} nodule (Fig. 2B) lacks the characteristic organization of lateral roots (Supplemental Fig. S3). To investigate the nature of these nodules we tested marker genes for lateral roots and nodules using quantitative RT-PCR (QPCR) from \textit{lin-4} and WT spot-inoculated roots. We used the \textit{Medicago truncatula} Gene Expression Atlas (MtGEA) database to identify two marker genes expressed in the root tip, \textit{Ferridoxin I} (Medtr2g006290) and a membrane protein (Medtr7g011090) that are not expressed in nodules (Holmes et al., 2008). Quantitative PCR showed that \textit{lin-4} nodules have significantly lower expression of these root tip marker genes compared to lateral roots (Fig. 3A). In addition, we tested expression of the nodulation specific transcription factor \textit{NIN} (Schauser et al., 1999; Marsh et al., 2007). \textit{NIN} gene expression was lower in \textit{lin-4} nodules than in WT nodules but this difference was not significant (P>0.05, \textit{t}-test). However, the expression of \textit{NIN} was significantly higher in \textit{lin-4} nodules than in the lateral roots (Fig. 3B). We also checked the expression of other nodulation marker genes in \textit{lin-4} nodules. \textit{ENOD20} has been shown to be induced in dividing cortical cells in nodule primordia, in cells containing infection threads, and later in nodules in the infection and nitrogen fixing zones (Vernoud et al., 1999) whereas \textit{HAP2.1} is reported to express in the nodule meristem (Combier et al., 2006; 2008). Relative transcript levels of \textit{ENOD20}, and \textit{HAP2.1} in developing nodules were not significantly lower in \textit{lin-4} than in the WT (data not shown).
Early abortion of rhizobial infection in the root hair leads to the development of nodules with central vascular bundles

To test whether the abnormal nodules that formed on lin-4 were a result of the premature termination of infection or a specific feature of the lin-4 allele, we studied some rhizobial and plant infection mutants where rhizobial infection aborts at a similar stage. First we tested the M. truncatula vapyrin-2, and lin-1 mutants. These mutants have a mixture of infections that either abort within root hairs or penetrate into the nodules (Kuppusamy et al., 2004; Murray, 2011). Nodule sectioning revealed these structures were devoid of bacteria (Fig 4A). When nodules were allowed to develop over several months, lin-1, developed a mixture of nodules having either central or peripheral vascular bundles (Fig. 4C and D). This contrasts with the kce (lin-4) phenotype which featured only centrally vascularised nodules. This difference may be due to the differing genetic backgrounds of these alleles (lin-1; Jemalong A17, lin-4; R108). We then examined the nodules more closely to determine if there was a relationship between the stage of rhizobial infection and vasculature pattern. For the lin-1 mutant, nodules with peripheral vasculature were almost always (95%, n=20) found to be associated with a greatly thickened infection thread either in the nodule epidermis or possibly in the outer cortex (Fig. 4C) while in the instances where nodules had central vascular bundles infection thread development was limited to root hairs and was not evident in nodule cross-sections (Fig. 4D). This contrasted with the lin-4 allele, in which infection threads always terminated in root hairs and never reached the base of the epidermal cell (Supplemental Fig. S1B). We then tested a S. meliloti mutant that is defective in the first step of exopolysaccharide biosynthesis, which leads to the abortion of rhizobial infection in the root hair curl and the occasional initiation of infection threads that abort in the root hair. Vascular bundles in the small nodules produced by the exoY mutant also developed in a central position (Fig. 4B).

Cytokinin signalling is not maintained in lin-4 nodule development

One of the earliest events during nodule organogenesis is cytokinin signalling. To test whether the development of centrally vascularised nodules is associated with decreased cytokinin signalling we tested the expression of the cytokinin receptor MtCRE1 and the type-A cytokinin response regulator MtRR4 in lin-4 nodules. We studied MtCRE1 expression by using a fusion of the MtCRE1 promoter with the β-glucuronidase (GUS)
reporter (Lohar et al 2006). During nodulation, cytokinin signalling is activated during early cell divisions and as the nodule develops, it is strongly maintained in the nodule meristem (Lohar et al 2006). We compared WT and mutant nodules of similar size which required using relatively old nodules from the mutant. Wild type nodules of a similar age would be senescent, but mutant nodules at this point were still growing, albeit slowly. In early development, the induction of MtCRE1 in lin-4 nodules was similar or slightly greater than in the WT (Fig. 5A and B), but in fully emerged nodules (45 days post inoculation) MtCRE1 gene expression was greatly reduced or absent as seen using promoter-GUS (Fig. 5C and D) and Q RT-PCR (Fig. 5E). MtRR4 gene expression was consistently lower in lin-4 nodules, and was significantly lower when nodules of a similar developmental stage (~ 1 mm in length) were compared (Fig. 5F).

**DR5::GUS is expressed ectopically in exoY induced nodules**

Since interactions between cytokinin and auxin are central to the development of vascular patterning, we used a line expressing the auxin reporter DR5::GUS to study the spatial distribution of auxin signalling. WT nodules exhibited GUS expression that was mostly limited to the phloem tissue of the nodule vascular strands, with no staining in the infection zone (Fig. 6A). Inoculation using the exoY mutant rhizobia which induces centrally vascularised nodules revealed DR5 expression in the vascular bundle and, surprisingly, in the surrounding cells (Fig. 6B). For comparison, we examined lateral roots from infected plants and found they had expression in the vascular bundle and in the epidermal cell layer, but no expression in the intervening cortical cell layers (Fig. 6C).

**DISCUSSION**

**Nodule Infection is required for normal vascular development in nodules**

We provide evidence that successful epidermal infection is required for normal nodule development during nodulation in *M. truncatula*. We found that nodules that form on the kce (lin-4) mutant have a central rather than peripheral vascular bundle. This configuration is similar to lateral roots and actinorhizal nodules and the rhizobia-induced nodules that develop on the non-legume *Parasponia*. This outcome is independent of the plant genotype as shown by the occurrence of nodules having either central or peripheral vasculature on the same plants on certain alleles of *vpy* and *lin* mutants in a manner that
directly corresponds to the extent of infection progression. In addition, rhizobial \textit{exoY} mutants that abort early in infection also produce nodules with a centrally-located vascular strand. The distinct structure of these growths and analysis of marker gene expression suggests that symbiotic identity is retained despite the fact that these nodules are not colonized.

This work implies that appropriate nodule development requires the ongoing infection of bacteria and in cases where bacterial infection is initiated but progression aborts, there is a direct impact many cell layers away in the developing nodule primordia. The fact that this impacts the final nodule structure highlights the level of control exerted by the progressing infection process on the developing cells of the nodule. Support for the idea that infection determines nodule development outcomes is provided by Hirsch et al. (Hirsch et al., 1985). They showed that \textit{Agrobacterium tumefaciens} and \textit{Sym}^{-} \textit{Rhizobium trifolii} transconjugants carrying nodulation sequences from \textit{S. meliloti} were able to induce nodules with peripheral vascular bundles on \textit{M. sativa}, but formed nodules with central vascularisation when white clover roots were inoculated. When the nodules were examined it was found that despite lacking infection threads the nodules formed on \textit{M. sativa} contained large pockets of intracellular bacteria, while the nodules on the white clover roots were not colonized. Although our experiments were limited to \textit{M. truncatula}, nodules with central vascular tissue were reported for soybean infected by a \textit{Bradyrhizobium japonicum} mutant that lacked the high molecular weight lipopolysaccharide I. These nodules also lacked bacteria (Stacey et al, 1991). \textit{L. japonicus aberrant localization of bacteria inside nodule 1 (alb1)} mutant appears to develop central vasculature in uncolonized nodules, which may suggest this phenomenon is widespread in legumes (Imaizumi-Anraku et al., 2000).

\textbf{A role for auxin and cytokinin in infection-dependent nodule development outcomes}

How might infection affect development in such a striking manner? There are several interesting exceptions to the link between infection and the development of peripheral vascular bundles that provide important clues to the mechanisms underlying this phenomenon. First, nodules induced by NFs alone can sometimes have normal positioning of the vascular strands (Truchet et al., 1991; Grosjean and Huguet, 1997). Also, application of exogenous cytokinin, or the \textit{L. japonicus snf2} mutant with constitutively active cytokinin signalling both produce nodules with peripheral vascular bundles in the absence of infection (Tirichine et al., 2006; Heckmann et al., 2011). Similarly, the \textit{snf1}
mutant also produces spontaneous nodules with normal architecture (Tirichine et al., 2006). Therefore, it appears that the requirement for infection can be obviated by activation of downstream processes, in particular cytokinin signalling. Counter to this, auxin transport inhibitors like NPA induce nodules that lack vascular strands altogether (Hirsch et al., 1989; Van De Wiel et al., 1990; Takanashi et al., 2011), or form nodules with centrally proliferating vascular tissue (Wu et al., 1996; Rightmyer and Long, 2011) underscoring the central role of polar auxin transport in vascular bundle formation. NFs applied to legume roots have a dramatic effect on polar auxin transport (Mathesius et al., 1998; Boot et al., 1999). Spot application of NFs or rhizobia on white clover roots has been shown to induce a strong local repression of expression from the promoter of the auxin reporter gene GH3 (Mathesius et al., 1998). It is not known how this effect is exerted, but cytokinin perception has been shown to be required for the rhizobia-induced changes in auxin transport that occur during nodulation (Plet et al., 2011), and flavonoids, which are essential for nodulation, also exert effects on auxin transport (Wasson et al., 2006; Subramanian et al., 2007; Zhang et al., 2009). We propose that the advancing infection thread results in local signalling outputs, most likely in response to Nod factor, that mediate appropriate cytokinin and auxin distribution in the developing nodule primordia.

NFs are produced by rhizobia enclosed in infection threads within the nodule (Marie et al., 1992) and these are likely to initiate the coordination of nodule development with infection progression. While NF-induced nuclear calcium oscillations accompany the advance of infection through the underlying cortical cell layers (Sieberer et al., 2012) NF signalling remains restricted to those cells in close contact with the invading rhizobia. Thus Nod factor signalling alone may not be sufficient to explain the coordination of infection and nodule development that are separated by multiple cell layers. Nod factor perception may activate a transcellular signal that drives developmental changes in the inner root cortex and such signalling must be maintained to ensure appropriate nodule development. It’s possible that the production of NFs within the centre of the nodule results in changes in cytokinin and auxin distribution that have developmental consequences. Auxin has a well-established role in vascular differentiation, and xylem formation in particular is associated with high levels of auxin stimulation in the presence of cytokinin (discussed in Aloni et al., 2006). This idea is consistent with the observation that expression of the auxin reporter GH3 is initially uniform within nodules prior to infection, but as the nodule develops GH3 expression becomes diminished at the centre of the nodule where infection occurs, and is limited to the nodule periphery and is especially high in the vascular bundles (Mathesius et al., 1998; Pacios-Bras et al., 2003; Takanashi et al., 2011). We find that marker genes for
cytokinin and auxin signalling associated are deregulated when infection is prematurely aborted. Since cytokinin can induce normal nodules while auxin cannot it seems most likely that the abnormal nodule development is ultimately a result of altered cytokinin signalling. However, auxin can directly repress cytokinin at the biosynthesis and signalling levels (Cheng et al, 2013; Zhao et al, 2010), so it remains possible that ectopic auxin signalling leads to the repression of cytokinin which in turn leads to abnormal vascular development.

Implications for nodule evolution

Nodules with central vascular bundles result from rhizobial infection of members of the non-legume genus *Parasponia* (Trinick, 1979; Lancelle and Torrey, 1985). The infection programme in *Parasponia* does not utilize root hairs; infection threads are formed only after penetration of the bacteria into the root cortex which leads to the formation of a small non-vascularised pre-nodule near to where the main centrally-vascularised nodule will emerge and eventually become infected (Bender et al., 1987). Similarly, nodules induced by *Frankia* involve the formation of a pre-nodule, so that the initial epidermal infection and lateral organ development programmes are separate both temporally and spatially. It has been proposed that these independent processes present in actinorhizal nodulation have been merged together in legumes (Gualtieri and Bisseling, 2000; Laplaze et al., 2000). Our finding that physical separation of infection from the emerging nodule leads to the development of nodules with a central vascular arrangement supports this hypothesis. These findings suggest that by achieving a better understanding of the impact of infection on nodule development we will gain insights into the evolution of this legume-specific innovation in nodule architecture.

MATERIALS AND METHODS

Plant Materials and Bacterial Strains

Seeds of Medicago truncatula cv Jemalong A17, *lin-1* and *M. truncatula* R108, *vapyrin-2*, and *kce (lin-4)* were scarified with concentrated sulphuric acid for about 10 mins, rinsed 6 times, surface-sterilized in 100% bleach for 2 mins, and rinsed 6 times, then imbibed in sterile water and plated on 1% (W/V) deionized water agar plates. Seeds were left at 4°C for one day and germinated on inverted agar plates at 23°C overnight. For hairy
root transformation, *M. truncatula* seedlings were transformed with *Agrobacterium rhizogenes* strain ARqua1 carrying the appropriate binary vector using standard protocols (Boisson-Dernier et al., 2001). For nodulation assays, either *S. meliloti* 1021, *S. meliloti* 1021 pXLDG4 or *S. meliloti* Rm7210 exoY210::Tn5 (Leigh et al., 1985) strain was used. The *DR5::GUS* marker line was produced by stable transformation of *M. truncatula* leaves using the *A. tumefaciens* strain EHA105 harbouring the *DR5::GUS* construct.

**Marker Gene QRT-PCR Assay**

The germinated *M. truncatula* seedlings were put on Buffered Nodulation Medium (BNM) containing 0.1 µM 2-aminoethoxyvinylglycine (AVG) for 2 days until the main roots grow to a length of about 3 cm. The roots were spot inoculated in the differentiation zone with 1 µL of *S. meliloti* 1021 pXLDG4 culture (OD600≈0.2). The site of inoculation was marked at the back of the plate. The inoculated section was harvested at different time points. For each time point, more than 6 root segments of about 1 mm in length were collected. As *kce* (*lin-4*) nodules emerge much slower than WT nodules, we attempted to account for this by isolating nodules from *kce* (*lin-4*) and emerging lateral roots from WT that were matched in size. For the size-matched samples, an emerged *lin-4* nodule (70 dpi) and a just-emerged lateral root tip, each 1 mm in length were excised.

RNA extractions and cDNA synthesis were carried out using the RNeasy® Micro kit (Qiagen) and SuperScript™ II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR reaction was done using LightCycler® 480 SYBR Green I Master (Roche Applied Science Ltd.) reagents and analyzed using LightCycler® 480 Real-Time PCR System (Roche Applied Science Ltd.) over 45 cycles of 95°C for 25s, 58°C for 25s and 72°C for 40s after an initial denaturation at 95°C for 2min. The internal control gene was *MtEF-1α*. Data from three technical replicates and three biological repeats were analyzed using the 2-ΔΔCt method. The standard Deviation (SD) values were calculated using Excel 2007 software. Primers used had PCR efficiencies greater than 90%. Means comparisons were based on RQ (relative quantity) values. Primer sets used in QRT-PCR: *MtRR4*: 5'-ATGCTTTTGTTCCGGGTTTA-3'; 5'-CTGCACCTTCTCCCAACAT-3'. *MtCRE1*: 5'-CACCACCTTTGGCTTCTAA-3'; 5'-CACTAAGTAGCGGCTTTCG-3'. *MtGH3*: 5'-ACTTAACGGTGCACCTGACC-3'; 5'-AACTGAGCAGGCTCATTTC-3'. *MtNIN*: 5'-CAGGACTACCATCGCTGCA-3'; 5'-CCACAGGTGCTTGGAGGT-3'. Root specific ferridoxin: 5'-GCAACCACACCTGCTTTGA-3'; 5'-GTGGTGGTGGCTGATTGACAC-3'. Root specific membrane protein: 5'-AGGAGCAGTGCTGGAATGAC-3'; 5'-
TGCTGACAAAAAGCAAACCA-3'. *MtEF-1α*: 5'-ATTCCAAAGGCGGCTGCATA-3'; 5'-CTTTGCTTGGTGCTGTTTAGATGG-3'

**Nodulation Assay**

*M. truncatula* seedlings were grown in 40 cell trays with a Terragreen (Oil-Dri UK Ltd.) and silver sand 1:1 mixture for 7 days before inoculation with rhizobia. A 24 h culture of rhizobia was spun down and resuspended in BNM to an OD<sub>600</sub> of 0.02 and 3 mL of the culture was used to inoculate each of the growing *M. truncatula* plants. Nodules and infection events were counted 6-90 days post inoculation.

**GUS Staining and LacZ Staining**

*pCRE1::GUS* construct was from Lohar et al. (2006). Hairy root transformation of *M. truncatula* and selection of kanamycin-resistant plants were performed as described above. Plants were transferred to a 50:50 Terra Green and silver sand mixture and inoculated with *S. meliloti* 1021 pXLDG4 as described above. GUS activity (Vernoud et al., 1999) and LacZ activity (Pichon et al., 1994) of whole roots and nodules were performed as previously described.

**Technovit Imbedding, Sectioning and Section Staining**

Collected tissues were fixed in 2.5% (V/V) glutaraldehyde as described in (Vernie et al., 2008). After fixation, tissues were embedded in Technovit 7100® (Kulzer GmbH, Germany) resin according to the manufacturer’s instructions and 10 µm transverse or longitudinal sections were taken. Sections were stained with toluidine blue O (0.5% (W/V) Toluidine blue ‘O’ in 0.5% (W/V) sodium tetraborate buffer) or 20 mg mL<sup>-1</sup> phloroglucinol in 20% HCl (V/V) before taking pictures under a Nikon Eclipse E800 microscope.

**Acknowledgments**

The authors would like to thank Kate VandenBosch for sending us the p*MtCRE1::GUS* construct and Allan Downie for critical reading of the manuscript and Susan Bunnewell for technical assistance.
LITERATURE CITED


Murray JD (2011) Invasion by invitation: rhizobial infection in legumes. Mol Plant Microbe Interact 24: 631-639


Rightmyer AP, Long SR (2011) Pseudonodule formation by wild-type and symbiotic mutant *Medicago truncatula* in response to auxin transport inhibitors. Mol Plant Microbe Interact 24: 1372-1384


Voroshilova VA, Demchenko KN, Brewin NJ, Borisov AY, Tikhonovich IA (2009) Initiation of a legume nodule with an indeterminate meristem involves proliferating host cells that harbour infection threads. New Phytol **181**: 913-923

Wasson AP, Pellerone FI, Mathesius U (2006) Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by Rhizobia. Plant cell **18**: 1617-1629


Figure Legends

Fig. 1. *kce* (*lin*-4) nodulation phenotype. (A and B) Nodules of WT (A) and *lin*-4 (B) 80 dpi with *S. meliloti* 1021. The white arrows in (A) and (B) highlight nodules. (C) Mean (± SD) number of infection threads and infection foci of WT and *lin*-4 6 and 14 dpi with *lacZ*-tagged *S. meliloti* 1021. (D) Mean (± SD) number of nodules of WT and *lin*-4 14, 30, 60, 90 dpi with *S. meliloti* 1021. (n=8). Scale Bars in (A) and (B) are 5 mm.

Fig. 2. *lin*-4 nodule vascular bundles are centrally localized. (A to D) Longitudinal (A and B) and cross (C and D) sections of WT (A and C) and *lin*-4 (B and D) nodules. Black arrows highlight nodule meristems and black arrowheads highlight vascular bundles. (E and F) lateral root cross sections of WT (E) and *lin*-4 (F). (A and B) are stained with toluidine blue, (C to F) with phloroglucinol. Scale bars are 100 µm.

Fig. 3. *lin*-4 nodules do not express lateral root marker genes. (A) Root-tip marker gene expression in WT and in size-matched *lin*-4 nodules and lateral roots measured by quantitative RT-PCR normalized using *EF1α*. Marker genes are *ferridoxin I* (*Medtr2g006290*) and a plant integral membrane protein (*Medtr7g011090*). (B) *NIN* gene expression in WT and *lin*-4 nodules and lateral roots measured by quantitative RT-PCR normalized using *MtEF-1α*. The results represent three biological replicates (±SD). A comparison of gene expression was made between *lin* nodules and the other tissues (Welch’s *t*-test, *P*<0.05). LR: lateral roots.

Fig. 4. Rhizobial infections that abort in the root hair curl lead to abnormal nodule organogenesis featuring a central vascular bundle. (A) A *vpy*-2 nodule. The section is stained with toluidine blue. (B) An *S. meliloti* 1021 *exoY* mutant-induced nodule on WT (ecotype R108). (C and D) *lin*-1 forms two types of nodules: (C) nodules with branched, peripheral vascular bundles; and (D) nodules with central vascular bundles. Note the thickened infection thread in the nodule outer cortex (white arrow, panel C) visualised by X-gal staining. Nodules were harvested at 60 dpi. Longitudinal nodule sections of 10 µM thickness are shown. Scale bars are 200 µm.
**Fig. 5.** Cytokinin signalling is not maintained in *lin*-4 nodule organogenesis. (A-D) WT (A and C) and *lin*-4 (B and D) transformed with pMtCRE1::GUS using the hairy root transformation system. (A and B) 6 dpi with *S. meliloti* 1021 carrying lacZ. (C) WT nodule 13 dpi with *S. meliloti* 1021 carrying lacZ. (D) *lin*-4 nodule 45 dpi with *S. meliloti* carrying lacZ. X-Gluc was used to stain for GUS activity in (A-D), seen as blue in (A-C). (E and F) MtCRE1 (E) and MtRR4 (F) gene expression in WT and *lin*-4 spot inoculated roots (0, 2, 4, 6, 14 dpi) and excised size-matched nodules (see materials and methods) measured by quantitative RT-PCR normalized using *MtEF-1α*. Results represent three biological replicates (±SD). Expression in *lin* vs WT nodules was compared at the different timepoints (Welch’s *t*-test, * P<0.05;** P<0.01). Infection foci and infection threads containing rhizobia were detected by LacZ activity using magenta-gal (indicated by the black arrows). Scale bars in A, B, D, 200 µM, in C, 100 µM.

**Fig. 6.** Auxin response is altered in central vascular bundle nodules. (A and B) Nodules cross sections of *DR5::GUS* stable transgenic plants inoculated with WT *S. meliloti* 1021 10 dpi (A) or *S. meliloti* 1021 exoY mutant 60 dpi (B). (C) Lateral root sections of *DR5::GUS* stable transgenic plants. Sections are 50 µm thick. Black arrows highlight vascular bundles. Scale bars are 100 µm.

**Supplemental Fig. S1.** kce (*lin*-4) rhizobial infection phenotype. (A and B) nodules with associated infection threads in WT (A) and infection foci in *lin*-4 (B) after inoculation with *S. meliloti* 1021 carrying lacZ, visualized using X-gal staining. Inset in B shows close-up of infection pockets. The black arrowheads in A and B highlight infection events. Scale bars are 0.2 mm.

**Supplemental Fig. S2.** Early cell divisions in WT (A) and *lin*-4 (B) longitudinal sections after *S. meliloti* 1021 inoculation. Scale bars 100 µm.

**Supplemental Fig. S3.** R108 (WT) lateral root longitudinal section stained with toluidine blue. Scale bar is 100 µm.
Fig. 1. kce (lin-4) nodulation phenotype. (A and B) Nodules of WT (A) and lin-4 (B) 80 dpi with S. meliloti 1021. The white arrows in (A) and (B) highlight nodules. (C) Mean (± SD) number of infection threads and infection foci of WT and lin-4 6 and 14 dpi with lacZ-tagged S. meliloti 1021. (D) Mean (± SD) number of nodules of WT and lin-4 14, 30, 60, 90 dpi with S. meliloti 1021. (n=8). Scale Bars in (A) and (B) are 5 mm.
Fig. 2. lin-4 nodule vascular bundles are centrally localized. (A to D) Longitudinal (A and B) and cross (C and D) sections of WT (A and C) and lin-4 (B and D) nodules. Black arrows highlight nodule meristems and black arrowheads highlight vascular bundles. (E and F) lateral root cross sections of WT (E) and lin-4 (F). (A and B) are stained with toluidine blue, (C to F) with phloroglucinol. Scale bars are 100 μm.
Fig. 3. lin-4 nodules do not express lateral root marker genes. (A) Root-tip marker gene expression in WT and in size-matched lin-4 nodules and lateral roots measured by quantitative RT-PCR normalized using EF1α. Marker genes are ferridoxin I (Medtr2g006290) and a plant integral membrane protein (Medtr7g011090). (B) NIN gene expression in WT and lin-4 nodules and lateral roots measured by quantitative RT-PCR normalized using MtEF-1α. The results represent three biological replicates (±SD). A comparison of gene expression was made between lin nodules and the other tissues (Welch’s t-test, * P<0.05). LR: lateral roots.
Fig. 4. Rhizobial infections that abort in the root hair curl lead to abnormal nodule organogenesis featuring a central vascular bundle. (A) A vpy-2 nodule. The section is stained with toluidine blue. (B) An S. meliloti 1021 exoY mutant-induced nodule on WT (ecotype R108). (C and D) lin-1 forms two types of nodules: (C) nodules with branched, peripheral vascular bundles; and (D) nodules with central vascular bundles. Note the thickened infection thread in the nodule outer cortex (white arrow, panel C) visualised by X-gal staining. Nodules were harvested at 30 dpi. Longitudinal nodule sections of 10 μM thickness are shown. Scale bars are 200 μm.
Fig. 5. Cytokinin signalling is not maintained in lin-4 nodule organogenesis. (A-D) WT (A and C) and lin-4 (B and D) transformed with pMCRE1::GUS using the hairy root transformation system. (A and B) 6 dpi with S. meliloti 1021 carrying lacZ. (C) WT nodule 13 dpi with S. meliloti 1021 carrying lacZ. (D) lin-4 nodule 45 dpi with S. meliloti carrying lacZ. X-Gluc was used to stain for GUS activity in (A-D), seen as blue in (A-C). (E and F) MICRE1 (E) and MIRR4 (F) gene expression in WT and lin-4 spot inoculated roots (0, 2, 4, 6, 14 dpi) and excised size-matched nodules (see materials and methods) measured by quantitative RT-PCR normalized using MIEF-1α. Results represent three biological replicates (±SD). Expression in lin vs WT nodules was compared at the different timepoints (Welch’s t-test, * P<0.05, ** P<0.01). Infection foci and infection threads containing rhizobia are detected by LacZ activity using magenta-gal (in A-C, black arrows). Scale bars in A, B, D, 200 μM, in C, 100 μM.
Fig. 6. Auxin response is altered in central vascular bundle nodules. (A and B) Nodules cross sections of DR5::GUS stable transgenic plants inoculated with WT S. meliloti 1021 10 dpi (A) or S. meliloti 1021 exoY mutant 60 dpi (B). (C) Lateral root sections of DR5::GUS stable transgenic plants. Sections are 50 μm thick. Black arrows highlight vascular bundles. Scale bars are 100 μm.