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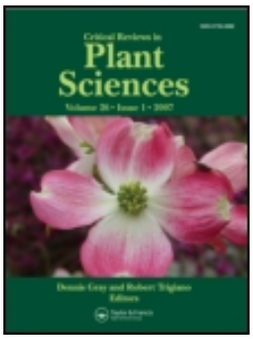
Spaink, H.P.; Carlson, R.W.



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Regulation of Plant Morphogenesis by Lipo-Chitin Oligosaccharides

Herman P. Spaink

Leiden University, Institute of Molecular Plant Sciences, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

Referee: Dr. R. W. Carlson, Complex Carbohydrate Research Center, The University of Georgia, 220 Riverbend Road, Athens, GA 30602–4712.

ABSTRACT: Lipo-chitin oligosaccharides (LCOs), produced by rhizobia, are causative agents of the formation of root nodules in leguminous plants. As outlined in this review, the root nodulation process presents a valuable model system to study plant morphogenesis. The knowledge that resulted from the studies of the biological function and biosynthesis of the rhizobial LCOs is summarized. It has been postulated that LCOs are representatives of a general class of signal molecules involved in plant and animal morphogenesis. Discussed is how the present knowledge can be used for future studies on the function of LCOs in morphogenesis and in the search for analogue signal molecules produced by plants and animals.

KEY WORDS: review, chitin, nodulation, host specificity, organogenesis.

I. INTRODUCTION

Lipo-chitin oligosaccharides (LCOs) (Figure 1) are signal molecules that were discovered as a result of the study of the root nodulation process in leguminous plants. Nitrogen-fixing root nodules are the result of an association of plants with bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, commonly called rhizobia. LCOs produced by the rhizobia, after induction by plant flavonoids, are key factors in the specific recognition processes that underlie the formation of root nodules and their bacterial infection (for recent reviews see: Dénarié and Cullimore, 1993; Carlson et al., 1994; Fisher and Long, 1992; Downie, 1994; Göttfert, 1993; Hirsch, 1992; Lerouge, 1994; Mylona et al., 1995; Schultze et al., 1994; Spaink, 1995.

The study of the biological activity of various purified or chemically synthesized LCOs has yielded information as to the role of variations in structures that are found in the signal molecules produced by different rhizobial strains. Substitutions at the oligosaccharide backbone structure can play a role in determining the host range of the rhizobia or might have a general role in the capacity of LCOs to induce plant morphogenesis. The present knowledge of the biosynthesis of LCOs, as outlined in this review, has been an additional basis for assigning biological functions to various substituents.

The results of several studies indicate that LCOs are representatives of a general class of signal molecules involved in plant and animal morphogenesis (De Jong et al., 1993; Röhrig et al., 1995; Semino and Robbins, 1995; Spaink et al., 1993). I dis-

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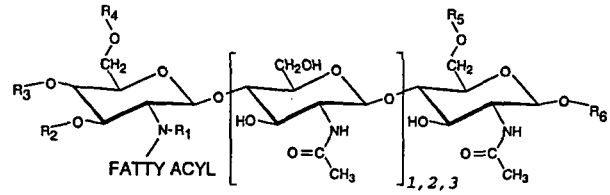
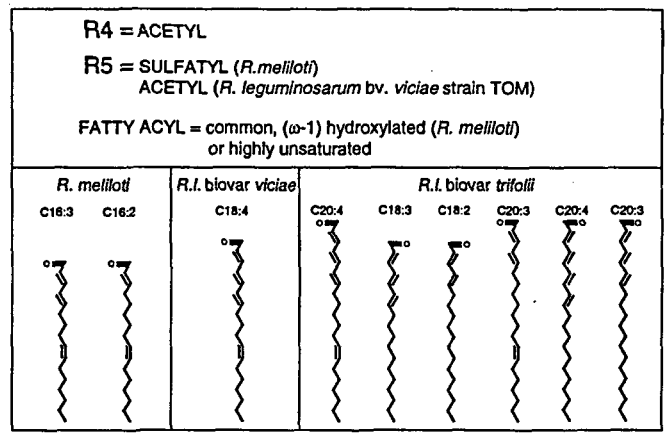


RHIZOBIA ASSOCIATED WITH INDETERMINATE NODULATING PLANTS



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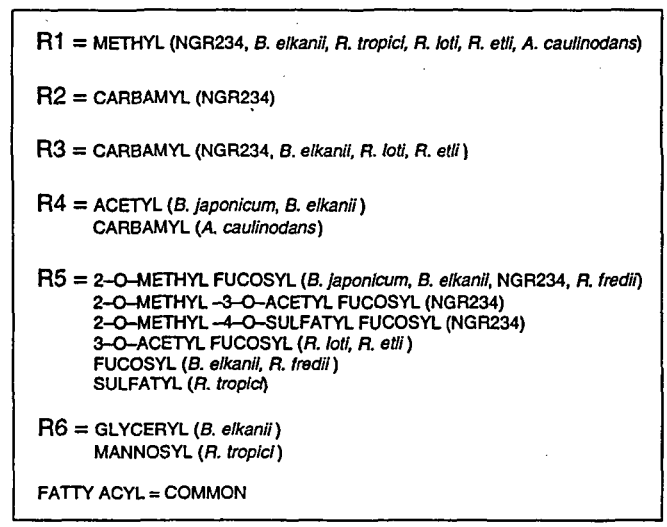

FIGURE 1. Structures of LCOs. When strain-specific modifications are not indicated, the groups R1 to R6 stand for hydrogen groups. The length of the sugar backbone can vary from 3 to 6 *N*-acetylglucosamine units (López-Lara et al., 1995b). The term common fatty acyl moieties is used for a variety of fatty acids that are commonly occur as part of the phospholipids of bacterial cell membranes. Rhizobia are divided on the basis of their ability to form successful nitrogen-fixing symbioses with plants forming either indeterminate or determinate nodules. The shown structures were reported by Bec-Ferté et al. (1994) for *R. fredii*, by Carlson et al. (1993) and Stokkermans et al. (1996) for *B. japonicum* and *B. elkanii*, by Demont et al. (1993) and Schultze et al. (1992) for *R. meliloti*, by Firmin et al. (1993) for *R. leguminosarum biovar* viciae strain TOM, by Mergaert et al. (1993) for *A. caulinodans*, by Folch-Mallol et al. (1996) and Poupot et al. (1993) for *R. tropicii* (which produces a similar mixture of LCOs as *Rhizobium* strain GRH2 [López-Lara et al., 1995b]), by López-Lara et al. (1995a) for *R. loti*, by Cardenas et al. (1995) and Poupot et al. (1995a) for *R. etli*, by Price et al. (1992) for *Rhizobium* strain NGR234, by Spaink et al. (1991) and Spaink et al. (1995) for *R. leguminosarum* biovar *viciae* strain RBL5560 and *R. leguminosarum* biovar *trifolii* strain LPR5045.pRtRF101.

cuss how the knowledge and technologies that resulted from the studies reviewed in this article can be used for future studies on the function of LCOs in morphogenesis and in the search for analogue signal molecules produced by plants and animals.

II. EFFECTS OF LCOs ON LEGUMINOUS PLANTS

Purified or chemically synthesized LCOs can elicit various effects on plant as outlined in Table 1. Most of the studies mentioned in Table 1 concern effects of LCOs on the roots of leguminous plants and their relationship to phenotypes observed during the interaction of rhizobia with the host plants. The host-specific aspects of the elicitation of these effects are discussed in a later section of this review.

Several fast responses of the root hairs to nanomolar concentrations of LCOs are most likely related to the process of root hair curling, an early step in the infection process. The earliest responses include depolarization of membrane potential (Ehrhardt et al., 1992; Felle et al., 1995; Kurkdjian, 1995), modulation of proton and calcium ion fluxes (Allen et al., 1994), induction of cytoplasmic streaming, deformation, and reinitiation of polar tip growth (Heidstra et al., 1994). The elicitation of the formation of root hairs has been reported in a number of plant species (Table 1). The new root hairs appeared to form very localized, preferentially opposite the cortical cells where nodule primordia are formed. However, it was not demonstrated whether the newly formed root hairs were the result of normal differentiation of epidermal trichoblast cells or, alternatively, were the result of polar tip growth of outer cortical cells as was shown to be an effect of LCOs in *Vicia sativa* (van Brussel et al., 1992). An example of a response that is obviously related to steps later in the infection process is the formation of preinfection threads in the outer cortex of roots of pea or vetch. The process of preinfection thread formation is probably related to the elicitation of polar tip growth of outer cortical cells mentioned above (van Brussel et al., 1992; van Spronsen et al., 1994).

The key role of LCOs in the formation of the root nodule has been clearly demonstrated in many leguminous plant species (Table 1). In order to understand the role of LCOs in nodule formation, it is important to consider the differences in development of the root nodule in the various plant species studied (Brewin, 1991; Franssen et al., 1992). In several plant species, exemplified in *Vicia* (Figure 2), nodule formation is initiated by the formation of primordia in the inner cortex. The formation of a nodule primordium leads to the formation of a persistent meristem that continues to grow during the processes of infection of the root nodule cells resulting in a protracting structure. This type of nodulation is therefore commonly classified as indeterminate nodulation. In other plant species, exemplified in *Phaseolus* (Figure 2), nodule formation is initiated by the formation of a primordium in the outer cortex. This nodule primordium gives rise to the formation of a round-shaped nodular structure that does not contain meristematic activity, and that therefore is called a determinate nodule. The correlation between the position of the nodule primordia (i. e., inner or outer cortex) and the subsequent development into either indeterminate or determinate nodules is not strict. For instance, in *Sesbania rostrata* the formation of determinate root nodules is preceded by the formation of nodule primordia in the inner cortex (at the basis of emerging lateral roots) (Ndoye et al., 1994). At micromolar concentrations, externally applied LCOs elicit the formation of nodule primordia in the cortex which are indistinguishable from the nodule primordia in the first stage of bacteria-induced nodule

TABLE 1
Effects of LCOs on Plants

Effect and tissue type	Plant species	Ref.
Root epidermis		
Formation of new root hairs	*Vicia*	van Brussel et al., 1992
	Sesbania	Mergaert et al., 1993
	Medicago	Roche et al., 1991b
	Lotus	López-Lara et al., 1995a
	Phaseolus	López-Lara et al., 1995b
Induction of *enod12*	*Medicago*	Pichon et al., 1992
Root hairs		
Deformation, branching, swelling	Many legumes	See Schultze et al., 1994
Formation of Shepherd's crooks	*Macroptilium*	Relic et al., 1993; Relic et al., 1994
Reinitiation of polar tip growth	*Vicia*	Heidstra et al., 1994
Stimulation of cytoplasmic streaming	*Vicia*	Heidstra et al., 1994
Depolarization of membrane potential	*Medicago*	Ehrhardt et al., 1992; Felle et al., 1995; Kurkdjian, 1995
	Vicia	Kurkdjian, 1995
Modulation of proton and calcium ion fluxes	*Medicago*	Allen et al., 1994
Root cortex		
Ethylene-related cell swelling	*Vicia*	van Spronsen et al., 1995
Polar tip growths of outer cortical cells	*Vicia*	van Brussel et al., 1992
Formation of preinfection threads	*Vicia*	van Brussel et al., 1992
Formation of nodule primordia (inner cortex)	*Vicia*	Spaink et al., 1991
	Medicago	Truchet et al., 1991
	Trifolium	Bloemberg et al., 1995a
	Sesbania	Mergaert et al., 1993
Formation of nodule primordia (outer cortex)	*Lotus*	López-Lara et al., 1995a
	Phaseolus,Acacia	López-Lara et al., 1995b
	Glycine	Stokkermans and Peters, 1994
Formation of complete nodules[a]	*Medicago*	Truchet et al., 1991
Induction of *enod5*, *enod12*, or *enod40*	*Pisum*, *Vicia*	Horvath et al., 1993; Vijn et al., 1995c
Induction of *enod12*	*Medicago*	Bauer et al., 1994; Journet et al., 1994
Local induction of cell cycle genes	*Vicia*	Yang et al., 1994
Induction of flavonoid synthesis genes	*Vicia*	Yang et al., 1994
Root pericycle		
Induction of *enod40*	*Vicia*	Vijn et al., 1995c
Whole root[b]		
Thick and short roots	*Vicia*	Spaink et al., 1991; van Spronsen et al., 1995
Production of additional flavonoids	*Vicia*	Spaink et al., 1991
Induction of a specialized chitinase	*Medicago*	Staehelin et al., 1995

TABLE 1 (continued)
Effects of LCOs on Plants

Effect and tissue type	Plant species	Ref.
Suspension cell cultures		
Induction of flavonoid synthesis genes	*Medicago*	Savouré et al., 1994
Induction of cell cycle genes	*Medicago*	Savouré et al., 1994
Complementation of embryogenesis mutant	*Daucus*	De Jong et al., 1993
Protoplast cultures		
Induction of mitosis	*Nicotiana*	Röhrig et al., 1995
Induction of an auxin-responsive gene	*Nicotiana*	Röhrig et al., 1995

[a] Complete nodules are distinguished from other externally visible nodular structures resulting from the formation of nodule primordia by the formation of a completed vascular system. Nodular structures that do not contain complete vascular bundles or that are not described as such are categorized as nodule primordia.
[b] The particular effect has not been attributed to a particular cell type.

organogenesis (Table 1). Like in rhizobia-induced primordia, the position of the LCO-induced nodule primordia is either in the inner or outer cortex, depending on the plant species. Furthermore, as in plants that are infected by rhizobia, the primordia are only induced at certain positions in the plant root, namely, the position where young root hairs emerge and are frequently opposite the protoxylem poles of the central cylinder. In early stages the inner cortical nodule primordia can be distinguished from lateral root primordia (which arise in the pericycle) by their broad shape and lower cell density (Figure 2). In most plant species tested the nodule primordia induced by LCOs do not proceed further than the stages shown in the third panels of Figures 2B and 2C. The early initiation of nodule vascular bundle differentiation has been reported for *Glycine soja* (Stokkermans and Peters, 1994). The formation of full-size nodular structures, including fully completed vascular systems, after LCO-induction, has been shown for *Medicago* plants. This exceptional ability might be related to the occurrence of natural genotypes of *Medicago,* which are able to form root nodules in the absence of added nodulation factors (Truchet et al., 1989).

Little is known about factors of the plant that determine the position of the formation of nodule primordia. The gradient hypothesis offers an explanation for the local reaction of particular cortical cells to the LCOs by postulating that a variation in concentration of a plant factor determines that only particular cortical cells respond by cell division (Libbenga et al., 1973). A factor from the central stele, which stimulated cell division in pea cortical root explants grown in the presence of auxin and cytokinin, has been purified and was shown to be uridine (Smit et al., 1995). In this study, uridine, which is active at nanomolar concentrations, was proposed to be an important regulator of cell division in the nodulation process. In *Medicago*, the nodule primordia-inducing effect of LCO can be mimicked by the addition of other compounds such as the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) (Hirsch et al., 1989). Furthermore, *R. meliloti* mutant strains that are unable to produce LCO can be restored for their nodulation phenotype by the introduction of the

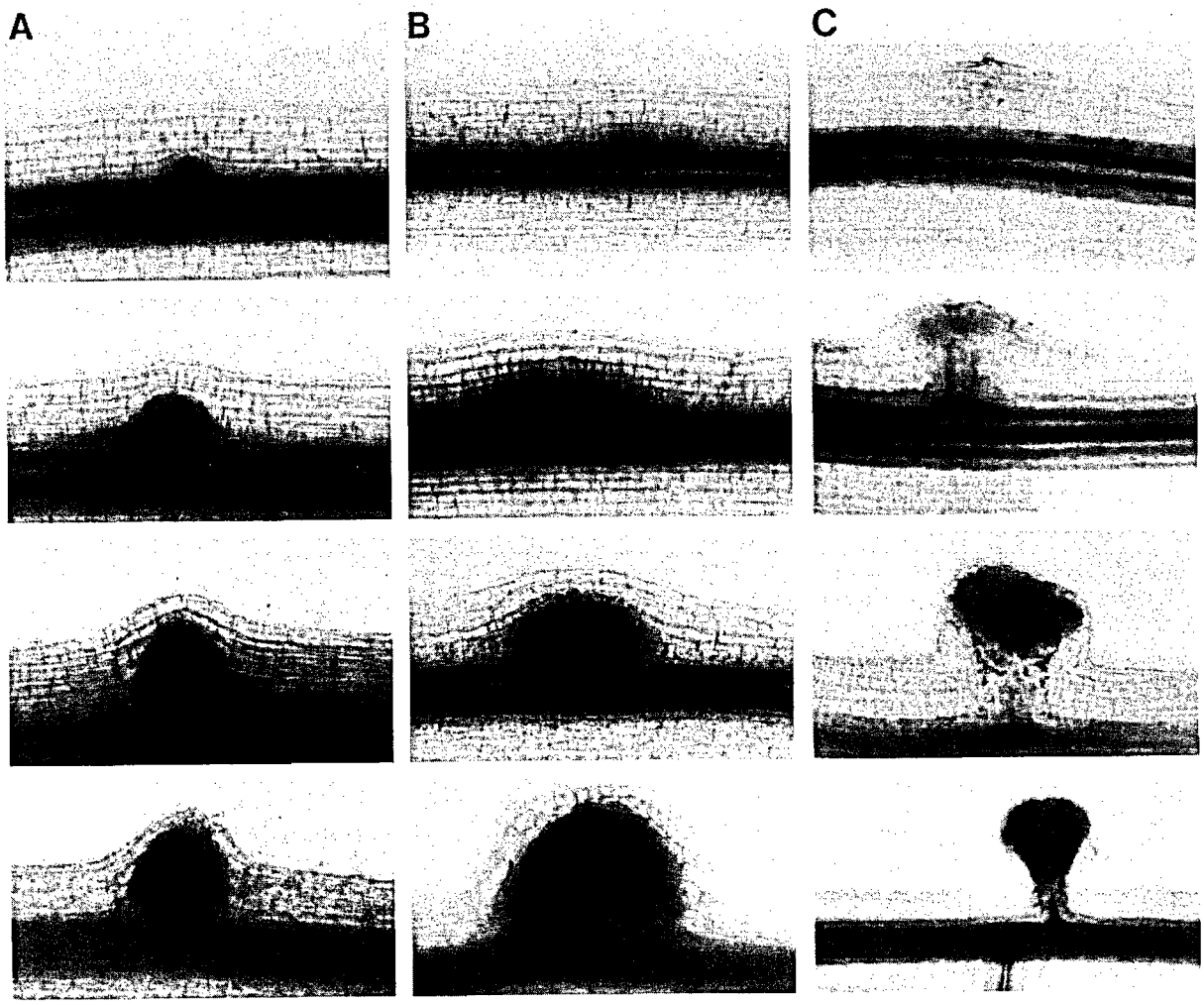

FIGURE 2. Various stages in the development of lateral roots (A), inner cortical root primordia (B), and outer cortical root primordia (C). Shown are roots of *Vicia hirsuta* (A and B) or *Phaseolus vulgaris* (C) bleached by hypochlorite treatment and stained with methylene blue according to the method of Truchet *et al*. Pictures are courtesy of Dr. A.A.N. van Brussel (Leiden University) and Dr. I.M. Lopez-Lara (Leiden University). For further details see (López-Lara et al., 1995b; Cardenas et al., 1995).

Agrobacterium tzs gene that results in the production of *trans*-zeatin (Cooper and Long, 1994). These results suggest that plant hormones other than uridine are also involved in the nodulation process.

At the genetic level, the induction of root nodule primordia by LCOs has been shown to be concomitant with the activation of various genes involved in the regulation of the cell cycle or responses that could be related to a plant defense. The latter responses have been reviewed recently in the context of other phytopathological aspects of infection and nodulation by rhizobia (Spaink et al., 1995). Savouré et al. (1994) have shown that LCOs also have various host-specific effects on gene expression in *Medicago* microcallus suspension cultures indicative for a stimulation of cell division and defense-related responses. Several plant genes,

such as *enod5* (Scheres et al., 1990b; Vijn et al., 1995b), *enod8* (Dickstein et al., 1993), *enod10* (Löbler and Hirsch, 1993), *enod12* (Bauer et al., 1994; Hirsch, 1992; Pichon et al., 1992; Scheres et al., 1990a; Vijn et al., 1995a), *enod40* (Asad et al., 1994; Crespi et al., 1994; Kouchi and Hata, 1993; Matvienko et al., 1994; Papadopoulis et al.; Roussis et al., 1995; Yang et al., 1993), *enodPRP4* (Wilson et al., 1994), *enodGRP3* (Kuster et al., 1995), and *didi-2,13,20* (Goormachtig et al., 1995) are differentially expressed during the early stages of nodule formation. Although the expression patterns of these so-called nodulin genes strongly suggest a possible function in infection or nodulation (or both processes), such a function has not been demonstrated. Csanadi et al. (1994) have obtained evidence that *enod12* is not required for the infection and nodulation processes. Introduction in *M. varia* of constructs that overexpress *enod40* resulted in overactive cell proliferation of the transformed explants (Crespi et al., 1994). Introduction of the antisense *enod40* construct resulted in an arrest of growth of the transformed explants. These results indicate that *enod40* plays an important role in plant development. Because significant conserved open reading frames were not found in *enod40* cDNAs of several plant species (Asad et al., 1994; Crespi et al., 1994; Matvienko et al., 1994) and because of the predicted capacity of *enod40* RNAs to form stable secondary structures (Crespi et al., 1994), it was suggested that they encode nontranslatable RNAs. However, recent comparisons by Vijn et al. (1995c) of the sequences of the *enod40* transcripts of *Vicia sativa, Pisum sativum, Medicago sativa,* and *Glycine max* show the presence of a conserved open reading frame encoding a peptide of 12 or 13 amino acids (consensus sequence: M**LCW***IHGS). Because these open reading frames are located close to the 5′ terminus of the mRNAs and are not preceded by another AUG start codon, the production of the predicted small polypeptides seems possible. The function of these open reading frames, as well as the remaining 3′ part (more than 90%) of the peculiar *enod40* mRNAs, remains to be elucidated.

III. STRUCTURES OF LCOs

The chemical structures of the LCOs produced by a large number of rhizobial strains have been determined (Figure 1; for references see legend). LCOs produced by all rhizobia consist of an oligosaccharide backbone of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc). A fatty acyl group is attached to the nitrogen of the nonreducing saccharide. Rhizobia appear to produce complex mixtures of LCO species. Differences in structures occur as the result of the following variations. (1) Variation in the number of GlcNAc units. Most commonly, LCOs varying in length between three and five GlcNAc units are produced. (2) Variation of the structure of the fatty acyl moieties attached. LCOs can be substituted by a variety of fatty acyl groups that also occur commonly as moieties of the phospholipids. In many cases the ratios of types of fatty acyl substituents found seems to reflect the composition of the fatty acyl pool present as components of the phospholipids. (3) The presence or absence of strain-specific substituents indicated as R1 to R6 in Figure 1. For instance, in a subset of LCO species produced by some rhizobia, an additional carbamyl unit can be attached to the nonreducing GlcNAc. (4) The presence or absence of special fatty acyl moieties. A special α,β-unsaturated fatty acyl moiety can be present in the LCOs produced by *R. meliloti* and *R. leguminosarum* biovars viciae and trifolii. In addition, C18-C26 (ω-1)-hydroxy fatty acyl, possible intermediates in the synthesis of C27 (ω-1)hydroxy fatty acyl groups found in the bacterial LPS, can be

present in the LCOs produced by *R. meliloti* (Demont et al., 1993). The relative abundance of LCOs containing a special fatty acyl (when compared with common fatty acyl moieties) in the mixtures produced is very different for *R. meliloti*, *R. leguminosarum* biovar vicia, and *R. leguminosarum* biovar trifolii (Bloemberg et al., 1995a; Demont et al., 1993; Demont et al., 1994; Firmin et al., 1993; Lerouge et al., 1990; Spaink et al., 1991; Spaink et al., 1995). Some researchers have not been able to detect LCOs that contain highly unsaturated fatty acyl moieties in *R. leguminosarum* biovar trifolii (Orgambide et al., 1995).

IV. BIOSYNTHESIS AND SECRETION OF LCOs

Rhizobial genes that are induced by the secretion of plant flavonoids or that have been shown to play a role in the nodulation process have arbitrarily been called *nod*, *nol*, and *noe* genes (for a review see Spaink, 1995). Several proteins encoded by these genes have been shown to play a role in the biosynthesis of LCOs (Table 2). For some of these proteins, detailed biochemical analyses have indicated their position in the biosynthetic pathway leading to the production of LCOs, including various strain-specific modifications (Figure 3). The NodC, NodB, and NodA proteins play a pivotal role in the synthesis of the LCO backbone structure by their presumed function as chitin oligosaccharide synthase, chitin oligosaccharide deacetylase, and acyl transferase, respectively (Atkinson et al., 1994; Geremia et al., 1994; John et al., 1993; Röhrig et al., 1994; Spaink et al., 1994b). These functions were demonstrated by *in vitro* enzymatic activity of purified protein in the case of the NodB protein only (John et al., 1993). Further biochemical studies on the function of NodC protein are in progress and could lead to further insights into the mechanism of chitin synthesis, a process that is still poorly understood (Deiannino et al., 1995; Kamst et al., 1995; Mergaert et al., 1995). The nature of the

TABLE 2
Biochemical Functions of Nod and Nol Proteins in the Biosynthesis of LCOs[a]

Protein	Function	Structural element	Ref.
NodA	Acyltransferase	Core structure	Atkinson et al., 1994; Röhrig et al., 1994
NodB	Chitin oligosaccharide deacetylase (D)	Core structure	John et al., 1993
NodC	Chitin oligosaccharide synthase (I)	Core structure	Deiannino et al., 1995; Geremia et al., 1994 Kamst et al., 1995
NodE	β-Ketoacylsynthase (I)	α,β unsat. acyl	Bibb et al., 1989; Spaink et al., 1991
NodF	Acyl carrier protein (D)	α,β unsat. acyl	Geiger et al., 1991; Ritsema et al., 1994
NodH	Sulfotransferase (D)	R6: sulfate	Ehrhardt et al., 1995; Schultze et al., 1995
NodL	Acetyltransferase (D)	R4: acetyl	Bloemberg et al., 1994
NodM	D-glucosaminesynthase (I)	Core structure	Baev et al., 1991; Marie et al., 1992
NodPQ	ATP sulfurylase and APS kinase[b](I)	R6: sulfate	Schwedock and Long, 1990
NodS	Methyltransferase (D)	R1: methyl	Jabbouri et al., 1995; Geelen et al., 1995
NodU	Carbamyltransferase (I)	R2–R4: carbamyl	Jabbouri et al., 1995; Geelen et al., 1996
NodX	Acetyltransferase (I)	R5: acetyl	Firmin et al., 1993
NodZ	Fucosyltransferase (D)	R5: fucose	Quinto et al., 1996
NolK	Sugar epimerase (I)	R5: fucose	Mergaert et al., 1996
NolL	Acetyltransferase (I)	R5: acetylfucose	Scott et al., 1995

[a] For the description of the substituents numbered R1 to R6 see Figure 1. Codes: I, function based on indirect evidence (e.g., homology studies, studies of LCO structures produced by mutants or complementation studies); D, function based on direct biochemical evidence using purified protein.

[b] These enzymes form subunits of a complex that leads to the production of the sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (Schwedock et al., 1994).

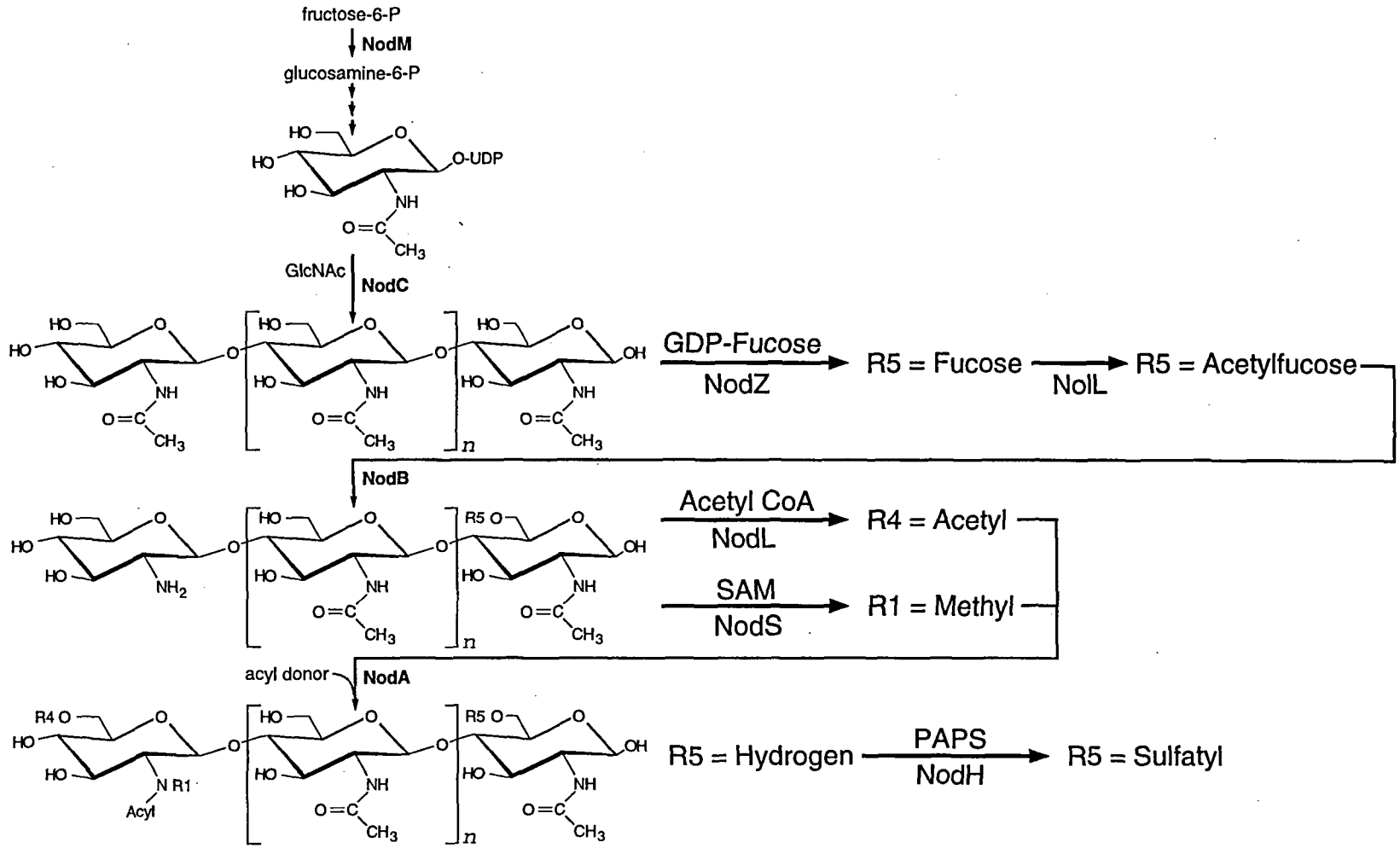

FIGURE 3. Model for the biosynthesis of LCOs. In the absence of strain-specific modifying enzymes, the groups R1, R4, and R5 represent hydrogen groups. The functions of the NodB from *R. meliloti* (John et al., 1993), NodZ from *B. japonicum* (Quinto et al., 1996), NodL from *R. leguminosarum* (Bloemberg et al., 1995b), NodS from *A. caulinodans* (Geelen et al., 1995), and NodH from *R. meliloti* (Schultze et al., 1995) proteins have been demonstrated by *in vitro* analysis of enzymatic activity of purified proteins. The functions of NodM from *R. meliloti* and *R. leguminosarum* (Baev et al., 1991; Marie et al., 1992), NodC from *R.fredii*, *A. caulinodans*, *R. leguminosarum* (Deiannino et al., 1995; Geremia et al., 1994; Kamst et al., 1995; Spaink et al., 1994b), NolL from *R. loti* (Scott et al., 1995), and NodA from *R. meliloti* and *R. leguminosarum* (Atkinson et al., 1994; Röhrig et al., 1994; Spaink et al., 1994b) have been inferred from indirect evidence.

substrate used by NodA as a source of the acyl moiety remains to be identified. It has been shown that NodA of *R. leguminosarum* is specialized for the transfer of particular fatty acyl groups (Ritsema et al., 1996).

Other plant-induced genes encode enzymes that are involved in the production of strain-specific modifications of the oligosaccharide backbone (Table 2). The enzymatic activity of purified protein was shown to be a transferase reaction in the cases of the NodL, NodH, NodS, and NodZ proteins (Figure 3; references are given in the legend). Several plant-induced proteins were shown to be involved in the synthesis of building blocks needed for the production of strain-specific modifications of the LCO. The NodF and NodE proteins probably function as an acyl carrier protein and a β-ketoacylsynthase, respectively, and are required for the biosynthesis of the special polyunsaturated fatty acid moieties shown in Figure 1. The NodP and NodQ proteins, which are encoded by members of a family of three sets of genes in *R. meliloti*, were shown to function together as sulfurylase and kinase, leading to the production of the sulfate donor of 3′-phosphoadenosine 5′-phosphosulfate (PAPS), which is used as a substrate for NodH. The NodM protein is a glucosamine synthase homologous to the chromosomally encoded glucosamine synthase (GlmS) counterpart. The presence of gene products that duplicate the normal household enzymatic functions, such as NodP and NodQ, is probably needed to overcome the limitation of building blocks needed for the biosynthesis of LCOs (Poupot et al., 1995b).

In most studies, LCOs were isolated from the spent culture for structural analysis, indicative for a secretion process (Bec-Ferté et al., 1994; Carlson et al., 1993; Demont et al., 1994; Firmin et al., 1993; Lerouge et al., 1990; Mckay and Djordjevic, 1993; Mergaert et al., 1993; Poupot et al., 1993; Poupot et al., 1995a; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Spaink et al., 1991; Spaink et al., 1992; Spaink et al., 1995). Because LCOs are hydrophobic amphipathic molecules, it can be speculated that they are present in the medium as multimeric forms or attached to small carrier compounds. Some LCO species have the tendency to accumulate in the membrane fraction (Mckay and Djordjevic, 1993; Orgambide et al., 1995), which was presumed to be a result of the formation of unsoluble complexes under overproduction conditions (Spaink et al., 1995; Spaink et al., 1995). Improved methods for the analysis of LCO secretion can overcome this technical problem (Cardenas et al., 1996; Fernandez-Lopez et al., 1996; Spaink et al., 1995). Using these methods, *nodI* and *nodJ* genes have been shown to play a role in the efficiency of secretion of LCOs (Spaink et al., 1995). NodI and NodJ proteins are similar to subunits of various ATP-dependent secretion protein complexes (Downie, 1991; Vásquez et al., 1993). Because NodT protein is similar to outer membrane proteins that form part of secretion complexes, it was suggested to be involved in LCO secretion in a complex with NodI and NodJ (Downie, 1994; Rivilla et al., 1995). More detailed studies on the secretion of LCOs could lead to the establishment of a useful model system for the study of secretion of small hydrophobic metabolites in general.

Several *nod*, *nol*, and *noe* genes apparently do not have a function in the synthesis or secretion of LCOs (e.g., Ardourel et al., 1995; Plazanet et al., 1995). Some of these genes, such as *nodO* of *R. leguminosarum* biovar *viciae* (Sutton et al., 1994) or the genes *nolXWBTUV* of *R. fredii* (Krishnan et al., 1995; Kovacs et al., 1995), play a role in the production or secretion of extracellular proteins.

V.FUNCTION OF STRUCTURAL DIVERSITY OF LCOs

The elicitation of root nodule formation by rhizobia is based on specific recognition, that is, each rhizobial strain has a characteristic host range and, conversely, each leguminous plant species has a characteristic guest range. The enormous number of different compatible rhizobial-plant interactions make general conclusions about determinants of specificity very difficult. The classification of two groups of guest bacteria (Figure 1), according to their natural association with indeterminate or determinate nodulating plant species, should therefore be regarded as tentative.

As outlined in the previous sections, all individual rhizobial strains produce a structurally diverse mixture of LCOs. In several strains this diversity can be attributed mainly to the diversity of the fatty acyl substituents. The LCOs produced by the rhizobia associated with indeterminate nodulating plants have in common that they can contain a special highly unsaturated fatty acyl moiety (Figure 1). The nature of the special fatty acyl moiety is determined by the *nodE* gene (Table 2), which has been shown to be a major determinant of host range (Spaink et al., 1989). Analysis of the activity of purified LCOs has demonstrated the importance of a highly unsaturated fatty acyl moiety in the induction of nodule primordia on *Vicia*, *Trifolium*, and *Medicago* (Table 1) (Bloemberg et al., 1995a; Truchet et al., 1991; Spaink et al., 1991). A NodH-determined sulfate group attached to the reducing terminal saccharide has been shown to be a major determinant of host range for *R. meliloti* (Lerouge et al., 1990; Roche et al., 1991a). This sulfate group is required to induce responses in the host plant *Medicago* (listed in Table 1) and to prevent activity on non-host plants such as *Vicia*.

The LCOs from bacteria that are isolated from plants that form determinate root nodules often contain an NodS-determined *N*-linked methyl substituent at the nonreducing saccharide (Figure 1) (Geelen et al., 1993; Geelen et al., 1995; Jabbouri et al., 1995). By using a gain of function approach, a NodZ-determined fucosyl residue (Figure 2) (López-Lara et al., 1996; Mergaert et al., 1996) was shown to extend host range of *R. leguminosarum* (a guest of the indeterminate nodulating plant *Vicia*) toward *Macroptilium* (a determinate nodulating host) (López-Lara et al., 1996). However, the LCOs of some broad host range bacteria, such as *R. fredii* or *R. tropicii* and *Rhizobium* strain GRH2 (which are known to nodulate a broad range of determinate nodule-forming plants species, including *Macroptilium*), do not seem to contain an *N*-methyl or additional saccharide moiety, respectively (Figure 1) (Bec-Ferté et al., 1994; Folch-Mallol et al., 1996; López-Lara et al., 1995b; Poupot et al., 1995a). These results indicate that specificity of nodulation of host plants such as *Macroptilium* cannot be attributed to a particular substituent or to a particular position of a substitution. Therefore, it can be hypothesized that different strain-specific substitutions, such as an *N*-methyl or fucosyl substituent, might have equivalent functions in signal transduction.

The following functions for strain-specific modifications are possible. (1) Protection against degradation by the host plant. LCOs are rapidly degraded by chitinases after contact with plants (Heidstra et al., 1994; Staehelin et al., 1994a; Staehelin et al., 1994b). The presence of specific modifications of LCOs has been shown to protect LCOs against degradation by the host plant (Staehelin et al., 1994b). Protection against degradation is therefore an obvious possible function of modifications of the chitin oligosaccharide backbone. (2) Optimalization of binding affinities for a specific plant receptor. Plant receptors for LCOs have not yet been identified making this possibility

entirely speculative. The absolute requirement of a sulfyl group to induce membrane depolarization in *Medicago* root hairs within several minutes indicates the presence of sulfyl-specific receptors (Felle et al., 1995). (3) Targeting of the LCOs or degradation products to receptors. Receptors for LCOs or degradation products might be difficult to reach from the positions where rhizobia make contact with the plant. Furthermore, the induction of root nodule primordia in the inner cortex when compared with the outer cortex (Figure 2) might force additional restraints on the structural requirements for the LCOs. Plant lectins might play a role in the facilitation of these entry processes, leading to a greater susceptibility for nonfunctional LCO molecules (Diaz et al., 1995). Recently, results were presented that indicate that the fatty acyl moiety of the LCOs play a role in targeting of the LCOs toward a receptor for chitin oligosaccharides (Spaink et al., 1994a).

III. INDICATIONS FOR GENERAL FUNCTIONS OF LCOs IN PLANT AND ANIMAL DEVELOPMENT

There are several indications that plants and animals are also able to produce and recognize signal molecules that structurally resemble the rhizobial lipo-chitin oligosaccharides. The most relevant indications are listed chronologically: (1) Schmidt et al. have shown that the *Rhizobium nodA* and *nodB* genes, when introduced singly or in combination into *Nicotiana* plants, have severe effects on plant development (Schmidt et al., 1993). A probable explanation for these results is that *nodA* (encoding an acyl transferase) and *nodB* (encoding a chitin oligosaccharide deacetylase) (Figure 3) interfere with the biosynthesis or structure of plant molecules that are involved in plant morphogenesis. These results also suggest that such plant molecule(s) have structural homology with the bacterial LCOs. A possible substrate for the NodA and NodB proteins in plants might be the recently discovered oligosaccharide moieties of nuclear pore complex glycoproteins or their precursors (Heese-Peck et al., 1995). (2) De Jong et al., 1993 have shown that the *Rhizobium* LCOs are able to rescue a temperature-sensitive somatic embryogenic mutant of *Daucus* (Table 1). After addition of the LCOs in nanomolar concentrations, the ability of the mutant to form embryos was restored. Chitin oligosaccharides were not active in this system, indicating that the fatty acyl moiety of the LCOs was essential for activity. However, the presence of other structural modifications, such as the *O*-acetyl moiety, did not influence activity. Complementation of the embryogenic mutant could be achieved as well by the addition of a 32-kDa endochitinase purified from wild-type *Daucus*. Because chitin and its derivatives are currently the only possible known candidate substrates for this enzyme, it is tempting to speculate that the function of this chitinase is to release LCO-like molecules from larger polymers produced by *Daucus* cells. (3) Chitinase-sensitive lipophilic molecules have been isolated from uninfected plant materials, such as flowers (Spaink et al., 1993). (4) Cell division of tobacco protoplasts is stimulated by synthetic LCOs (Röhrig et al., 1995). Furthermore, LCOs induce the expression of auxin-responsive promoters in these protoplasts. Because the used synthetic LCOs were active at concentrations up to 10^{-13} *M*, these results also indicate that nonleguminous plants can regulate cell division through high-affinity receptors for LCO molecules. (5) The significant similarity of NodC protein, responsible for the oligomerization of the sugar backbone of the LCO (Figure 3), with the DG42 protein, which is transiently expressed during embryogenesis of the frog, suggests that chitin-like molecules might even play a

role during embryogenesis in vertebrates (Bulawa and Wasco, 1991; Rosa et al., 1988; Sandal and Marcker, 1990). Recently, it was demonstrated that the frog or zebrafish DG42 protein *in vitro* can function as a chitin oligosaccharide synthase, giving further support for this hypothesis (Semino and Robbins, 1995; Semino et al., 1996).

VII. FUTURE PROSPECTS FOR THE STUDY OF LCO SIGNAL TRANSDUCTION

Considering the complexities of organogenesis, further study of the signal transduction pathways underlying the process of nodule formation faces many extremely difficult challenges. However, because LCOs, the key factors in root nodule formation, are expected to be of a general nature, any knowledge arising from further study of this system will be of great importance. Several general questions that have to be answered are discussed in this section. These questions can only be addressed by making optimal use of the tools arising from the studies outlined above in combination with various additional modern technologies mentioned below.

What is the fate of LCOs in plants and what is the nature of receptors for (L)COs? Methods for the radiolabeling of LCOs and chitin oligosaccharides to high specific activity have been reported and can be used to further study the characteristics of the reported binding sites of LCOs or chitin oligosaccharides (Baureithel et al., 1994; Bono et al., 1995; Bourdineaud et al., 1995). Furthermore, the techniques available for organic synthesis of LCOs will make it possible to design various derivatives containing fluorescent groups or photo-activated cross-linkers (Ikeshita et al., 1994; Nicolaou et al., 1992; Stokkermans et al., 1995; Tailler et al., 1994). When such derivatives become available, they will open the way for studies of the interaction of LCOs with plant components. In these studies, sophisticated techniques based on laser technology, such as fluorescent lifetime imaging microscopy (FLIM), are applicable (Gadella, Jr. et al., 1993). The role of degradation of LCOs could be studied elegantly when chemical synthesis of a variety of analogues of LCOs that are no longer susceptible to chitinase degradation will become possible.

Alternative approaches are based on genetic techniques. Loci of leguminous plants that have been shown to play a role in the response to LCOs, such as *sym2* (Kozik et al., 1995), could be analyzed further and might eventually lead to identification of gene(s) involved in recognition of LCOs. Because nonleguminous plants also seem to contain receptors for LCOs (De Jong et al., 1993; Röhrig et al., 1995; Staehelin et al., 1994a), *Arabidopsis* could be considered the most versatile system for identification of genes involved in the LCO signal transduction.

What is the function of substituents of the oligosaccharide backbone and chitin oligosaccharide chain length? The present knowledge of the biosynthesis and chemical synthesis of LCOs makes detailed analyses of the function of structural characteristics possible. Early responses of root hairs, detectable by electrophysiological techniques (Ehrhardt et al., 1992; Felle et al., 1995; Kurkdjian, 1995), are excellent markers for functional analyses. Further refinements of the used technology, for example, by the use of laser surgery (de Boer et al., 1995), might facilitate these studies. With respect to a function of substituents in targeting of the signal molecules, microballistic targeting techniques can be applied (Sautter et al., 1991; Spaink et al., 1994a).

How are the LCO signals transduced to changes in the cytoskeleton observed during root hair curling, infection thread for-

mation and nodule primordium formation? For this objective, future studies can make optimal use of the tools developed for studying signal transduction in animal systems. Various probes are available that might recognize components of signal transduction pathways in plants, such as MAP kinases, or for observing changes in the cytoskeleton at a molecular level (Aderem, 1992; Cooper, 1987; Heard and Dunn, 1995).

What is the function of the nodulin genes and which other plant genes are involved in nodulation? An obvious candidate for further studies is the *enod40* gene. Analysis of the function of a possibly translated polypeptide is presently under study (Vijn et al., 1995c). In order to identify other plant genes involved in nodulation, such as genes encoding unique transcription factors, screening procedure using reverse transcription PCR can be undertaken. Considering the possibilities of *Lotus japonicus* as a system for stable transformation (Handberg and Stougaard, 1992), promoter tagging approaches, as have been carried out in the model organism *Arabidopsis thaliana* (Koncz et al., 1992), are also technically possible. For these tagging experiments use could be made of reporter genes such as luciferase or green fluorescent protein (Haseloff and Amos, 1995).

What is the function of uridine and other plant hormones? These questions should be addressed at various stages of the research objectives described above. Considering the effects of auxin transport inhibitors in nodulation (Hirsch et al., 1989) active transport processes of hormones are expected to play a role in nodulation. If fluorescently labeled functional derivatives of hormones become available, they will be useful in future studies of transport in combination with confocal laser scanning microscopy and FLIM.

How general is the occurrence of LCOs and their biosynthetic genes? Research aimed at solving this question can make optimal profit from the knowledge obtained from the study of LCO biosynthesis in rhizobia (Figure 3; Table 2). Nod enzymes can be used for specific radioactive labeling of putative substrates present in plant or animal tissues or suspension cell cultures. Considering the differences in substrate specificities for the purified NodL, NodS, NodH, and NodZ proteins (Figure 3), a combination of these enzymes can be used to probe for various types of chitin oligosaccharide derivatives. The *NodC* and *NodB* genes belong to a multigene family for which a large number of genes have been identified (Spaink et al., 1993) (Dr. D. Kafetzopoulos, personal communication). Eukaryotic and prokaryotic members of the NodC and NodB families are sufficiently conserved to make the cloning of plant homologues using PCR technology seem possible.

What is the function of putative plant and animal signal molecules analogues to LCOs? Detailed expression studies performed with the *Xenopus laevis* DG42 gene have given strong indications for a role of DG42 in the gastrulation stage of embryogenesis (Rosa et al., 1988). Gene inactivation experiments are needed to demonstrate a function of DG42 in development of vertebrates. Identification of plant proteins homologues to NodC or NodB could be followed by gene inactivation experiments in *A. thaliana*. Suitable test systems for the study of somatic embryogenesis in *A. thaliana* are presently under development (Pillon et al., 1996). Recently, a class of nuclear pore complex glycoproteins have been identified in *Nicotiana tabacum* that contain a novel oligosaccharide that contains a terminal *N*-acetylglucosamine and that has a degree of polymerization of larger than five (Heese-Peck et al., 1995). It would be of interest whether these oligosaccharides are similar to the oligosaccharide backbone of the LCOs, as this could indicate possible similarities in function.

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