Genes and signal molecules involved in the rhizobialeguminoseae 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.

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Abbreviations

CPS	capsular polysaccharide
EPS	exopolysaccharide
GlcA	glucuronic acid
GlcNAc	N-acetylglucosamine
KDO	2-keto-3-deoxyoctonic acid
KPS	KDO rich polysaccharide
LCO	lipo-chitin oligosaccharide
LPS	lipopolysaccharide
NPA	naphthylphtalamic acid

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•,3–5], 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 Mathesius *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••]. These

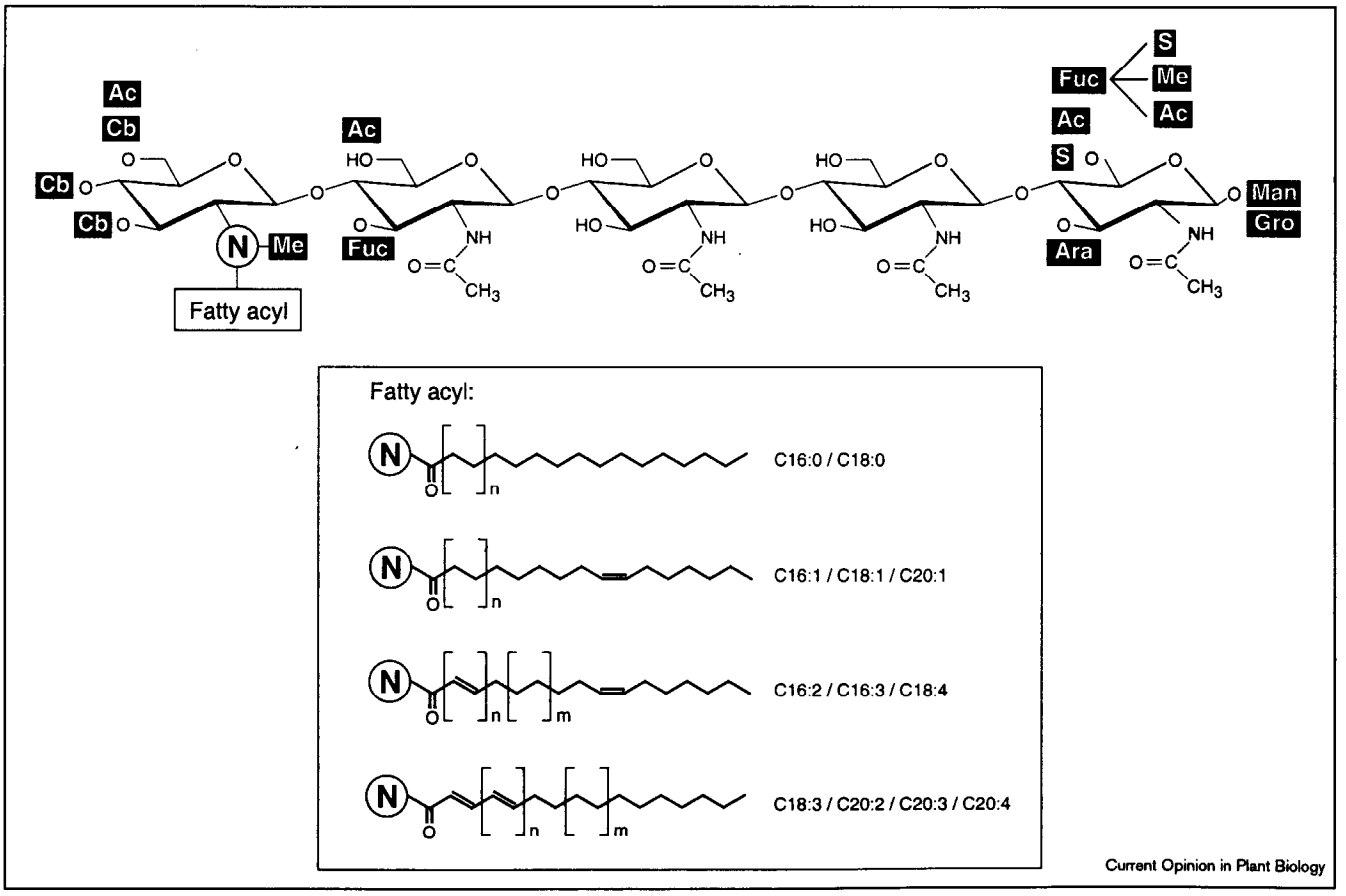
results correlate well with the earlier conclusions of Jacobs and Rubery on a role of flavonoids in auxin transport inhibition [11].

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



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.

gene 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-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 embryogenic 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•,25••,26].

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 *lpsβ1* and *lpsβ2* 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/II$^-$ mutant promoted the infection of alfalfa [32]. An *exoH* 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 time 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 Enod8. 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*, Szczyglowski *et al.* [38] identified several new nodule-specific genes.

enod40

enod40 is one of the nodulin genes that is expressed very early in nodulation. *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 also *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 $p34^{cdc2}$ like proteins for activation of the cell cycle from the G_1/G_0 or G_2 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 proliferation of explants at sub-nanomolar concentrations [52]. There are indications that uridine is required for induction of cortical 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. Crystallographic 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 *enod40* 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].

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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.

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