CALCIUM, KINASES AND NODULATION SIGNALLING IN LEGUMES

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Several genes have recently been identified using legume mutants that are defective for nodulation signalling. The proteins they encode include novel types of receptor-like kinase that are predicted to recognize bacterial nodulation (Nod) factors, a leucine-rich-repeat receptor kinase, a putative ion channel and a predicted Ca^{2+}/calmodulin-dependent protein kinase. The identification of these gene products provides new insights into the legume signalling responses to rhizobial signals.

BACTEROID
A terminally differentiated form of rhizobial bacteria that reside inside the nodule and fix nitrogen.

SYMBIOSOME
A bacteroid that is surrounded by a specialized plant membrane, and that is the site of nitrogen fixation and nutrient exchange.

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It is estimated that about one third of the food that is required to sustain the present world population depends on industrally produced nitrogen fertilizer[1]. This is needed because the growth of most plants is limited by the availability of nitrogen. Many legumes can bypass this limitation by entering into a symbiosis with nitrogen-fixing bacteria that can reduce nitrogen to ammonia. Nitrogen fixation in free-living bacteria is limited by the availability of carbon and by the inhibition by oxygen of the enzyme that is responsible for nitrogen fixation. Legumes overcome these constraints by creating specialized plant organs (nodules) within which bacteria are provided with a carefully regulated oxygen and carbon supply, which allows the bacteria to reduce nitrogen efficiently.

To initiate the symbiosis, *Rhizobium* bacteria usually attach to the root-hair cells of legumes and induce root-hair deformation. In a few cases the root hairs deform in such a way as to entrap bacteria within a curl. Infection is initiated from these curled root hairs, with infection threads growing as tunnel-like invaginations of the host cell from the centre of the curl (FIG. 1a,b). The infection thread is reminiscent of a growing cylinder of plant cell wall in which rhizobia replicate to remain at the growing tip[2]. In parallel with this infection process, cell division is initiated in the cortical cells of the root, and this leads to the formation of a nodule. The infection thread is invasive and traverses several cells in the root cortex to reach the newly dividing cells (FIG. 1a,d). When the infection threads enter these cells, the bacteria are budded off into the plant cytoplasm and enveloped by a plant membrane. The bacteria then enlarge and differentiate into nitrogen-fixing forms that are known as BACTEROIDS. These bacteroids, which are surrounded by the plant membrane (which also undergoes significant changes), are known as SYMBIOSOMES. In several regards, symbiosomes can be thought of as organelles that are similar to mitochondria or chloroplasts; they are surrounded by a specialized plant membrane across which there is metabolite exchange, including uptake of dicarboxylic acids, export of ammonia and cycling of amino acids[3]. However, instead of reducing oxygen or carbon dioxide as occurs in mitochondria or chloroplasts, respectively, these symbiosome 'organelles' reduce nitrogen.

Establishing this symbiosis requires an exchange of molecular signals between the plant and the bacteria. This review focuses on a number of recent advances in our understanding of how the plant perceives a critical bacterial signal (nodulation (Nod) factor) and transduces the signal for the activation of downstream responses, leading to infection and nodule morphogenesis. Genes and proteins from four different legume species are described in this article and to avoid confusion, the initials of the species precede the gene names: *Lj*, for *Lotus japonicus*; *Ps*, for *Pisum sativum* (pea); *Mt*, for *Medicago truncatula* (barrel medic); and *Ms*, for *Medicago sativa* (alfalfa).

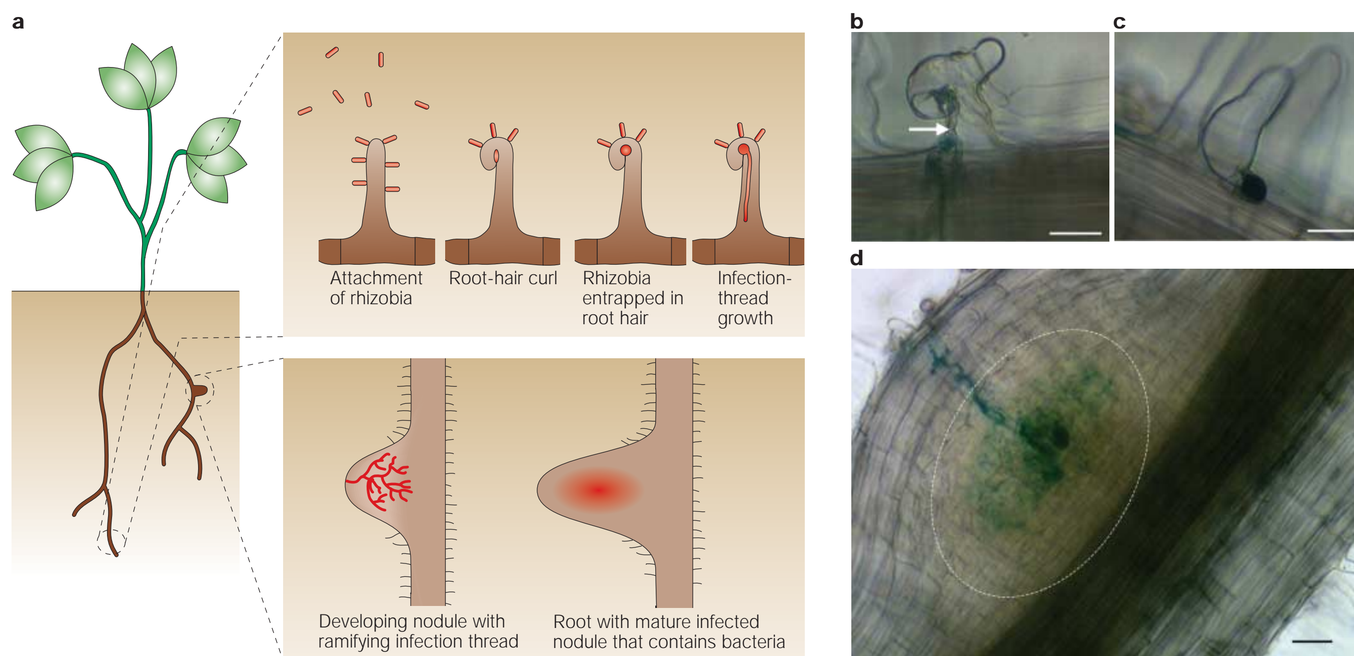


Figure 1 | **Legume infection by *Rhizobium leguminosarum*. a** | The early stages of infection of root hairs by rhizobia and the growth of a nodule on roots. The images in parts **b**, **c** and **d** were obtained by inoculating *Pisum sativum* or *Vicia hirsuta* with *Rhizobium leguminosarum* biovar *viciae* carrying a constitutively expressed *lacZ* gene. The root was histochemically stained for β-galactosidase, which shows the infecting bacteria in blue. **b** | A normal infection thread is shown initiating from a *P. sativum* root hair, which bent back on itself and trapped bacteria to form an infection focus. The infection thread (indicated by an arrow) has grown out of such an infection point. **c** | Some mutants of *R. leguminosarum* biovar *viciae* form infection foci but do not form an infection thread as shown here with a *nodO nodE* double mutant of *R. leguminosarum* biovar *viciae*[44]. **d** | Infection threads are invasive and grow through root cells to the growing nodule primordium (circled). The image is of a very young nodule on *V. hirsuta* that was infected as explained above. (Images courtesy of Simon Walker, John Innes Centre, UK.) The bars in parts **a** and **b** represent 20 μm and in part **c** represents 100 μm.

Nod-factor signalling

The first step in the molecular dialogue between the plant and the bacteria is the detection by rhizobia of FLAVANOIDS and related molecules that are secreted from the legume roots. The various legume species release different arrays of phenolic signals, and this accounts for at least some of the specificity that is observed in this symbiotic interaction[4]. The flavanoid signals are recognized by rhizobial NodD proteins, which are transcriptional regulators that bind directly to a signalling molecule and, in doing so, are able to activate gene expression[5]. Multimers of NodD bind to the promoter regions and thereby regulate the expression of a range of nodulation-related (*nod*) genes[4,6].

Nod factors (FIG. 2) are essential signalling molecules that are synthesized by the products of some of these *nod* genes. Nod factors usually comprise four or five β1–4-linked *N*-acetyl glucosamine residues with a long acyl chain that is attached to the terminal glucosamine. Many Nod factors from different rhizobial species have been identified and shown to differ with regard to the number of glucosamine residues, the length and saturation of the acyl chain and the nature of modifications on this basic backbone[7,8]. These so-called host-specific modifications include the addition of sulphuryl, methyl, carbamoyl, acetyl, fucosyl, arabinosyl and other groups to different positions on the backbone, as well as differences in the structure of the acyl chain. These variations define much of the species specificity that is observed in this symbiosis[4]. Although these differences are essential, a striking aspect is the overall similarity among all Nod factors: they all consist of essentially the same structure, with a number of subtle, but highly important modifications.

Host plants are able to perceive Nod factors at concentrations as low as 10^{-12} M, which indicates that they must bind to a high-affinity Nod-factor receptor. Isolated Nod factors induce many of the plant responses that are observed during the early stages of the symbiosis except the development of infection threads[9]. Nod factors are able to induce changes in actin filaments near root-hair tips[10] and the formation of pre-infection structures, which implies a role in infection-thread initiation[11]. However, the bacteria themselves are needed for the appropriate development of infection threads. This might be due, in part, to the directional supply of Nod factor from bacteria that reside on the root hair, and cause cytoskeletal changes that are required for appropriate curling[12]. In addition, the lack of infection by bacterial mutants that are defective for the normal exopolysaccharide structure indicates that additional rhizobial polysaccharide signals have an important role in infection[13,14].

Ion fluctuations

When added to legume roots, Nod factors induce two phases of ionic changes that can be observed in root-hair cells. One is a rapid influx of Ca^{2+} (Ca^{2+} flux),

FLAVANOID
A phenolic compound that is produced by the plant and that activates symbiotic responses in free-living rhizobial bacteria.

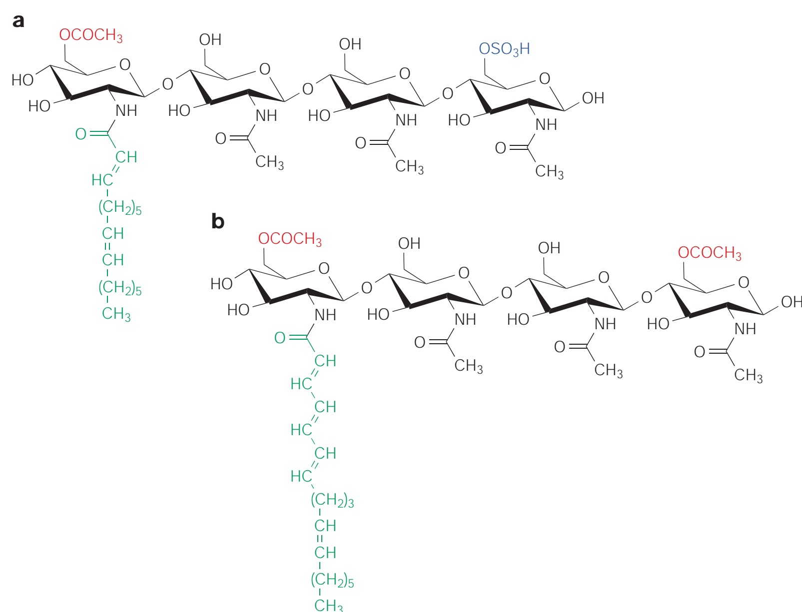


Figure 2 | **Structure of Nod factors.** Nodulation (Nod) factors from *Sinorhizobium meliloti* and *Rhizobium leguminosarum* biovar *viciae* nodulate *Medicago* spp. or *Pisum* and *Vicia* spp., respectively. The backbone of β1–4-linked *N*-acetyl glucosamine residues can carry many different substituents depending on the rhizobial species. **a** | *S. meliloti* Nod factors carry a sulphate group (blue), which requires the bacterial proteins NodH, NodP and NodQ[42]. **b** | At the equivalent location, a *R. leguminosarum* biovar *viciae* Nod factor carries an acetyl group (red), which requires NodX. This modification is specifically required for the nodulation of some types of *P. sativum*, which are homozygous for the *Ps SYM2A* locus[47]. The NodF- and NodE-dependent acyl chains (green) can vary in their length and degree of saturation. Although the acetyl groups that are attached to the same residue by NodL are seen in Nod factors from both strains, they are absent from Nod factors of other rhizobial species. Other substitutions, which are not shown here, can be present in Nod factors from other rhizobia[4].

which is immediately followed by membrane depolarization. Some minutes later, oscillations in the cytosolic Ca^{2+} concentration (this phenomenon is known as Ca^{2+} spiking) are induced.

Calcium flux. Using ion-specific micro-electrodes, Felle *et al.*[15,16] observed a rapid Nod-factor-induced Ca^{2+} influx followed by the efflux of Cl^-, then K^+ and an alkalinization of the cytoplasm[17]. These ion movements occurred within 1 minute of adding Nod factor and explained earlier observations of membrane depolarization and pH changes[18–20]. Membrane depolarization was induced over a range of Nod-factor concentrations (10^{-10}–10^{-7} M) with half-maximal induction at 10^{-9} M and no response at 10^{-11} M (REFS 15,16,21). These Nod-factor concentrations might not reflect a true K_D for receptor binding, as Nod factor accumulates in the plant cell wall and this might enhance the actual concentration at the plasma membrane[22]. The Ca^{2+} influx might trigger the activation of an anion channel that allows Cl^- efflux and K^+ might serve as a charge balance, which eventually stops the depolarization and initiates repolarization[15,18].

Rapid increases in cytosolic Ca^{2+} concentrations have also been observed using Ca^{2+}-sensitive dyes[23–27], and this approach has provided much information on the relative positions within the cell of these changes in Ca^{2+} concentrations. Growing root hairs have increased Ca^{2+} concentrations at the tip and this establishes a gradient of Ca^{2+} down the root hair. Adding Nod factor accentuates this gradient[23] and induces a wave of Ca^{2+} that migrates down the shaft of the root-hair cell towards the nucleus[26]. Isolated regions of high Ca^{2+} concentrations are observed in the root hair during this wave[23], and these might reflect localized Ca^{2+} influx from the exterior of the cell or the local release of Ca^{2+} from internal stores. Nod-factor-induced Ca^{2+} flux has been observed in a diversity of legumes (*P. sativum*, *M. sativum*, *M. truncatula*, *Phaseolus vulgaris*). This indicates that this response is commonly found[23–26], although the induction is variable and might depend on the developmental status of the root-hair cell[26,28]. Together, the data can be incorporated into a model in which Nod factors activate a Ca^{2+} flux at the tip of root-hair cells, with at least some of this Ca^{2+} originating from the external medium.

Recent advances indicate an essential role for reactive oxygen species (ROS) in the generation of the Ca^{2+} gradient in growing root-hair cells[29]. An important question that remains to be resolved is whether the Nod-factor-induced Ca^{2+} flux uses Ca^{2+} channels that are associated with the developmental gradient and could therefore potentially alter ROS production. Recent studies indicate that Nod factors induce a rapid decline in H_2O_2 production[30], followed much later by the induction of H_2O_2 levels[31,32]. These two responses probably have different functions and the rapid decline in H_2O_2 might relate to the modulation of root-hair growth.

Calcium spiking. Oscillations in cytosolic Ca^{2+} (Ca^{2+} spiking) have been observed in legume root-hair cells following the addition of Nod factor[23–25,33,34] (FIG. 3). Nod-factor-induced Ca^{2+} spiking occurs with a lag of approximately 10 minutes following the application of either Nod factor or rhizobia[35]. The Ca^{2+} spikes are predominantly restricted to the region of the cytosol that is associated with the nucleus[24,25], although there is some proliferation of the signal from the nuclear region up to the tip of the root-hair cell[26]. Individual Ca^{2+} spikes have a very rapid initial Ca^{2+} increase, followed by a more gradual decline. This can be interpreted to indicate the opening of a Ca^{2+} channel on internal stores, which allows a flow of Ca^{2+} down its concentration gradient into the cytosol, followed by the closure of the channel and a slower, active re-uptake of Ca^{2+} into the internal store[24]. Consistent with this model are pharmacological studies that indicate a role for Ca^{2+} channels and pumps in the spiking response[36].

It seems likely that the lag period of approximately 10 minutes between Ca^{2+} influx and the initiation of Ca^{2+} spiking could reflect changes in Ca^{2+} homeostasis that occur before the induction of Ca^{2+} spiking[26]. However, it is clear that Ca^{2+} spiking and the Ca^{2+} flux can be uncoupled under different experimental conditions. Some modified Nod factors (that either lack or carry an altered acyl group) can activate Ca^{2+} spiking without activating a flux[25,26]. Furthermore, a subset of

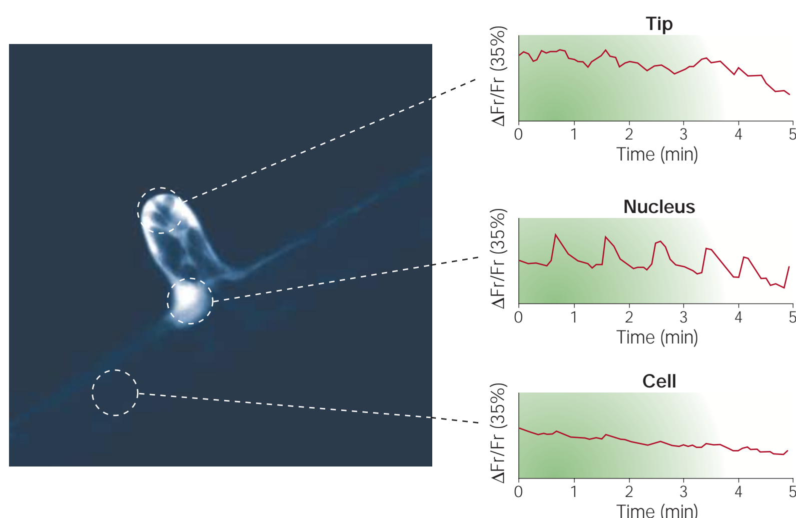


Figure 3 | **Calcium spiking in a root-hair cell.** The microscopic image shows fluorescence from a *Pisum sativum* root-hair cell that was injected with the Ca^{2+}-sensitive fluorescent dye Oregon green. The fluorescence intensity was measured from three different regions of the cell as indicated. The traces shown are recordings of 'raw' fluorescence intensity corrected for background (x-axis) and reflect the changes in intracellular Ca^{2+} as detected by Oregon green over time (y-axis). The traces were recorded about 30 minutes after the addition of nodulation (Nod) factor. Ca^{2+} spiking is most clearly seen around the nuclear region. The Ca^{2+} spikes are detected as a characteristic rapid increase in fluorescence followed by a slower decay of fluorescence. (Image courtesy of Simon Walker, John Innes Centre, UK.)

mutants of *M. truncatula* can activate the flux without inducing spiking[26] (see below). These observations suggest that Ca^{2+} spiking and the Ca^{2+} flux are independent to some extent and might be involved in activating different (but possibly overlapping) responses.

Assigning functions to Nod-factor-induced cellular responses is still a significant challenge. In animal systems, Ca^{2+} spiking has been shown to regulate gene expression, with much information encoded in the amplitude of, and period between, spikes[37,38]. In addition, work in plants has shown that Ca^{2+} spiking can directly regulate stomatal closure, and the nature of the response is defined by the period of spiking[39]. This indicates that Ca^{2+} spiking has the capacity to transduce information from ligand perception to downstream responses and therefore might have a similar role during Nod-factor signalling. The Ca^{2+} flux might also have a direct signalling function. Alternatively, it might coordinate cytoskeletal changes that are required for root-hair deformation or it could have a more indirect role.

When considering the possible function of the two different Ca^{2+} responses, it is important to note that the Ca^{2+} flux requires concentrations of Nod factor that are considerably higher than those required for the induction of root-hair deformation and gene expression[26]. By contrast, the minimal concentration of Nod factor that is required to induce Ca^{2+} spiking is similar to that required for root-hair deformation and gene induction[40]. This suggests that the flux is not essential for these downstream events but that Ca^{2+} spiking might be required. Interestingly, application of a Ca^{2+} chelator, EGTA, to the external medium blocked both membrane depolarization and expression of nodule-specific genes, and this indicates an essential role for external Ca^{2+} in several Nod-factor responses[16,41]. However, the effect of EGTA on the induction of Ca^{2+} spiking has not been measured and so these data cannot be used to discriminate between the Ca^{2+} flux and Ca^{2+} spiking.

Nod-factor perception

Rhizobial mutants that generate altered Nod factors have provided much information on the relative importance of the diverse moieties on the Nod-factor molecule in relation to its perception by plants. *Sinorhizobium meliloti* strains that are mutated in the *O*-sulphur transferase gene, *nodH*, generate a Nod factor that lacks the sulphate group (FIG. 2). These mutants are unable to activate any of the early responses in the host plants *M. truncatula* or *M. sativa*, which indicates that this modification is required in host plant perception[42]. By contrast, *S. meliloti* strains that contain mutations in the *nodL* and *nodF* genes generate a sulphated Nod factor that lacks the acetyl attachment on the non-reducing terminal sugar and has a $C_{18:1}$ *N*-acyl attachment, rather than the standard $C_{16:2}$ attachment. This double mutant can activate all the early responses and can form infection foci in root hairs, but the infections abort at this very early stage[43].

An analogous phenotype has been observed with *R. leguminosarum*, in which *nodE* and *nodO* single mutants produce normal infections, whereas the *nodO nodE* double mutant induces many infection foci on *P. sativum* and *Vicia hirsuta* (vetch), but does not often produce infection threads[44] (FIG. 1c). The *R. leguminosarum nodE* mutant produces only Nod factors that carry a $C_{18:1}$ *N*-acyl group, rather than the mixture of Nod factors with $C_{18:1}$ or $C_{18:4}$ groups that are produced by the wild-type strain[45]. The *nodO* gene encodes an exported protein that forms cation-selective pores in membranes[46]. In addition, the *Ps SYM2A* allele has been shown to be crucial for perceiving the presence of an acetyl group on the Nod factor that is made by some strains of *R. leguminosarum*[47] (FIG. 2). This perception is important for infection-thread growth[48] but is not required for early responses[25]. Taken together, these studies indicate that two specificities for Nod-factor perception exist. Initially, a less stringent perception is required for early responses. A second, more stringent, perception occurs during infection, and this requires the appropriate *N*-acyl attachment coupled with further Nod-factor modifications (such as sulphate or acetate attachments), or action of Nod proteins.

These studies with different Nod factors have led to the hypothesis that two receptors exist for Nod factor, a low-stringency 'early' receptor and a high-stringency 'entry' receptor[43,44,49]. However, others have proposed an alternative model to explain the data in which the differential activation of a single receptor can lead to alternative outcomes with regard to downstream events. In support of this hypothesis are experiments in which the different *S. meliloti* Nod factors were tested for their ability to induce Ca^{2+} spiking in *M. truncatula*[40]. This study showed that unsulphated Nod factor had a 30,000-fold-reduced capacity to induce Ca^{2+} spiking,

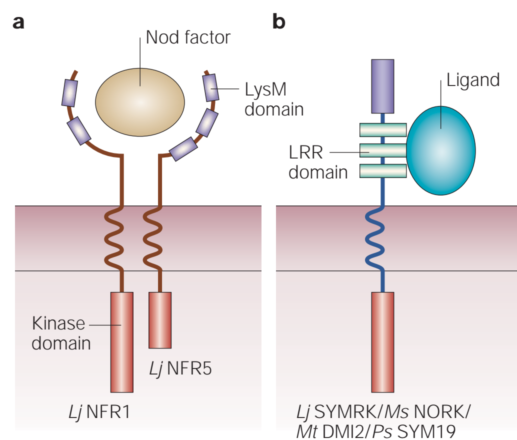


Figure 4 | **Predicted kinases that are required for Nod-factor signalling. a** | *Lotus japonicus Lj NFR1* and *Lj NFR5*, which both encode extracellular LysM motifs, are thought to function in nodulation (Nod)-factor binding. The effects of mutations in these genes support a role in Nod-factor recognition. According to a simple model, the Nod-factor receptor is a heterodimer that consists of the two receptor-like kinases, *Lj* NFR1 and *Lj* NFR5. The kinase domains (red) might be involved in signal transduction; whereas *Lj* NFR1 is predicted to have an intact kinase domain, *Lj* NFR5 lacks a kinase-activation loop in the kinase domain. Several closely linked genes that are strongly related to *Lj* NFR1 have been identified in both *L. japonicus* and *Medicago truncatula*, and so it is possible that types of complex other than those shown here could occur. Figure modified from REF. 52. **b** | Plants use leucine-rich-repeat (LRR) receptor-like kinases (LRR-RLKs) in various signal-transduction pathways. The LRR-RLKs are related to Toll receptors in *Drosophila melanogaster* and Toll-like receptors in animal cells. The LRR domain is often involved in protein–protein interactions and the kinase domain is involved in protein phosphorylation. The product of *M. truncatula Mt DMI2* and its orthologues in *M. sativa* (*Ms NORK*), *L. japonicus* (*Lj SYMRK)* and *Pisum sativum (Ps SYM19)* belong to this class of proteins. It has been proposed that this protein might interact with an (unidentified) extracellular protein and mediates the phosphorylation of some component that has yet to be identified. Figure modified from REF. 84.

whereas a Nod factor that is equivalent to a *nodF nodL* double mutant showed a 100-fold reduction in activity compared with wild-type Nod factor. The simplest explanation for these data is that the different Nod factors cause different levels of inducing activity on a single receptor or receptor complex that activates Ca^{2+} spiking. This differential activation of the pathway might be sufficient to explain the different stringencies that are observed between early and late responses.

In search of the Nod-factor receptor

A genetic strategy to identify the Nod-factor receptor has focused on legume mutant phenotypes that lack all Nod-factor responses or show altered Nod-factor perception[25,50–52]. Two genes in *L. japonicus*, *Lj NFR1* and *Lj NFR5*, that were predicted to function in Nod-factor perception, both encode receptor-like kinases with LysM DOMAINS in the predicted extracellular domain (FIG. 4a)[51,52]. The *P. sativum*[51] and suspected *M. truncatula* orthologues of *Lj NFR5* are *Ps SYM10* and *Mt NFP*, respectively. In *M. truncatula*, two additional receptor-like kinase genes (*Mt LYK3* and *Mt LYK4*) that encode LysM domains have been identified, and they are thought to be orthologous to *Ps SYM2A*[53]; both of these show strong similarity to *Lj NFR1*. LysM domains are present in the *Escherichia coli* MltD protein that binds PEPTIDOGLYCANS. The LysM domains are the binding sites for peptidoglycan and binding seems to be to the *N*-acetyl-glucosamine-*N*-acetylmureine backbone[54]. In addition, LysM domains are present in two proteins that are known to bind CHITIN[55], which is chemically identical to the Nod-factor *N*-acetylglucosamine backbone. Furthermore, chitin oligomers can induce Ca^{2+} spiking in legumes[25,40]. The analogy to Nod-factor binding is striking and the LysM-receptor-like kinases seem excellent candidates for Nod-factor receptors. However, the binding of Nod factor to these LysM-receptor-like kinases has yet to be shown — and would ultimately be required to indicate their role in Nod-factor perception.

According to a simple biological model, the Nod-factor receptor in *L. japonicus* might be a heterodimer that comprises the two LysM-receptor-like kinases *Lj* NFR1 and *Lj* NFR*5* (REF. 52,56). Mutations in either gene cause analogous phenotypes (defects in all early responses), and this indicates that both are equally essential for early Nod-factor perception. Furthermore, *Lj* NFR5 lacks a kinase-activation loop, and so it probably forms a multimer with another protein, possibly *Lj NFR1*, which supplies the kinase-activation domain[51].

Within the *Ps SYM2A* SYNTENIC region of *M. truncatula* there are seven receptor-like-kinase (RLK) genes that encode proteins with LysM domains and all these show close homology to *Lj NFR1*. The RNA-interference-mediated suppression of two of these genes, *Mt LYK3* and *Mt LYK4*, caused an infection defect[53]. It is possible that the Nod-factor receptor in *P. sativum* and *M. truncatula* consists of several heterodimers that always contain *Ps* SYM10 or *Mt* NFP (the *Lj* NFR5 orthologues), but the second component could be equivalent to *Mt* LYK3, *Mt* LYK4 or perhaps other RLKs that are encoded by the closely related gene family. This hypothesis requires that many heterodimers exist for the Nod-factor receptor and these might show slight differences in Nod-factor perception. This would satisfactorily explain the complexity of Nod-factor perception and the differences that are observed in the stringency requirements at early and later stages of the symbiosis.

Activation of calcium spiking

New genes that are linked to calcium responses. In addition to candidates for the Nod-factor receptor, mutant screens have identified several other loci, which might function in Nod-factor signal transduction (FIG. 5). *M. truncatula* plants that are mutated in *Mt DMI1*, *Mt DMI2* or *Mt DMI3* do not show root-hair curling or infection, but do induce a swelling at the tip of root-hair cells in response to Nod factor. However, a recent study indicates that the root-hair-swelling phenotype in *Mt dmi2* is caused by an enhanced sensitivity

LysM DOMAIN
A domain that is proposed to be involved in binding β1–4-linked *N*-acetylglucosamine residues.

PEPTIDOGLYCAN
A proteinacious polysaccharide that is found in bacterial cell walls.

CHITIN
A polysaccharide that is made up of β1–4-linked *N*-acetyl glucosamine residues and is found in arthropod exoskeleton and some plants and fungi.

SYNTENIC
A region of the genome that is conserved between different species.

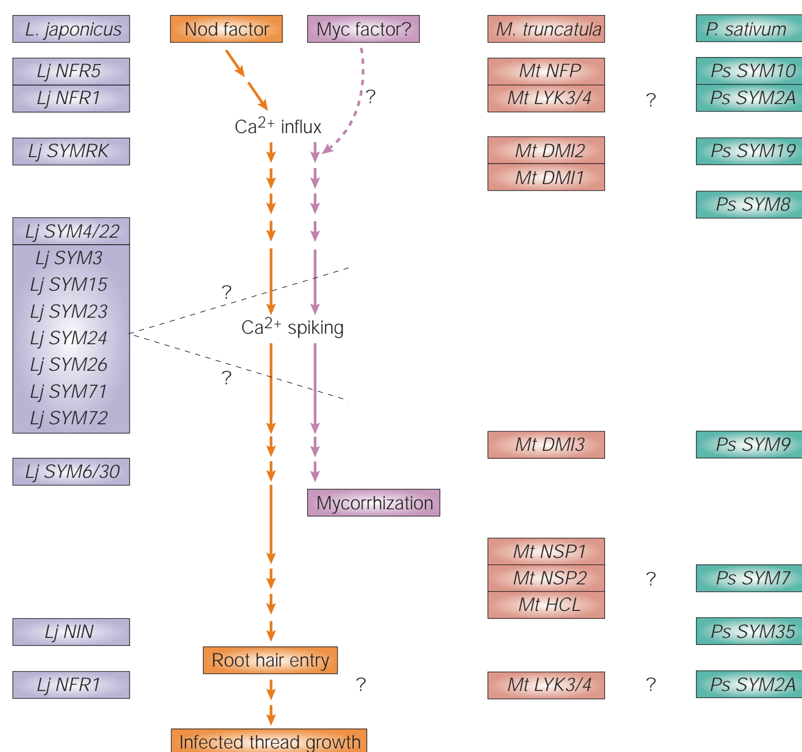


Figure 5 | **Orthologous legume genes and their predicted roles in early signalling events.** Genes from *Lotus japonicus* (*Lj*; purple), *Medicago truncatula* (*Mt*; red) and *Pisum sativum* (*Ps*; green) that are involved in nodulation (Nod)-factor signalling are shown. Genes that are aligned horizontally are either true orthologues (based on sequence comparisons) or are postulated to be orthologous (marked '?') on the basis of relative map locations and similarities in phenotype. Genes are located relative to their predicted position in the signalling pathway, which is indicated by arrows. So, mutations in *Lj NFR5*, *Mt NFP* and *Ps SYM10* are orthologous and the proteins they encode block both the Nod-factor-induced Ca^{2+} influx and Ca^{2+} spiking. Several *L. japonicus SYM* genes cannot yet be placed upstream or downstream of Nod-factor-induced Ca^{2+} spiking. A number of genes are required for both nodulation and infection by mycorrhizal fungi. The suspected orthologous *Lj NFR1*, *Mt LYK3*, *Mt LYK4* and *Ps SYM2A* loci are represented at two locations because mutation of *Lj NFR1* blocks early signalling, whereas the defects in *Ps sym2A*, *Mt lyk3* and *Mt lyk4* occur at the infection stage. This figure is based on proposals that were presented previously[25,100]. However, a recent alternative hypothesis is that the *Mt DMI1*, *Mt DMI2* and *Mt DMI3* genes (and their orthologues) are on one branch of a signalling pathway that is required for induction of early nodulation genes, but are not on the branch of a pathway that is required for root-hair deformation[57].

of this mutant to touch[57]. This seems to occur independently of the symbiotic phenotype, and the authors of this study report that if Nod factor is applied to the plant without disturbance to the root hairs, then normal deformation is observed. The fact that the root hairs respond in some way to Nod factor in the *Mt dmi1*, *Mt dmi2* and *Mt dmi3* mutants indicates that Nod factor is perceived, but it seems that most of the signal transduction is blocked in these mutants[58]. These mutants are also defective for MYCORRHIZAL symbioses, which indicates a shared signalling pathway between these two symbiotic interactions[58] (BOX 1). Mutations in three loci in *P. sativum*, *Ps SYM8*, *Ps SYM19* (a *Mt DMI2* orthologue) and *Ps SYM9* (a *Mt DMI3* orthologue), cause similar phenotypes[59–62].

Analysis of the Ca^{2+} responses in the *dmi* and *sym* mutants of *M. truncatula*, *P. sativum* and *L. japonicus* has been highly informative with regard to the nature and potential function of the different Nod-factor-induced Ca^{2+} effects (FIG. 5). The *Mt nfp*, *Ps sym10*, *Lj nfr1* and *Lj nfr5* mutants show no Ca^{2+} responses[25,50,52]. By contrast, the *Mt dmi* mutants show a Ca^{2+} flux when treated with Nod factor[26]. However, *Mt dmi1* and *Mt dmi2* mutants show a reduced Ca^{2+}-flux response, and from this study it is apparent that the Ca^{2+} flux is a biphasic response that can be separated into an initial rapid Ca^{2+} increase and a secondary, plateau-like increase that is maintained for approximately five minutes. *Mt dmi1* and *Mt dmi2* mutants show only the first phase of this response, whereas the *Mt dmi3* mutant shows the full Ca^{2+} flux[26]. This mutant analysis shows a correlation between the initial phase of the Ca^{2+} flux and root-hair swelling. This might indicate a role for the initial phase of the flux in regulating root-hair growth and could be consistent with a role for Ca^{2+} in coordinating growth of the root-hair tip.

In addition, mutation of *Mt DMI1*, *Mt DMI2*, *Ps SYM8* and *Ps SYM19* blocks Ca^{2+} spiking, whereas *Mt dmi3* and *Ps sym9* mutants show normal Ca^{2+} spiking when treated with Nod factor[25,33]. This indicates that

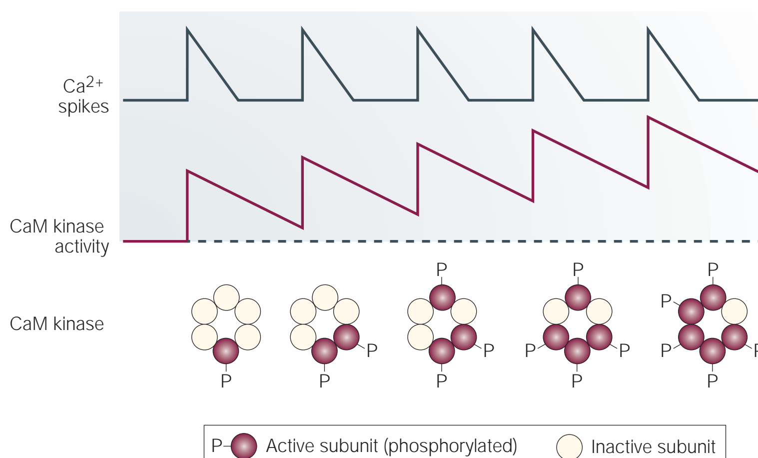
MYCORRHIZAE
Fungal species that form symbiotic interactions with plants and that assist in the uptake of nutrients from the soil.

Box 1 | Shared components between Nod-factor signalling and the mycorrhizal symbiosis

Most plants can establish symbioses with mycorrhizal fungi. Such symbioses are ancient, and the fungi can translocate nutrients, such as phosphate and organic nitrogen, into the root[81,82]. By contrast, symbiotic nitrogen fixation evolved more recently and is restricted to relatively few plant genera[83,84]. However, some legume mutants that are defective for nodulation are also defective for the mycorrhizal symbiosis (FIG. 5), which implies that there are common signalling steps (reviewed in REF. 84). Several genes (such as *Medicago truncatula* (*Mt*) ENOD11) that are induced during nodulation are also activated during mycorrhizal infection[62,85–87] and there are parallels with some aspects of both types of infection[84].

Three loci (*Mt DMI1*, *Mt DMI2* and *Mt DMI3* in *M. truncatula* and *Ps SYM8*, *Ps SYM19* and *Ps SYM9* in *Pisum sativum*) are required for the early stages of infection by mycorrhizal fungi[58,59], and several nodulation mutants of *Lotus japonicus* are also defective for the mycorrhizal symbiosis (FIG. 5)[88–91]. Therefore, there might be at least five to seven loci that are involved in signalling in the symbioses in both plant species (FIG. 5). Some of these mutants from both plant species (*Mt dmi1*, *Mt dmi2/Ps sym19* and *Ps sym8*) block Ca^{2+} spiking, whereas others (*Mt dmi3/Ps sym9*) do not[25,33]. This has been taken to suggest that Ca^{2+} spiking is a signalling step during the mycorrhizal symbiosis. However, this has yet to be established experimentally. The fact that early signalling genes are conserved between these two symbiotic interactions indicates that the rhizobial symbiosis evolved using a pre-existing signalling pathway that is involved in the mycorrhizal symbiosis[92,93]. Presumably, there are mycorrhizal-specific components of the signalling pathway, and these could reflect a receptor for the mycorrhizal signal(s) and genes downstream of *Mt DMI3* that specifically activate mycorrhizal responses.

Box 2 | **Proposed mechanism of action of calcium/calmodulin-dependent protein kinases**



Calmodulin (CaM)-dependent protein kinase II (CaMKII) has been shown to have the capacity for frequency-dependent activation by Ca^{2+} oscillations[94]. Indeed, it was shown that the relative kinase activity is directly related to the frequency of Ca^{2+} spiking. CaMKII activation involves CaM binding that activates autophosphorylation, which releases autoinhibition, thereby allowing kinase activity (see figure; filled purple circle)[95]. An important mechanism in CaMKII activity is the fact that CaM binding is enhanced 1,000 fold by autophosphorylation, which is activated by CaM binding[96]. In essence, CaMKII entraps CaM as a result of autophosphorylation. Therefore, CaM can remain bound even as Ca^{2+} concentrations fall. In addition, CaMKII exists as a multimeric complex, such that the regulation state of different members of this complex can differ (see bottom panel). The autophosphorylation that follows CaM binding occurs by a monomer that phosphorylates its neighbour in the complex, and this *trans*-phosphorylation event requires that both monomers are bound to CaM. If one considers low-frequency Ca^{2+} spiking, most of the CaM will dissociate from the multimeric protein before the next oscillation, and therefore kinase activity will drop to baseline levels between each spike. In high-frequency oscillations (see top panel in figure), CaM will not be fully dissociated before the next spike and the kinase activity will gradually increase between spikes (see middle panel). Maximal kinase activity is achieved through incremental step-like increases between each spike. The timing to maximal activity or the ultimate level of catalytic activity is directly linked to the frequency of oscillations[95].

Like CaMKII, CaM binding is essential to activate the kinase domain of Ca^{2+}/CaM-dependent protein kinases (CCaMKs)[97]. In addition, CCaMKs show enhanced CaM binding following autophosphorylation[98,99]. However, it is Ca^{2+} binding within the visinin-like domain of CCaMKs rather than direct CaM binding, as occurs in CaMKII, that activates autophosphorylation[97]. The figure is modified from REF. 95.

Mt DMI1, *Ps* SYM8 and *Mt* DMI2/*Ps* SYM19 lie upstream of Ca^{2+} spiking (FIG. 5) and provides the strongest evidence so far that Ca^{2+} spiking is a component of the Nod-factor signal-transduction pathway. *Mt* DMI3/*Ps* SYM9 must lie downstream of Ca^{2+} spiking and might have a role in perceiving the Ca^{2+} signal (the recent cloning and identification *Mt DMI3* (REFS 63,64) supports this hypothesis; see BOX 2). During these studies it was found that *Mt dmi2* mutants showed broad Ca^{2+} fluctuations in the absence of treatment with Nod factor[26,33]. These observations might well be related to the enhanced touch sensitivity in these mutants[57]. It is likely that the micro-injection of dyes into *Mt dmi2* mutants will strongly activate touch responses and these could modify (or block) the Ca^{2+}-spiking response independent of any block in Nod-factor signalling. Therefore, we might need to be cautious when interpreting the Ca^{2+} responses in legumes that carry mutations in *Mt DMI2* (or its orthologues).

New proteins in Nod-factor signalling. On the basis of these mutant phenotypes, we can conclude that *Mt* DMI1, *Ps* SYM8 and *Mt* DMI2/*Ps* SYM19 have a role in the activation of Ca^{2+} spiking and maintenance of the Ca^{2+} flux and perhaps other, as yet unidentified, downstream events. *Mt DMI2*, *Ps SYM19*, *Lj SYMRK* and *Ms NORK* all seem to be orthologues that encode a receptor-like kinase with leucine-rich-repeat domains in the predicted extracellular region[65,66] (FIG. 4b). Receptor-like kinases with leucine-rich-repeat domains have been identified in a number of plant signalling pathways, including perception of pathogen signals, BRASSINOSTEROID signalling and signalling from the CLAVATA RECEPTOR complex[67]. Leucine-rich-repeat domains seem to have a role in protein–protein or protein–ligand interactions. It is possible that the *Mt* DMI2/*Ps* SYM19/*Lj* SYMRK/*Ms* NORK receptor-like kinase perceives a secondary signal that is generated by Nod-factor recognition. Alternatively, this receptor-like kinase might be part of a large complex at the membrane that includes the Nod-factor receptor and *Mt* DMI1.

Mt DMI1 is predicted to encode a transmembrane protein that is conserved across plants, and it is present in only two of the eubacteria that have been sequenced — *Mesorhizobium loti* and *Streptomyces coelicolor*[68]. From phylogenetic analysis it seems that *Mt DMI1* is a plant-specific innovation, which was acquired by *M. loti* and *S. coelicolor* through horizontal gene transfer. *Mt* DMI1 has proline-rich and leucine-zipper domains, which mediate interactions with other proteins, and has weak but broad similarity to the NAD-binding TrkA domain of bacterial K^{+} channels. It has

BRASSINOSTEROIDS
A group of naturally occurring plant polyhydroxysteroids that function as plant hormones.

CLAVATA RECEPTOR
A receptor that is involved in meristematic identity.

proved inherently difficult to predict the ions that are translocated by cation channels and transporters purely on the basis of sequence comparisons. Indeed, the weak homologies between *Mt* DMI1 and bacterial K^+ channels are only insightful in defining a possible role in cation translocation.

It is tantalizing to predict that *Mt* DMI1 might be directly involved in the Ca^{2+} influx or the K^+ efflux that is part of the initial membrane depolarization. The *Mt dmi1* mutations cause defects in the Ca^{2+} flux, but it is not known whether K^+ efflux is modified. Clearly, defining these early responses in more detail in the mutants and assessing *Mt* DMI1-specific cation translocations in heterologous systems will shed light on the function of this protein. It is interesting to note that the *R. leguminosarum* cation-selective channel NodO[46] is able to modify Nod-factor perception in *P. sativum* and might achieve this by mimicking the activity of *Mt* DMI1.

On the basis of their mutant phenotypes we know that several genes, including at least *Mt DMI1*, *Mt DMI2* and their orthologues, must be involved in the activation of Ca^{2+} spiking. In mammalian systems, the predominant mechanism for the induction of Ca^{2+} spiking is through phospholipid signalling that is driven by phospholipase C. There is already evidence which indicates that components of this pathway are conserved in Nod-factor signalling, despite the fact that the genes cloned so far have no homologies with genes that are involved in phospholipid signalling. Studies with pharmacological inhibitors indicate that phospholipase C is involved in Nod-factor-induced Ca^{2+} spiking and expression of Nod-factor-induced genes[36,41]. Biochemical evidence indicates that both phospholipase C and phospholipase D are activated by Nod factor[69,70]. Furthermore, mastoparan — a peptide from wasp venom with G-protein-agonist activity — can activate expression of Nod-factor-induced genes. This has been taken as evidence for a role for heterotrimeric G-proteins (which in animal systems are important for the activation of phospholipase C) in Nod-factor induction of gene expression[41]. However, recent work indicates that mastoparan can activate plant mitogen-activated protein kinase (MAPK) signalling independently of heterotrimeric G-proteins[71]. Interestingly, this study indicates a role for a Ca^{2+} flux in MAPK activation by mastoparan. Furthermore, mastoparan activates Ca^{2+} fluxes in a number of plant systems[72,73]. Taken together, these studies indicate a possible role for phospholipase C, which, in combination with phospholipase D, could be involved in the generation of phospholipid signals, which, in turn, might induce Ca^{2+} release. It is important to strengthen these observations on phospholipase C, and ultimately link the induction of Ca^{2+} spiking to the components of early Nod-factor signalling that have recently been cloned.

Perception of calcium spiking

The Ca^{2+} spiking signal must be perceived and transduced to the downstream responses in Nod-factor signalling. The position of *Mt DMI3* in the pathway and the fact that signal transduction seems to be completely blocked in this mutant make it a likely candidate for fulfilling this role. *Mt DMI3* and the orthologous gene *Ps SYM9* encode proteins with strong homology to chimeric Ca^{2+}/calmodulin (CaM)-dependent protein kinases (CCaMK)[63,64]. The proteins are multifunctional, with a kinase domain and a CaM-binding domain that has strong homology to the CaM-binding domain of CaM-dependent protein kinase II (CaMKII) in animals[74]. In addition, CCaMKs also have a Ca^{2+}-binding domain that is homologous to the neuronal Ca^{2+}-binding protein VISININ, with three EF-hand domains within this region[74]. This family of proteins seems to be unique to plants and, invariably, single copies have been found in various plant species[75].

CCaMKs have many similarities to CaMKII, which has the capacity to dissect Ca^{2+} oscillations (BOX 2). However, unlike CaMKII, CCaMKs can be modified by Ca^{2+} in two forms, either through direct binding to the visinin-like domain or as a complex with CaM. Perhaps the essence of CCaMK activity is entrenched in a subtle interaction between these two sites for Ca^{2+} binding. If, as is probably the case, the visinin domain has greater affinity for Ca^{2+} than CaM, then the binding of Ca^{2+} to the visinin domain will lower the relative concentration of Ca^{2+} that is required for CaM binding to CCaMK (BOX 2). This would have the effect of enhancing the entrapment of CaM beyond that which can be achieved through CaM binding alone — as in the case of CaMKII. CCaMKs are probably functional analogues of CaMKII, and have the capacity for frequency-dependent activation by Ca^{2+} oscillations, but perhaps to greater levels of sensitivity or regulation.

Activation of gene expression

Presumably, *Mt* DMI3/*Ps* SYM9 must phosphorylate downstream protein(s) that transduce the signal to activate the cascade of events leading to infection and nodule development. Acting later than *Mt DMI3*, but still important for Nod-factor signalling, are the *Mt NSP1* and *Mt NSP2* loci (FIG. 5). Mutations in these loci cause very similar phenotypes: Nod-factor-induced gene expression is greatly reduced, root-hair deformation is modified and nodule-primordia initiation and infection are blocked[58,76]. Both *Mt nsp1* and *Mt nsp2* mutants show normal Ca^{2+} flux and Ca^{2+} spiking. The *Ps sym7* mutant has a broadly similar phenotype[25] and *Ps SYM7* might be orthologous to *Mt NSP2* on the basis of map locations[58,76,77]. These data indicate that *Mt* NSP1 and *Mt* NSP2 (and their orthologues) probably function downstream of Ca^{2+} spiking, but upstream of gene expression and cortical cell division, and possibly function immediately downstream of *Mt* DMI3/*Ps* SYM9.

It is presumed that one of the last stages of the Nod-factor signalling pathway is the activation of gene expression through the induction of transcription factor(s). *Lj* NIN and its orthologue *Ps* SYM35 (REF. 78) might have a role in Nod-factor signalling and have some molecular hallmarks of transcriptional regulators[79]. *Lj nin* mutations block rhizobial infection and early nodule development, and this suggests an essential

VISININ
A Ca^{2+}-binding protein in animals.

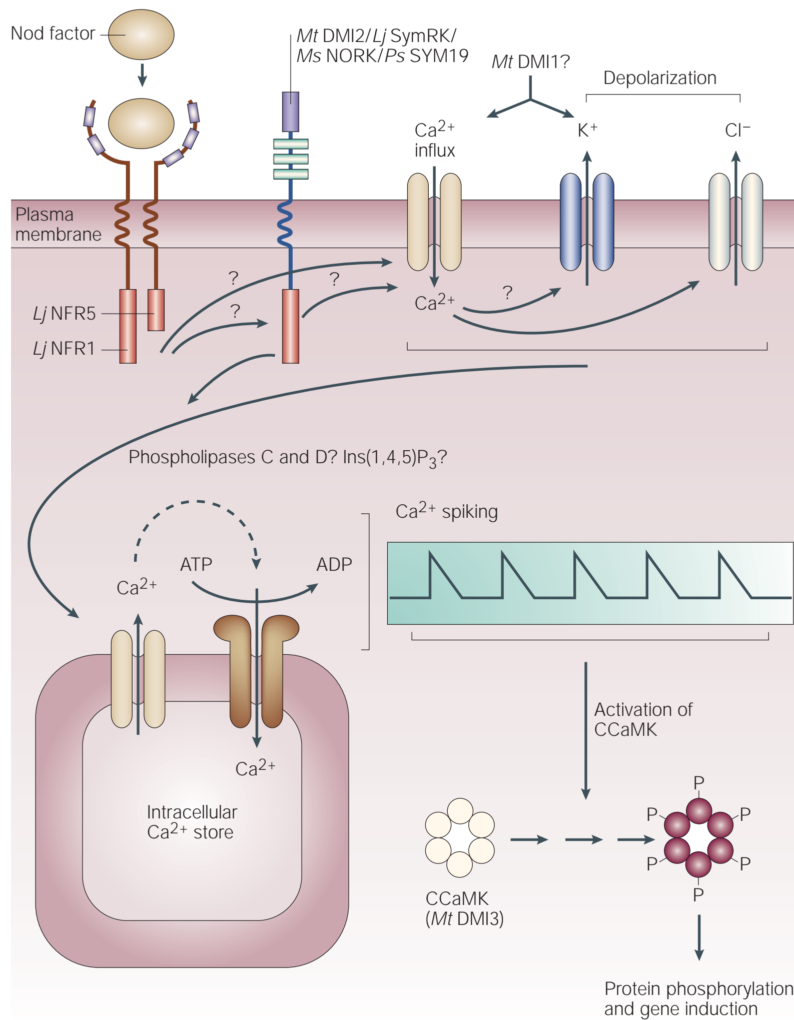


Figure 6 | **The Nod-factor signalling pathway in legumes.** It is thought that nodulation (Nod) factors are recognized by a receptor-like-kinase complex that contains *Lotus japonicus* (*Lj*) NFR1 and *Lj* NFR5 (REF. 51,52), although other components such as *Medicago truncatula* (*Mt*) LYK3 and *Mt* LYK4 might also be involved[53]. The subsequent protein phosphorylation might result in the activation of a Ca^{2+} channel in the plasma membrane. It remains to be determined whether this involves the *Mt DMI2/M. sativa* (*Ms*) *NORK/Lj SYMRK/Pisum sativum* (*Ps*) *SYM19*-encoded protein; a mutation in *Mt DMI2* does not block the Ca^{2+} influx, but the increased intracellular Ca^{2+} concentration is not sustained in the mutant[26]. The influx of Ca^{2+} directly or indirectly results in the opening of cation- and anion-selective channels, which results in a partial depolarization of the root-hair plasma membrane. *Mt* DMI1 has weak similarity with some cation channels[68] and could therefore be a Ca^{2+} or K^+ channel. The influx of Ca^{2+} and membrane depolarization could contribute to the induction of Ca^{2+} spiking, possibly as a result of the activation of phospholipases C and D and the production of inositol 1,4,5-trisphosphate (Ins $(1,4,5)P_3$). This, in turn, could result in the opening of a Ca^{2+} channel in a membrane that contains stores of intracellular Ca^{2+}, coupled with the action of a Ca^{2+} pump leading to the characteristic spikes in intracellular Ca^{2+}. *Mt DMI3* encodes a protein with similarity to Ca^{2+}/calmodulin-dependent protein kinases (CCaMKs)[63] and is a good candidate for integrating the Ca^{2+}-spiking signal into the activation of gene expression.

role for *Lj* NIN during the symbiosis. However, the mutants show excessive root-hair curling in response to rhizobia, which indicates additional roles for this protein during root-hair deformation. *Lj* NIN has the characteristics of a transmembrane protein with a potential nuclear localization signal and a predicted DNA-binding domain, which indicates a role for *Lj* NIN in gene regulation[79]. It has a domain that shows similarity to the Mid regulators of mating type in *Chlamydomonas* sp., and its overall structure has some characteristics that are shared by transmembrane proteins that are proteolytically cleaved to release a transcriptional regulator[79]. Schauser *et al.*[79] proposed a model in which membrane-bound *Lj* NIN is proteolytically cleaved, and the relocation of the DNA-binding domain to the nucleus allows gene regulation in response to rhizobia. However, this model has yet to be substantiated with experimental data. *Mt hcl*, a mutant of *M. truncatula*, has a similar phenotype to *Lj nin*: a block in infection and nodule-primordia initiation and excessive root-hair curling[80]. A detailed analysis of this mutation indicates microtubule cytoskeleton defects during root-hair curling, and the authors conclude that this protein has a root-hair-growth function. Defining the molecular identity of this gene might clarify its relationship with *Lj NIN*. *Lj* NIN might function downstream of the *L. japonicus* orthologues of *Mt* DMI3, and possibly *Mt* NSP1 and *Mt* NSP2, in Nod-factor signal transduction, or might have a role beyond the initial signal-transduction pathway, perhaps analogous to that proposed for *Mt* HCL.

FIGURE 6 shows a diagrammatic representation of the nodulation signal-transduction pathway. Presumably, binding of Nod factor to the receptor complex activates an influx of Ca^{2+} and the movement of other ions, which results in membrane depolarization. It is possible that the *Mt* DMI1 protein could be one such channel. Either as a result of the membrane depolarization, or some other event, it is thought that the *Lj SYMRK/Ms NORK/Mt DMI2/Ps SYM19* gene product will be activated and influence the induction of Ca^{2+} spiking. There could be several gene products that are required for this, on the basis of the number of additional mutations in *L. japonicus* that influence early signalling events (FIG. 5). Once Ca^{2+} spiking has been initiated, the putative CCaMK that is encoded by *Mt DMI3/Ps SYM9* could integrate this signal, which results in the phosphorylation of regulatory proteins that influence gene induction.

Concluding remarks

Recent advances in the field of Nod-factor signal transduction have been highly illuminating. Receptor-like kinases with LysM domains in the extracellular portion probably represent the Nod-factor receptor, and the complexity of this gene family might explain the complexity of Nod-factor perception. Downstream of these receptors are a receptor-like kinase with leucine-rich-repeat domains in the extracellular portion and a protein with weak, but broad homology to bacterial K^+ channels. These proteins must function in the activation of Ca^{2+} spiking, and defining the role of these proteins during signal transduction is a challenge for the future. Acting immediately downstream of Ca^{2+} spiking is a CCaMK that is an excellent candidate for dissecting the Ca^{2+}-spiking signal. There are still a number of genes with a role in Nod-factor signal transduction that remain to be cloned. These genes will provide further insights into this signalling pathway.

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Competing interests statement

The authors declare that they have no competing financial interests.

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