Genes and signal molecules involved in the rhizobia–Leguminoseae symbiosis

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The symbiosis between Rhizobium bacteria and their host plants is dependent on the specific recognition of signal molecules produced by each partner. Many players in the signal exchange have been identified. Among them are signal molecules such as flavonoids, LCOs, auxin, cytokinin, ethylene and uridine and genes such as Enod40, Enod2 and Enod12. Their interconnection, however, is only starting to be understood. The most recent insights into their interconnection include: advances in the use of transgenic leguminous plants containing reporter gene constructs for studying the effect of the signal molecules; novel methods for delivery of signal molecules using ballistic microtargeting; and the discovery of the role of chitin oligosaccharides in animal embryogenesis.

Introduction

The interaction between bacteria of the genera Rhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium and Sinorhizobium, hereafter called rhizobia, and their host plants is dependent on the specific recognition of signal molecules by each partner. After attachment of the bacteria to the plant root, the root hairs start to deform and a tubular structure, called the infection thread, grows through the root hair towards the simultaneously formed nodule primordium. This primordium is formed by mitotically activated cortical cells. In this primordium the rhizobia are released into the cytoplasm of the plant cells and develop into an endosymbiotic form, the bacteroid, while the nodule primordium develops into a mature nodule. The bacteroids are able to fix nitrogen from air into nitrogen salts that are useful for the plant. In return, the plant provides the bacteroids with various nutrients.

In the early steps of the interaction, secretion of flavonoids by the plant leads to the synthesis of lipo-chitin oligosaccharides (LCOs) by the rhizobia. After synthesis, the LCOs are secreted into the environment, where they trigger various responses on plant roots. The genes involved in the synthesis of the LCOs have been studied intensively and for many of them we know their function in some detail. In addition to flavonoids, other plant signal molecules also play an important role in the development of root nodules. For several signal molecules we know when and where they are produced and what physiological changes they induce, but their exact function and relation to each other remains obscure. Since several reviews on this subject have already been written [1,2*], we will discuss mainly the very recent findings on flavonoids, LCOs and other carbohydrates and their interconnection with lectin and genes regulated by conventional plant hormones.

Flavonoids: a role in auxin transport

Flavonoids have a diverse variety of functions. They function as precursors for various pigments, they play a role in pollen tube growth and are suggested to be active as defence compounds, for instance the soybean phytoalexin glyceolin (reviewed in [6*]). In nodulation they are the inducers of LCO synthesis. In turn, LCOs trigger flavonoid secretion by the plant and are able to induce the flavonoid pathway [3,7,8]. This is a good explanation for the observation that externally applied LCOs have less effects than the invading bacteria which are subject to a positive feedback mechanism. Recently, it was suggested by Mathijs et al. that flavonoids play a direct role in nodulation by inducing a modulation of auxin distribution [9]. This hypothesis was formed on the basis of GUS assays when GUS was used as a reporter gene fused to the auxin inducible promoter GH3. Because spot inoculation of flavonoids resulted in the same GUS expression pattern as obtained with the synthetic auxin transport inhibitor naphthylphtalamic acid (NPA), it was concluded that the change in auxin distribution was the result of auxin transport inhibition. Introduction of flavonoids inside the roots using ballistic microtargeting had a similar effect. In both series of experiments the most active flavonoids appeared to be quercetin, fisetin, kaempferol, apigenin and naringenin. The glycosidic forms of these flavonoids and the isoflavonoid genistein were inactive, both after direct application or after microtargeting [10**].
results correlate well with the earlier conclusions of Jacobs and Rubery on a role of flavonoids in auxin transport inhibition [11].

**Structure and biosynthesis of LCOs**

The structure of LCOs produced by many rhizobial strains has been reported [2,3,4,7]. Most rhizobia produce a mixture of LCOs (Figure 1). In most cases the length of the chitin backbone is four or five N-acetylglucosamine (GlcNAc) residues long, but very recently a minimal LCO structure with a backbone of only two GlcNAc residues was identified in *Mesorhizobium loti* strain NZP2213 [12].

In the same strain another novel LCO was detected as the major product, namely the first LCO structure reported to have a substituent on one of the non-terminal GlcNAc residues. The substituent found was a fucose residue α-1,3-linked to the GlcNAc residue proximal to the non-reducing terminus [12]. The α-(1→3) fucosyltransferase responsible for this substituent still has to be identified.

The biosynthesis of LCOs has been investigated extensively during the past few years. The core of the LCOs is synthesized by NodA, NodB and NodC. Many of the other Nod proteins are involved in the attachment of various substituents. For instance, Quinto *et al.* [13] showed that NodZ is an α-(1→6)-fucosyltransferase. This enzyme has a high specificity for chitin-like molecules, but it also fucosylates molecules with at least one N-acetylglucosamine at the reducing end, albeit with a much lower efficiency. NodC, which is responsible for the synthesis of the chitin-oligosaccharide backbone, has recently been shown to be an important determinant of the chitin-oligosaccharide chain length [14] and biochemical studies showed that the direction of the synthesis of this chain is from the reducing to the non-reducing terminus [15]. A NodC homolog, DG42, was identified in zebrafish embryos, that is involved in early embryo development. Functional analysis of this protein pointed towards chitin oligosaccharide synthetase activity [16,17,18], but data indicating a function as a hyaluronate synthetase are also published [19]. Hyaluronate is a polymer of β(1→3)- and β(1→4)-linked GlcNAc and glucuronic acid (GlcA) residues. The actual biochemical function of the DG42

![Figure 1](image-url)

**Figure 1**

LCO structure with known modifications. Shown is an LCO with five GlcNAc residues. This is the most common chain length but backbones containing two to six GlcNAc residues have also been reported. Ac, acetyl; Ara, arabinosyl; Cb, carbamoyl; Fuc, fucosyl; Gro, glycerol; Man, mannosyl; Me, methyl; S, sulphate. Some known fatty acyl groups of LCOs are illustrated in the inset.
genes family members is one of the important questions that still has to be explored further. Furthermore, it remains of interest to analyse which structural feature determines whether an enzyme belonging to the nodC gene family synthesizes a polymer or an oligomer and what determines their substrate specificity.

Most rhizobia produce LCOs of which the fatty acid moiety is a common fatty acid such as C18:1—the most abundant fatty acid in Rhizobium membranes. Many of them also synthesize LCOs with highly unsaturated, and sometimes long-chain fatty acids, for example, C16:2 and C16:3 for Rhizobium meliloti and C18:3, C20:2, C20:3 and C20:4 for Rhizobium leguminosarum bv. trifolii. NodA is involved in the transfer of the fatty acid to the chitin backbone from a donor molecule, which is acyl-acyl carrier protein (acyl-ACP) in the case of the common fatty acids, or acyl-β-NodF in case of the (long-chain) unsaturated fatty acids [1,20].

The enzymes involved in the synthesis of LCOs have also been used to good effect for studies in animals—Bakkers et al. [16] have shown that chitin derived molecules important in developmental processes are also produced by vertebrates in the embryonic stage. When they microinjected the fucosyl transferase NodZ or antibodies against the glycosyl transferase DG42 into zebrafish embryos, the formation of a tail was completely inhibited. These results suggest a general role for chitin oligosaccharides and their derivatives in plant and vertebrate development.

**LCO receptors in plant cells**

The interaction between rhizobia and plants is very specific and is dependent on the LCO structure. Because of the low concentration at which the LCOs are active, it seems reasonable to assume that high affinity Nod factor receptors are present. Not all plant responses, however, have the same structural requirements for LCOs. For instance, a Sinorhizobium meliloti nodF/nodL double mutant, lacking highly unsaturated fatty acids or O-acetyl substituents on its LCOs, was able to elicit multiple deformations of single root hair cells. In the root cortex starch granule accumulation was observed, together with decrease of vacuole volume, increase of the nucleus size and development of cytoplasmic strands, processes that are often observed after the addition of wild-type nod factors [21]. The receptors involved in these responses do not necessarily have to be root hair specific, since the same mutant was able to trigger deformations of unhaired epidermal cells from Medicago roots. The nodF/nodL mutants, however, were not able to infect the roots. The authors, therefore, proposed a mechanism with two kinds of receptors, a signalling receptor involved in root hair deformation and an entry receptor with more stringent specific requirements for LCOs, which mediates the initiation of the infection process. Recently, a gene from Afghanistan pea, sym2, was identified as a possible entry receptor. The data are not sufficient, however, to definitely assign a receptor role for the sym2 product [22]. As an alternative for the presence of multiple receptors a mechanism with only one kind of receptor, the activity of which is dependent on the LCO structure, can be proposed [23]. The acyl chain could serve to anchor the LCO in the membrane close to a receptor [23,24*].

After biosynthesis of the LCOs, they are secreted into the rhizosphere, where they trigger various responses on plant roots, such as the formation of nodule primordia. Several studies have approached the question whether the LCOs bind to the root surface and act by eliciting a second signal in the plant root or whether the LCOs themselves enter the root. O-acetylated chitin oligosaccharides are also able to induce nodule primordia, provided that they are targeted inside the cells [25]. This indicates that the chitin backbone of LCOs has to enter the roots in order to be biologically active. In addition, it was shown that fluorescent labelled LCOs or LCO derivatives were transported into the root (hair) cells [24]. Since in these experiments only the chitin backbone was labelled it is unknown whether also the lipid part was cotransported. Recently, Timmers et al. have obtained evidence using antibodies against LCOs that they are internalized into the plant cell wall [27].

**Other rhizobial carbohydrates important for infection**

LCOs are not the only sugar compounds involved in nodulation. Several features of other carbohydrates, including their importance in nodulation, have been reviewed previously [28,29]. A Rhizobium etli strain lacking the plasmid-located lpsB1 and lpsB2 genes involved in the biosynthesis of lipopolysaccharides (LPSs) was unable to infect nodules [30]. Furthermore, Rhizobia produce several groups of secreted polysaccharides—exopolysaccharides (EPSs) have little or no cell adhesion and can be found in the environment as slime. The main form of rhizobial EPS is a succinoglycan called EPS I. Depending on the growth conditions this can be low (LMW) or high molecular weight (HMW) EPS. Under special circumstances some bacteria can produce an alternative EPS, which is a galactoglucan called EPS II. This type of EPS can also have variations in its molecular weight. EPSs have traditionally been thought to function in processes like attachment to surfaces and protection from the environment. It is now clear that they also have an important function in symbiosis—EPS from Rhizobium leguminosarum bv. viciae are required for infection thread formation in its host plant [31]. Purified LMW-EPS II added together with an EPS II− or EPS I− mutant promoted the infection of alfalfa [32]. An expH mutant of R. meliloti which lacks the succinyl group on its succinoglycan was unable to invade nodules [33] which was correlated with the absence of low molecular weight EPS I. The molecular weight of EPS seems to be an especially important factor.
Some bacteria produce acidic KDO-rich polysaccharides (KPS) that are analogous to the group II K-antigens of *E. coli*. These polysaccharides are tightly associated with the bacterial cells, forming a capsule around the bacterium and are, therefore, also called capsular polysaccharides (CPS). The K-antigen subgroup of CPS induced the expression of genes related to isoflavonoid production in the interaction between *Rhizobium meliloti* and alfalfa [34]. Interestingly, Savouré et al. [35] showed the increased expression of genes encoding isoflavonoid biosynthetic enzymes also upon incubation of a *Medicago* cell suspension with chitin oligosaccharides.

**Are nodulin genes specific?**

During the infection and nodule development process several plant genes are activated. Depending on the point of activation, these genes are called early or late nodulin genes. Three well characterized early nodulin genes include enod2, enod12 and enod40 which will be discussed below. Examples of late nodulin genes with known function are leghemoglobin and uricase. In contrast to the early nodulins which seem to have a role in the early signalling events and the infection process, the late nodulin proteins seem to mainly assist the settlement of the bacteria inside the nodule, to promote the conversion of bacteria into bacteroids and to support the nitrogen fixation process.

Many of the nodulin genes seem to be nodule specific, but an increasing number of genes also appear to be expressed in other stages of plant growth, like lateral root or pollen tube growth, or appear to be homologs of known household genes. Wu et al. [36] have identified a gene in pollen of alfalfa, which was 38% identical to Eno8. It is very well possible that infection thread growth and pollen tube growth share some physiological and biochemical characteristics. Homologs of the nodulin genes have also been found in non-leguminous plants like tobacco. Recently, five novel glycine rich proteins, both early and late expressed in nodulation, were identified [37]. By the use of a sequence tag library of *Lotus japonicus*, Szczygłowski et al. [38] identified several new nodule-specific genes.

**enod40**

*enod40* is one of the nodulin genes that is expressed very early in nodule development. *enod40* sequences from several plant species have been determined. *Medicago sativa* and *Lotus japonicus* appear to contain two different copies of the *enod40* gene ([39]; P Katinakis, personal communication). All *enod40* sequences have two highly conserved regions—region one (*enod40-1*) has been suggested to code for a short peptide of approximately ten amino acids which demonstrated biological activity [40]. Although doubts now exist about these experiments, other independent tests of the significance of ENOD40-like peptides in nonlegume species have been carried out; for example, tomato suspension cultures exposed to tomato ENOD 40 peptide are altered in their response to auxin (T Bisseling, personal communication). Region two (*enod40-2*) also has biological activity [41], but an open reading frame was missing and it was proposed to act on the RNA level as a regulating sequence, because computer analyses indicated a stable RNA structure [42]. Ballistic targeting experiments indicated that both regions of the *enod40* transcript have a biological function in cell division [41]. *enod40* is not only expressed in nodules, but also in non-symbiotic tissue, even in uninoculated roots [39] and lateral root primordia [39,43]. It has also been suggested that cells of a tobacco protoplast suspension culture produce Enod40 [40].

**enod2 and enod12**

The early nodulin genes *enod2* and *enod12* are also expressed in tissues other than nodules, for example, in lateral root primordia [44]. Both *Enod2* and *Enod12* are (hydroxy)proline-rich cell wall proteins. A role for (hydroxy)proline-rich proteins in forming the oxygen barrier in nodules was suggested by Minchin [45]. It was proposed that by crosslinking these proteins with glycoproteins, lectins and isoflavonoids, a water filled gel would be formed in the intercellular spaces, leading to a high resistance barrier for gases. This hypothesis was supported when transgenic plants containing *enod2* were used. It was shown that these plants were affected in their oxygen barrier [46]. *enod2* transcripts were also detected in the alfalfa–mycorrhizae symbiosis and in uninoculated roots upon cytokinin treatment [47], indicating that several molecular events are conserved between these symbiotic interactions. Since there is no evidence for an oxygen barrier in mycorrhizal symbioses, *Enod2* might also have a different function than serving as an oxygen barrier.

**The role of phytohormones in nodulation**

Phytohormones such as auxin and cytokinin play an important role in the nodulation process. The *enod2* and *enod12* genes are induced by cytokinins [39,44,47] and *enod40* was suggested to be induced in the protoxylem poles and surrounding cell layers upon accumulation of cytokinin [39]. The results of Mathesius et al. suggest that the induction of *enod40* expression is correlated with a local change of auxin concentration. With spot inoculation or microballistic targeting of *Enod40* peptide on transgenic GH3:GUS roots as probes for auxin concentration, however, they could not detect any changes in auxin distribution [10]. Using the same assay it was shown that application of rhizobia, LCOs or auxin transport inhibitors leads to a modulation of the auxin concentration between the application spot and the root tip [9,10]. The resulting localized increase in auxin concentration at the application spot may also be the result of local auxin synthesis. Auxin is known to be involved in cell division, especially in lateral root formation [48]. A high concentration of auxin is necessary for accumulation of p34cdc2 like proteins for activation of the cell cycle from the G1/G0 or G2 phase into mitosis [49]. Recently, a gene from *Medicago*...
which belongs to the RACK1 subfamily of WD-repeat proteins, Msgbl, was identified [50]. WD-repeat proteins regulate cellular functions, including cell division and transmembrane signalling [51]. Msgbl was induced upon cytokinin treatment of roots, indicating that this gene is also involved in hormone mediated cell division.

Smit et al. identified a compound in a stele extract of pea plants, that was able to enhance the activity of auxin in pea root cortex explants. They identified this compound as uridine which was able to enhance hormone-mediated cell division. For instance, in microballistic targeting experiments, O-acetylated chitin oligosaccharides were only active when uridine was cotargeted [25**].

**Lectins play an important role in nodulation and infection**

Plant lectins have been proposed to play an important role in the nodulation and infection process. They are defined as sugar binding proteins and it is possible, therefore, that they interact with LCOs (reviewed in [53*]). Two out of three tested lectin genes from *Medicago truncatula* were transcribed upon induction with *Sinorhizobium meliloti* or purified Nod factors [54]. Diaz et al. [55] showed that introduction of the pea lectin gene in clover roots allows the heterologous infection by *Rhizobium leguminosarum* bv. *viciae*. By the use of a mutant pea lectin van Eijsden et al. [56] showed that the sugar binding activity of pea lectin is essential for this activity. Recent experiments showed that pea lectin makes white clover plants susceptible for heterologous mitogenic activity of LCOs and chitin oligosaccharides. It is also likely, however, that this activity is not due to binding of LCOs to lectin, because of presumed steric hindrance of O-acetyl modifications (C. Diaz, personal communication). This discrepancy could be explained in the following way — many lectins seem to be able to interact with glycoproteins [57,58] or lipids [59]. These interactions could be more important physiologically than the binding activity of lectins to sugars. Crystallagographic studies showed that, next to the sugar binding site, a very hydrophobic site is present that in theory is able to bind to adenine or cytokinin [60]. This last compound is especially interesting with regard to the requirement of the high cytokinin concentration for evodh40 expression and the general role of phytohormones in cell division.

**Conclusions**

This review has clearly illustrated that the complete signal transduction pathway involved in the rhizobia–plant symbiosis is far from elucidated. Many factors are involved and every month new factors, which could play an important role, are discovered. Although it is known for some of them what physiological changes they induce, their exact function is still obscure. A main goal in future research is the identification of receptors for the known signal molecules involved in nodulation. It is clear that plant hormones play an important role in the nodulation process and more knowledge on the mechanism of auxin and cytokinin signal transduction is essential for further understanding of the role of these receptors. A great help in this research is the genetic and technological progress by the use of transgenic *Lotus japonicus* and *Medicago truncatula* plants, improved techniques for the local delivery of compounds by microballistic targeting and the recent advances in non invasive fluorescence imaging techniques with especially fluorescent proteins as reporters [61].

**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


This is a complete overview of the biosynthesis of LCO molecules by rhizobia. All possible variations in LCO structure and the genes that are responsible for this are discussed.


In the rhizobia-plant interaction flavonoids produced by the plant are the inducers of LCO synthesis by the bacteria. This is not the only function of flavonoids in this symbiosis. The author discusses functions of flavonoids in relation to nodulation and presents the hypothesis that flavonoids have been chosen in evolution as inducers of nod-genes, because they are unique secreted markers for the hormonal balance of the root which, therefore, indicates the position of the root which is susceptible for the induction of cell division leading to root nodules.


This paper gives a description of the synthesis and biological activity of fluorescent LCOs or chitinoligosaccharides and the specificity of the uptake of these compounds in legume roots.


The authors give an overview of the involvement of plant hormones in plant–microbe associations, mainly focused on cytokinin, by the use of enod40 expression or enod40 promoter-GUS fusions. They also present a model on the hormone flows in uninoculated and inoculated roots.


Plant-microbe interactions


Several reports have been presented that suggest that a change in auxin concentration in the root is responsible for the induction of cortical cell divisions leading to nodular formation. In this paper the authors show that application of LCOs and flavonoids result in a change of auxin concentration. Using microtargeting techniques it was shown that also O-acetylated chitin oligosaccharides were active.


12. Olsthoorn MMA, López-Lara IM, Petersen BO, Bock K, Havrankova J, Spahn HP, Thomas-Oates JE: Novel branched nod factor structure results from α-(1→3) fucosyl transferase activity: the major lipo-chitin oligosaccharides from Mesorhizobium loti strain NZP2213 bear an α-(1→3) fucosyl substituent on a non-terminal backbone residue. Biochemistry 1998, in press. This is the first report describing an LCO structure with modifications on one of the major LCO families.


Using radiolabelling and a sensitive bioassay the authors show that zebrafish embryos are capable of the biosynthesis of chitin oligosaccharides. Using microtargeting techniques it was shown that chitin-like molecules also play a role in eucaryotic embryo development.


This paper gives a description of the synthesis and biological activity of fluorescent LCOs or chitinoligosaccharides and the specificity of the uptake of these compounds in legume roots.
The authors describe the isolation of an enod40 homolog from tobacco. This homolog and the soybean enod40 were both active in conferring tolerance of tobacco protoplasts to high auxin concentrations. Although doubts now exist about this assay, other independent tests have been carried out. For example, tomato suspension cultures exposed to tomato ENOD40 peptide are altered in their response to auxins (T. Bisseling, personal communication).

41. Charon C, Johansson C, Kondorosi E, Kondorosi A, Crespi M: **enod40 induces differentiation and division of root cortical cells in legumes.** Proc Natl Acad Sci USA 1997, 94:8901-8906. In this article the authors show that enod40-overexpressing transgenic plants or plants bombarded with enod40 exhibit extensive cortical cell division in the roots under nitrogen-limiting conditions, indicating an association of enod40-expression with root nodule development.


43. Papadopoulou K, Roussis A, Katinakis P: **Nod genes and cytokinins induce similar cortical cell division, amyloplast deposition and msenod12a expression patterns in alfalfa roots.** Plant J 1996, 10:91-105.


