Plant responses to nodulation factors
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The focus of research on signalling in Rhizobium–legume interactions has moved from understanding the structure and synthesis of rhizobially made Nod factors, towards an analysis of how they function in plants. Nod-factor-induced changes in ion fluxes across membranes, followed by establishment of an oscillation of intracellular Ca^{2+} concentration, point to the involvement of a receptor-mediated signal transduction pathway. Progress towards the identification of components in this pathway is being made by identifying Nod-factor binding proteins, isolating plant mutants that are defective in signalling and analysing plant responses to Nod factors.

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Abbreviations
ENOD early nodulation
LCO lipo-chitin oligomer
LNP lectin nucleotide phosphohydrolase
NFBS Nod-factor-binding site

Introduction
Nod factors, which are signalling molecules made by rhizobia, initiate nodule development in legumes. They are composed of lipo-chitin oligomers (LCOs) that usually comprise four or five β, 1–4 linked N-acetyl glucosamine residues, in which the N-acetyl group of the terminal (non-reducing) sugar is replaced by an acyl chain. Many Nod factors, made by a diverse range of rhizobia, have been characterised and it is evident that specificity (i.e. the types of legume hosts nodulated by given rhizobia) is determined, at least in part, by chemical modification of the Nod factors. These modifications include the attachment of sulphate, acetate, carbamoyl groups; addition of other sugars such as arabinose, mannose or fucose (and substituted derivatives of fucose); changes to the acyl chain; and variation of the chitin oligomer length (Figure 1). (The role of rhizobial nod gene products in Nod factor biosynthesis has been reviewed in detail [1,2,3•].)

In the appropriate legume, Nod factors can induce a variety of effects including deformation of root hairs, division of root cortical cells, and nodule morphogenesis [1,4•,5••]. Some of these responses are induced at concentrations as low as 10^{-12}–10^{-13} M Nod factor; this finding alone points to the probable existence of high-affinity receptors. Other components may act to enhance nodulation; thus, for example, a protein secreted by some rhizobia can extend the range of legumes nodulated [6,7] possibly by forming ion channels in the plant membranes [8]. Proteins secreted by other rhizobia are also likely to play a role in nodulation [9], although nothing is known about how such secreted proteins act. There is indirect evidence for the uptake of Nod factors into plant cells [10•,11] raising the possibility that Nod factor recognition could occur within cells as well as at the membrane surface. Analysis of infection by rhizobial mutants that do not make fully substituted Nod factors has suggested that there may be different types of Nod factor receptors, one controlling early responses and another controlling invasion events [12,13]. Root chitinases, some of which are specifically induced during the symbiosis, can degrade Nod factors and may play an important role in the regulation of their activity [14].
Our review primarily addresses recent work on Nod-factor-induced changes to ion movements in and around legume root hairs and evaluates recent biochemical and genetic experiments that may lead to an understanding of the signalling events several of which are summarised in Figure 2. (Note that several reports on effects of Nod factors on cell division in non-legumes have recently been withdrawn [15•].) We have not described work on the identification of the many genes induced by Nod factors or on the role of plant hormones in nodule development [16,17]; recent reviews [4•,5•,18,19•,20•] describe in detail various aspects of the plant responses to Nod factors that are not included here.

**Roles for calcium**

Nod factors induce a rapid and transient depolarisation of the plasma membrane of root hairs [21–23] and an associated transient extracellular and sustained intracellular alkalinisation [24,25••]. A few seconds after Nod factor exposure, there is a transient influx of Ca\(^{2+}\), which is rapidly followed by a Cl\(^{-}\) efflux, and then efflux an of K\(^{+}\) to balance the charge (Figure 2i). This K\(^{+}\)/Cl\(^{-}\} movement and
the associated movement of H+ ions seem to account for the membrane depolarisation.

Calcium-sensitive fluorescent dyes have been used to monitor intracellular Ca2+ in root hairs. Dyes linked to high molecular weight dextran must be microinjected but are often preferred as they remain within the cytoplasm. Acetoxyethyl esters of dyes are permeant and so can be loaded into cells by diffusion but imaging can be problematic because they are present at only small intracellular concentrations, diffuse out of cells and can become localised inside organelles (particularly in plant cells). Microinjection of Calcium Green/dextran into alfalfa root hairs revealed that Nod factors induced regular periodic increases in intracellular calcium that were localised mostly around the nucleus [26]. These increases occurred about once per minute and the rapid rise and defined cut-off to the decrease in Ca2+ defined these as Ca2+ spikes (Figure 2iv). The spiking initiated ~10 minutes after Nod factor addition and continued for at least three hours. Since Ca2+ spiking did not occur in a non-nodulating alfalfa mutant and was not induced by Nod factors lacking a sulphate group that is essential for alfalfa nodulation, it is very likely to form part of the normal plant response. Moreover, similar Ca2+-spiking responses have been observed in different spp. of Vicia, Lotus and Medicago (S Long, personal communication) and in pea root hairs (SA Walker, unpublished data). Ca2+ spiking plays an important role in developmental gene regulation in mammalian cells [27•]. Analogous systems have yet to be defined in plants, although Ca2+ oscillations are proposed to have a role in regulating the opening of the stomatal guard cell aperture and are also associated with pollen tube growth [28,29].

Other studies using Ca2+-responsive dyes suggest that the dye type and/or its method of delivery may influence the observable effects of Nod factors. Microinjection of dextranlinked dyes revealed Ca2+ spiking, whereas buffered loading of acetoxyethyl derivatives in cowpea revealed a rapid plateau-like increase in Ca2+ at the root hair tip, but Ca2+ spiking was not observed [30]. In vetch, Nod factors induced swelling of root hair tips and inhibited root hair growth; after ~1-1½ hrs exposure to Nod factor, root hair growth resumed and enhanced concentrations of Ca2+ were seen (as detected by the Ca2+-sensitive dye Indo 1) in the swollen root hair tip and in the newly formed growing root hair tip [31•]. The relationships between, first, Nod-factor-induced Ca2+ influx measured using microelectrodes, second, the onset of Ca2+ spiking and third, the accumulation of Ca2+ at the growing tip of the root hair have yet to be resolved. The reasons for the different observations made by different groups could relate to differences in methodology. Microinjection may disturb the ionic equilibrium and/or the cytoarchitecture such that calcium accumulation at the tip does not occur. The age and growth phase of the root hair may be important and the temporal resolution of imaging techniques may be insufficient to allow the observation of the Ca2+ spiking (discussed in [20•,31•]).

Nod factors have been shown to induce disintegration of the actin cytoskeleton and inhibit root hair tip growth within 10 mins of Nod factor addition to Phaseolus vulgaris [32•]. At the subsequent branch-like outgrowth of the root hairs in vetch, actin filaments reappear [33•] in the region where there is an increased level of Ca2+ (Figure 2vi) [31•] and it was concluded that fine bundles of actin filaments promote polar growth by releasing Golgi vesicles to a vesicle-rich region of the growing root hair tip [33•]. Cytoskeletal changes may also be associated with the Nod factor induced reactivation of the cell cycle; outer cortical cells enter the cell cycle but do not divide, whereas inner cortical cells divide (Figure 2ix), entering the cell cycle from the G2/G1 phase [34]. At a later stage in the interaction, a transient disorganisation of microtubules was correlated with the appearance of Nod factors within infected cells, suggesting that Nod factors may also be involved in cytoskeletal rearrangements that occur during the differentiation of infected cells [10•].

The effects of pharmacological agents on the expression of the reporter gene MtENOD12A-GUS, which is induced in response to Nod factors, provided indirect evidence for a role for Ca2+ in a G-protein-mediated signal transduction pathway [35••]. Mastoparan, a G-protein agonist induced MtENOD12A in a similar way to Nod factor, whereas the G-protein antagonist, pertussis toxin, inhibited Nod factor and mastoparan-induced gene expression. Inhibitors of phospholipase C, such as neomycin, and of Ca2+ influx/release such as ethylene glycol tetraacetic acid (EGTA), La3+ and ruthenium red, also blocked both the Nod factor and mastoparan-induced gene expression. Although these results are consistent with a mammalian-type G-protein signalling pathway, coupled to the activation of phosphoinositide and Ca2+ as a second messenger (Figure 2iii), great care has to be taken with the interpretation of data generated by such pharmacological techniques that may be having multiple effects. The combination of such studies with the use of plant mutants in which signalling is blocked in the early stages should, however, give an insight into the nature of the pathways involved. The identity of the Nod factor receptors are of key interest.

Nod-factor-binding proteins

Radioactively labelled LCOs were used to identify Nod factor binding proteins from Medicago truncatula. A protein with a relatively low affinity (Kd = 86 nM) for the Nod factor was found but is unlikely to play a role in the symbiosis [36]. A second component, NFBS2 (Nod-factor-binding site 2), from a plasmalemma fraction [37••] has high affinity (Kd = 4 nM) for Nod factors. Several synthetic LCOs with structural similarity to Nod factors were used in competitive binding assays to determine the effects of structural modifications on relative binding efficiency. Substitutions on the non-reducing terminal sugar (i.e. the presence of an O-acetate group, or the type of fatty acid) and the oligomer length both contributed to optimal binding but the presence
or absence of a sulphate group on the reducing sugar did not affect Nod factor binding. This sulphate group is essential for normal Nod factor activity on *Medicago* spp. and so the binding specificity of NFBS2 did not correspond with what would be predicted (on the basis of *in vivo* studies) for a putative Nod factor receptor. Thus, if NFBS2 does correspond (part of?) a receptor, the perception of the sulphate group by the Nod factor receptor would be complex, and could possibly involve another protein that confers additional specificity.

Isolation of proteins that bind chitin oligomers led to the identification of a Nod factor binding lectin with apyrase activity [38••]. As this lectin hydrolyses the phosphoanhydride bonds of nucleoside phosphates, it was called lectin nucleotide phosphohydrolase (LNP). LNP, which was isolated from the legume *Dolichos biflorus*, has much greater affinity for Nod factors than chitin oligomers and has greatest affinity for those Nod factors from rhizobia that can nodulate *D. biflorus*. Furthermore, the apyrase activity of LNP is enhanced by Nod factors. These results, together with the observations that LNP is located on root hairs and that antibody to LNP inhibited root-hair deformation, suggest a role for LNP in the nodule symbiosis, but as with NFBS2 a direct role for LNP in signalling has not yet been established.

Although LNP is a lectin (as defined by its sugar-binding capability), its sequence shows that it does not belong to the large family of typical legume lectins [39,40]. Several years ago it was proposed that such ‘typical’ lectins might play a role in recognition by acting as a kind of surface-binding receptor, and several studies, including addition of lectins and demonstration of their presence on root hair tips, supported a role for lectins in the early stages of infection [40]. Furthermore, transgenic clover plants expressing a pea lectin gene can be nodulated by *Rhizobium* but root hair deformation and curling occur, thereby potentiating a weak signal and stimulating the early stages of nodule morphogenesis, even by heterologous Nod factors. This model illustrates that there could be multiple inputs into recognition between rhizobia and legumes and that dissecting out the signalling pathway from other recognition events may not be simple.

**Legume nodulation mutants**

Many nodulation-defective legume mutants have been isolated but many are in species that do not lend themselves to gene isolation. Nevertheless, these mutants have been used to gain insight into the events that occur during nodulation. Nod− mutants of several species do not form a mycorrhizal symbiosis [43]. The pea early nodulation transcripts *ENOD5* and *ENOD12A*, which are induced in response to *Rhizobium*, are also induced by the endomycorrhizal fungus *Gigaspora margarita*; no expression was seen in a Nod− *sym8* pea mutant when inoculated with either symbiont [44•]. Other studies using transgenic peas expressing an *ENOD12A–GUS* reporter gene demonstrated that the induction of the pea *ENOD12A* gene is also blocked in the *sym19* mutant [45]. Many gene transcripts are induced early in nodulation [5••,19•,20•] and knowledge of which genes are switched off in various mutants may help to determine the sequence of events that are initiated after Nod factor perception, and where divergence between endomycorrhizal and rhizobial symbioses occurs.

One of the key transcripts that is induced during rhizobium–legume symbiosis is *ENOD40*, which is expressed prior to initiation of pericycle cell division opposite the protoxylem poles and is also expressed in the dividing cells [46•,47]. Transgenic legumes expressing *ENOD40* initiate cortical cell division at multiple sites indicating that this gene influences the differentiation and division of root cells [48]. Sequence comparisons led to the realisation that *ENOD40* encodes a short (10–13 residue) peptide and a conserved non-coding RNA domain that may play a regulatory role [46•,47]. Each region seems to contribute to nodule development. The conservation of this gene in non-legumes [46•] suggests that nodule signalling may have evolved from a more ancient pathway conserved in non-legumes. Nod factor was reported to induce a *Medicago truncatula* *ENOD12* gene in rice [49•], although this observation has not yet been verified by other laboratories.

Recently, there has been a focussed effort on investigating the genetics of ‘model’ legumes, an experimental counterpart to *Arabidopsis* for the study of symbioses. These legumes are diploid, have relatively small genome sizes, are easy to transform and have high seed yield. Such properties lend themselves to gene-tagging strategies and positional cloning. One candidate gene for positional cloning affects the ethylene sensitivity of nodulation [50]; the mutant of *M. truncatula* which does not express this gene has enhanced nodulation and altered position of nodule development. These effects are due to its insensitivity to ethylene which normally down-regulates nodule development [17]. A bacterial artificial chromosome library of *M. truncatula* genomic DNA has been created and used to identify ethylene responsive genes [51]. Other approaches to gene identification involve a root-hair-specific cDNA library from *M. truncatula* [52•]. In *Lotus japonicus*, an essential nodulation gene *Nin* has been identified by gene tagging following mutagenesis using the maize transposable element *Ac* [53••]. The mutant is not infected by rhizobia, but root hair deformation and curling occur,
indicating that the *Nig* gene is unlikely to encode the primary Nod factor receptor. The *Nin* gene product is predicted to be a membrane protein and four similar *Arabidopsis* proteins (of unknown function) were identified in database searches. Similarity was also found with a *Chlamydomonas* gene product (the so-called *minus*-dominance protein) that regulates mating type during gametogenesis. All of these proteins share a putative DNA-binding dimerisation domain and so a regulatory role for the *Lotus* nodulation gene was proposed, although the mechanism of regulation and the regulated genes are not yet known. This first characterisation of an essential nodulation gene suggests that we are now on the threshold of a phase identifying genes required to establish symbiotic interactions.

**Conclusions**

There is strong evidence to suggest that the early events in Nod factor recognition involve movements of ions across the plant plasma membrane, followed by the establishment of intracellular Ca$$^{2+}$$ oscillations. Circumstantial evidence suggests that a G-protein-type receptor that could act via an inositol-phosphate and phospholipase based signalling pathway may be involved. Much work remains to be done, however, to integrate the various observations that have been made on the legume symbiosis. The key objective in the future will be to exploit various nodulation mutants to clone and characterise genes that are essential for nodule development. The ease of the isolation (and maintenance) of legume nodulation mutants, the availability of the Nod-factor-signalling molecules, and the ability to analyse ion movements in epidermal cells mean that nodule development is a particularly good system for the analysis of a developmental pathway. Understanding how nodules are formed may well give us an insight into how other morphogenetic events are activated in plants.

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**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


The very earliest plant responses to Nod factors are investigated. Nod factors induce a rapid influx of Ca2+ that is followed by Cl− and K+ efflux. These latter ion movements can explain the Nod-factor induced depolarisation of the root hair plasma membrane.


It has long been suspected that oscillations in intracellular calcium may play a role in regulating the expression in mammalian cells. This commentary highlights the importance of two papers published in the same issue describing the first hard evidence to support this hypothesis.


32. Cárdenas L, Vitali L, Dominguez J, Pérez H, Sánchez F, Hepler PK, Quinto C: Characterization of a chitin-binding protein fortuitously led to the identification of this novel type of lectin that has both a high affinity for Nod factors and an apyrase activity. It is structurally distinct from the large family of legume lectins previously described and a potential signalling receptor.


An expressed sequence tag (EST) library of root hair genes has been established and is growing rapidly. This paper describes many genes that are expressed in root hairs.

The first paper to describe cloning of a gene essential for some of the earliest stages in the rhizobia-legume symbiosis. Transposon-tagged mutants of Lotus japonicus led to the isolation of a gene required for both bacterial entry into the host plant and the establishment of the nodule primordium. The encoded protein contains transcription factor-like domains and could define a new class of developmental regulators.