

Role of rhizobial lipo-chitin oligosaccharide signal molecules in root nodule organogenesis

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Abstract

The role of oligosaccharide molecules in plant development is discussed. In particular the role of the rhizobial lipo-chitin oligosaccharide (LCO) signal molecules in the development of the root nodule indicates that oligosaccharides play an important role in organogenesis in plants. Recent results of the analyses of structures and of the biosynthesis of the LCO molecules are summarized in this paper. The knowledge and technologies that resulted from these studies will be important tools for further studying the function of LCO signals in the plant and in the search for analogous signal molecules produced by plants.

Introduction

The mechanisms underlying the formation of organs are, although one of the most intriguing problems in biology, still poorly understood. In animals, various genes and signal molecules involved in the local differentiation and dedifferentiation processes leading to organogenesis have been identified. However, in plants far less is known about organogenesis. Although several plant hormones, such as auxin and cytokinin, have been known for decades and have been studied intensively, their role in plant differentiation is still very poorly understood at the molecular level. The fact that they play a general role in many – if not all – morphogenic processes of the plant, as well as their apparent lack of specificity at a molecular level, strongly suggests that, like in animals, other, more specific (as yet undiscovered)

signal molecules also have to play a role in plant development. During the last decade evidence has accumulated that several classes of oligosaccharides, called oligosaccharins, have strong effects on plant development (see [1, 17]). Recently, a novel class of oligosaccharin signal molecules has been discovered which plays a role in the host-specific interaction between rhizobial bacteria and leguminous plants leading to the nitrogen-fixing root nodules (see [22, 26, 61, 62, 71]). These signal molecules, which were shown to be lipo-chitin oligosaccharides (LCOs), are the first plant organogenesis-inducing factors discovered. Several results indicate that plants, and perhaps even animals, also use LCO analogues as signal molecules (see [62]). In this paper the role of the LCOs in plant development is discussed in the context of the oligosaccharin concept. Furthermore, the knowledge of the chemical struc-

tures and biosynthesis of LCOs is summarized since this will be an important tool in the future search for novel plant signal molecules.

The oligosaccharin concept

Oligosaccharins are defined as particular oligosaccharides which, at low concentrations, exert biological effects on plant tissue other than as carbon or energy sources [17]. The oligosaccharin concept emanates from the original discovery that a certain class of oligosaccharides acts as a potent elicitor of the plant defense response [4]. This concept was shown to be of a more general nature by the discoveries of various other classes of oligosaccharide elicitor molecules. Recently, it has been shown that even in animals, oligosaccharides can have a very strong signalling function (see [48]). This was demonstrated by Velupillai and Harn [70], who showed that the pentasaccharide LNFP-III produced by schistosome parasites is able to specifically trigger the production of cytokines by spleen B cells. For plants, the oligosaccharin concept is built around the assumption that hydrolytic enzymes of plant or parasitic origin are involved in the release of oligosaccharides from cell wall polysaccharides [17]. Indeed, some of the molecules derived by enzymatic treatment of cell wall material of the pathogenic fungus *Phytophthora megasperma* have been shown to be active elicitors of the hypersensitive response of the host plants. In this case the smallest active component appeared to be a branched heptasaccharide consisting of *D*-glucose [57]. Also in other cases oligosaccharins which elicit a defense response have been shown to be released by enzymatic treatment of cell wall material of the parasite or host plant [7, 11, 17, 23].

The function of oligosaccharins is not limited to that of signal molecules with a role in disease resistance. Some oligosaccharins have effects on plant development which are not obviously related to elicitor activity [39, 75]. Good examples are the xyloglucan-derived oligosaccharins which antagonize the growth promotion of pea stem

segments by auxin at nanomolar concentrations [27, 75]. Although the effects of xyloglucan oligosaccharides are well documented in *in vitro* systems it has not been shown whether such molecules indeed play a role in the growth of intact pea plants [1].

The recently discovered LCOs produced by rhizobial bacteria are by definition oligosaccharins since they elicit various discernable effects on plants at low concentrations (see below). One of the effects that LCOs have in common with other oligosaccharins is that they can induce various effects on suspension-cultured plant cells in a species-non-specific way which, for instance, can be measured using electrophysiological techniques ([1], B. van Duijn and H.P. Spaink, unpublished results). A published example for such an effect of LCOs is the transient alkalization of suspension cultures of tomato cells which occurs within 5 min after addition of LCOs to the culture medium [64]. However, LCOs differ from other classes of oligosaccharins discovered until now in the following respects: (1) LCOs are apparently not derived from a larger precursor by proteolytic cleavage; (2) the oligosaccharide is linked to a fatty acyl group; (3) LCOs are very plant species-specific in their activity on the (intact) host plant.

Structures and biosynthesis of rhizobial LCO

As indicated in Fig. 1, the LCOs produced by *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* bacteria, collectively called rhizobia, uniformly consist of an oligosaccharide backbone of β -1,4-linked *N*-acetyl-*D*-glucosamine, varying in length between three and five sugar units. To the nitrogen of the non-reducing sugar moiety a fatty acid group is attached, the structure of which is variable (see [22, 26, 62]). In the cases of the LCOs produced by *R. meliloti* [35] and *R. leguminosarum* biovar. *viciae* [60] a special α,β -unsaturated fatty acid moiety can be present (for an example see Fig. 1). In the LCOs of other rhizobial species such a polyunsaturated fatty acyl group is not present but instead fatty acyl moieties are found

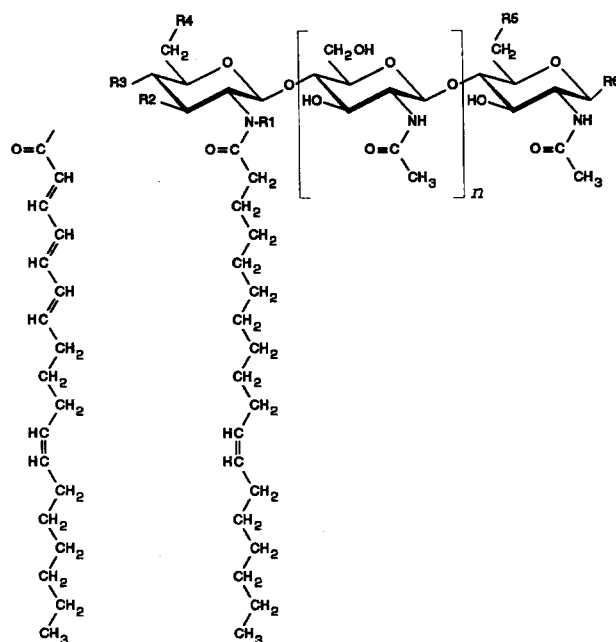


Fig. 1. Chemical structures of the rhizobial LCOs. The length of the chitin oligosaccharide backbone varies between 3 and 5 sugar units. The attached fatty acid shown is *cis*-vaccenic acid. At the left is indicated the highly unsaturated ($C_{18:4}$) moiety produced by *R. leguminosarum* biovar. *viciae* as an example of a special α,β -unsaturated fatty acyl moiety. The nature of other fatty acid moieties and of the substituents indicated by R1 to R5 is indicated in Table 1.

of the classes which also commonly occur in the phospholipids of the cell membrane. The presence of other substitutions on the chitin backbone is dependent on the rhizobial strain (Table 1). Substitutions which have been found are: sulphate, acetyl, carbamoyl, glycerol and sugar moieties such as arabinose, 2-*O*-methylfucose or fucose. The latter two moieties can also contain additional acetyl or sulphate modifications. In the LCOs of some species an *N*-linked methyl group can also be present.

Most of the proteins encoded by the rhizobial *nod* genes play a crucial role in the biosynthesis of the LCOs [61]. The NodA, NodB and NodC proteins, which are called common Nod proteins because they are present in all rhizobia and are not involved in the determination of host specificity, are sufficient for the production of a basic LCO structure [60]. Recent results have given strong indications that the NodC and NodB proteins function as a chitin synthase and a chitin deacetylase, respectively [3, 13, 14, 18, 32, 33, 63] (Fig. 2). Since the NodA protein is essential for the production of LCOs, this protein has been

Table 1. Comparison of LCO structures produced by various rhizobia¹.

Producing strain	Specific lipid	Other substituents	Reference
<i>R.l.</i> bv. <i>viciae</i> RBL5560	$C_{18:4}$	R4, <i>O</i> -acetyl	[60]
<i>R.l.</i> bv. <i>viciae</i> TOM	$C_{18:4}$	R4, <i>O</i> -acetyl; R5, <i>O</i> -acetyl	[25]
<i>R. meliloti</i> 2011	$C_{16:2}$	R4(+/-), <i>O</i> -acetyl; R5, sulphate	[35, 67]
<i>R. meliloti</i> AK41	$C_{16:2}$ or $C_{16:3}$	R5, sulphate	[54]
<i>Rhizobium</i> NGR234	-	R1, <i>N</i> -methyl, R2 and R3(+/-), <i>O</i> -carbamoyl R5, 2- <i>O</i> -methylfucose or 2- <i>O</i> -methyl-3- <i>O</i> -sulphofucose or 2- <i>O</i> -methyl-4- <i>O</i> -acetyl fucose	[42]
<i>R. tropicii</i> CFN299	-	R1, <i>N</i> -methyl; R5(+/-), sulphate	[41]
<i>R. fredii</i> USDA257	-	R5, 2- <i>O</i> -methylfucose or fucose	[8]
<i>B. japonicum</i> USDA110	-	R5, 2- <i>O</i> -methylfucose	[50]
<i>B. japonicum</i> USDA135	-	R4(+/-), <i>O</i> -acetyl; R5, 2- <i>O</i> -methylfucose	[15]
<i>B. japonicum</i> USDA61	-	R1(+/-), <i>N</i> -methyl; R2 or R3 or R4(+/-), carbamoyl; R4(+/-), <i>O</i> -acetyl; R5, 2- <i>O</i> -methylfucose or fucose; R6(+/-), glycerol	[15]
<i>A. caulinodans</i> ORS571	-	R1, <i>N</i> -methyl; R4, <i>O</i> -carbamoyl; R5, <i>D</i> -arabinose	[38]

¹ Reference is made to the groups indicated in Fig. 1. A minus indicates that no α,β -unsaturated fatty acyl group is present but a common fatty acyl group like the *cis*-vaccenic acid moiety indicated in Fig. 1. If not indicated otherwise, R1 stands for hydrogen and R2, R3, R4 and R5 stand for hydroxyl groups. (+/-) indicates that such a group is not always present. Abbreviation: *R.l.*, *R. leguminosarum*.

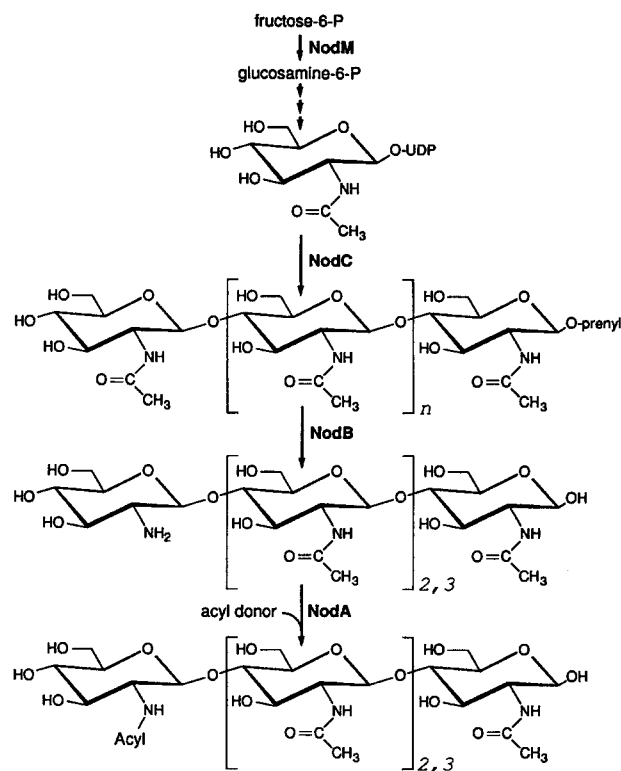


Fig. 2. Model for the functions for the NodA, NodB, and NodC proteins in the synthesis of rhizobial LCOs based upon data described in references [3, 13, 14, 18, 32, 33, 63]. The function of the NodM protein is postulated on basis of the results of Baev *et al.* [5] and Marie *et al.* [37]. The occurrence of oligosaccharide metabolites attached to a prenyl carrier postulated to be produced by NodC protein has not been confirmed by structural analysis. NodC protein has also been indicated to produce free chitin oligomers ($n = 1, 2, 3$) in low quantities [63]. In addition to its deacetylating activity, NodB plays a major role in determining the quantity of produced oligosaccharides [63].

postulated to be involved in the addition of the fatty acyl moiety [10] (Fig. 2).

Other Nod proteins are involved in the synthesis or addition of various structural modifications as indicated in Table 2. These functions are in good agreement with their important roles in the determination of host-specificity. For some of these gene products their enzymatic function has also been shown by using *in vitro* test systems. In *R. meliloti* the NodP and NodQ proteins were shown to function together as ATP sulphurylase and adenosine 5'-phosphosulphate (APS) kinase, leading to the production of the sulphate donor 3'-phosphoadenosine 5'-phosphosulphate (PAPS) [55, 56]. The NodH protein acts as a sulphotransferase involved in the transfer of the sulphate moiety of PAPS to the reducing terminal sugar of the LCO acceptor [2, 35, 46]. The NodL protein, which is produced by various rhizobial species, is an acetyl transferase which is involved in the addition of the *O*-acetyl moiety to the non-reducing terminal sugar [10]. In addition to showing the biochemical functions of these Nod proteins, these results from *in vitro* analyses have also yielded valuable systems for obtaining radiolabelled derivatives of the LCO molecules which can be used in future studies devoted to their function in the plant.

Effects of LCOs on the host plant

At micromolar concentrations, externally applied purified LCO molecules can elicit in the inner

Table 2. *nod* or *nol* genes which have been shown to be involved in the addition of LCO substituents.

LCO substituent ¹	Gene involved	Reference
α,β -unsaturated fatty acid	<i>nodF</i> and <i>nodE</i>	[21, 29, 58, 60]
Sulphate (R5)	<i>nodP</i> , <i>nodQ</i> and <i>nodH</i>	[2, 46, 55, 56]
<i>O</i> -acetyl (R4)	<i>nodL</i>	[10, 60]
<i>N</i> -methyl (R1)	<i>nodS</i>	[28]
<i>O</i> -acetyl (R5)	<i>nodX</i>	[25]
2- <i>O</i> -methylfucose (R5)	<i>nodZ</i>	[65]

¹ Reference is made to the R groups indicated in Fig. 1.

cortex the formation of nodule primordia which are indistinguishable from the nodule primordia in the first stage of normal nodule organogenesis [60, 67]. Furthermore, as in plants which are infected by rhizobia, the primordia are only induced at certain positions in the plant root, namely the position where young root hairs emerge, opposite (or almost opposite) the protoxylem poles of the central cylinder [60, 67]. In the case of *Medicago* the nodule primordia are capable of further developing into full-grown nodules which have the anatomical and histological features of genuine rhizobium-induced nodules, such as apical meristems and peripheral vascular bundles and endodermis [67]. In *Vicia* this was never observed but instead the development of the nodules stops at a stage at which small outgrowths are externally visible on the roots [62]. Besides their role in the formation of the root nodule primordia, LCOs also seem to be involved in the bacterial infection process, as suggested by the induction of pre-infection thread structures in the outer cortex of *Vicia* roots by mitogenic LCOs in the absence of bacteria [69]. These pre-infection thread structures are characterized by the formation of so-called cytoplasmic bridges in the outer cortex which are radially aligned, giving the impression of cytoplasmic threads which cross the outer cortex. The formation of these structures, which are indistinguishable from those observed after infection with *R. leguminosarum* biovar. *viciae* bacteria, always precedes the formation of infection threads, and therefore they were named pre-infection thread structures. The formation of cytoplasmic bridges in vacuolated cells is preceded by polarization of the cell in which the nucleus moves to the centre of the cell just as in cells which are about to divide [6]. The process of pre-infection thread formation can therefore be interpreted as being the result of activation of the cell cycle as is the case of the formation of the nodule primordium in the inner cortex. The final result apparently is determined by the position of the cells in the cortex. An explanation for the local reaction of particular cortical cells to the rhizobial signals is given by the gradient hypothesis which postulates that a variation in concentration of a

plant factor determines that only particular cortical cells respond towards the rhizobial signals [36, 69]. A factor from the central stele, which stimulated cell division in pea root explants at nanomolar concentrations has now been purified in our institute and was shown to be uridine ([59], G. Smit and J. Kijne, personal communication).

External application of LCOs, in concentrations varying between 10^{-8} and 10^{-12} M, can also elicit effects on root hairs of the respective host plants (see [22, 26, 61]). These effects, such as depolarization of membrane potential [24], curling, branching and swelling of the root hairs are probably related to the process of root hair curling which is also observed very early during the rhizobial infection process. Although the biological relevance of these phenotypes is not yet clear they have been shown to be very useful as semi-quantitative bioassays [30, 45].

At the molecular genetic level, several effects of LCO signals are observed which also occur during the rhizobial infection process. These effects include the induction of nodulin gene expression, for instance of the early nodulins ENOD12, ENOD5 and ENOD40, of which the expression in time and place is strongly correlated with the early steps in the symbiosis [31, 34, 52, 71, 74]. Transgenic plants which contain ENOD12-GUS reporter gene fusions have been constructed, providing a valuable molecular marker for studying LCO signal transduction in the plant [40]. Another effect of LCOs is the induction of flavonoid synthesis genes such as those encoding phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS). Flavonoid synthesis is condition-dependent since it is only detectable in roots not shielded from light [43, 44, 60, 68]. This induction process is correlated with the production of various new flavonoids which are capable of inducing the transcription of the *nod* genes [43, 44]. Savouré *et al.* [51] have shown that the cognate (*R. meliloti*) LCO signals also have various host-specific effects on gene expression in *Medicago* microcallus suspension cultures. At nanomolar concentrations a host-specific effect on the cell cycle was observed as was demonstrated by an increased expression of histone *H3-1*, *cdc2Ms*

and the cyclin encoding gene *cycMs2*. A stimulation of the cell cycle was also indicated by enhanced thymidine incorporation, elevated number of S-phase cells and an increase in kinase activity of p34^{cdc2}-related complexes [51]. At higher concentrations (10^{-6} M) LCOs also induced the expression of the flavonoid synthesis gene encoding isoflavone reductase (IFR) [51].

There are several structural requirements that an LCO molecule has to fulfil in order to elicit biological effects on plant roots. The necessity of substituents, such as *O*-acetyl [60], sulphate [46] or 2-*O*-methylfucose [65], is dependent on the type of bioassay and the plant species tested. The presence of the fatty acyl substituent seems always to be required since chitin oligomers are inactive in several of the above-mentioned bioassays (see [61]). The host-specific unsaturated fatty acyl substituents are required in order to obtain nodule primordia on the roots of *Medicago* and *Vicia* plants [60, 67]. However, a special fatty acyl moiety is not required for other effects such as root hair deformation. Surprisingly, the presence of a fatty acyl substituent seems neither to be required to obtain nodule primordia when chitin oligosaccharides are delivered by ballistic microtargeting into the plant tissue (C. Sautter and H.P. Spänk, unpublished results). These results suggest that the fatty acyl group is involved in the delivery of the signal molecules inside the plant tissue.

Do plants produce chitin-derived signal molecules?

There are several indications that plants contain signal molecules that structurally resemble the rhizobial lipo-oligosaccharides.

1. In alfalfa a certain proportion of wild-type plants can spontaneously develop genuine root nodule structures in the absence of *Rhizobium* bacteria [66]. Since the number of root nodules as well as their position on the root are indistinguishable from those observed in the infected situation, this indicates that the plant is able to trigger the genes involved in the nodule formation process in the same way as *Rhizobium* bacteria

do. Therefore it is possible that similar signal molecules are involved in the induction process in both cases.

2. Schmidt *et al.* [53] have shown that the *Rhizobium nodA* and *nodB* genes, when introduced singly or in combination into *Nicotiana* plants, have severe effects on plant development. One of the effects which was observed is that *nodB*-containing transgenic plants have abnormally formed leaves and flowers. Since these *nod* genes have an essential function in the biosynthesis of LCOs (Fig. 2), these results indicate that these *nod* genes interfere with the biosynthesis or structure of plant molecules which are involved in plant morphogenesis. They also suggest that such plant molecule(s) have structural homology with the bacterial LCOs.

3. De Jong *et al.* [20] have shown that the *Rhizobium* LCOs are able to rescue a temperature-sensitive somatic embryogenic mutant of *Daucus*. After addition of the LCOs in nanomolar concentrations, the ability of the mutant to form embryos was restored. In this heterologous test system the fatty acyl moiety of the LCOs was essential for activity. However, the presence of other structural modifications, like the *O*-acetyl moiety, did not influence activity [20]. Complementation of the embryogenic mutant could also be achieved by the addition of a 32 kDa endochitinase purified from wild-type *Daucus* [19]. Since 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. The observation that the expression of several other plant chitinases is correlated with plant development also indicates that chitin-like molecules occur in uninfected plants and could play a role in plant development (see [16, 62]).

Preservation of chitin-synthesis ability in various organisms

Since rhizobial bacteria and perhaps also plants are able to synthesize chitin oligosaccharides it is

tempting to speculate about a general occurrence of chitin in nature. Hardly anything is known about the occurrence of chitin derivatives in plants. In immunogold-labelling studies, using chitinase or wheat germ agglutinin as probes, Benhamou and Asselin [9] have obtained results which suggest that lipophilic chitin derivatives also occur in secondary plant cell walls of various plant species. Furthermore, using radioactive labelling studies we have recently obtained evidence that lipophilic molecules, which are susceptible to chitinase degradation, also occur in flowering *Lathyrus* plants ([62], Spaink *et al.*, unpublished results). In animals, improved methodologies for detecting chitin and chitin synthase genes has yielded results which also show that the classical notion that chitin only occurs in fungi and non-deuterostome animal taxa should be revised [73]. This is clearly indicated by the finding of chitin in the pectoral fins of the fish *Paralipophrys* [72]. The significant similarity of NodC protein, responsible for the oligomerization of the sugar backbone of the LCO (Fig. 2), with the DG42 protein, which is transiently expressed during embryogenesis of the frog [12, 47, 49, 62] suggests that chitin-like molecules might even play a role during embryogenesis in vertebrates.

Future prospects

The rhizobial LCO molecules are the first discovered examples of a novel class of signal molecules involved in plant organogenesis. Several lines of evidence indicate that plants and animals also produce chitin-derived oligosaccharide molecules. There are even indications that these molecules might play a role in the embryogenesis of plants as well as animals. Since plants are different from animals in that plants are continuously able to form new organs, it is not too far-fetched to speculate upon a generally conserved role of LCOs in the establishment of cell polarity and cell division, leading to the formation of new organs. The knowledge and the tools which have resulted from the study of the signal exchange in the nodulation process will be useful in the future search

for such putative novel plant and animal signal molecules.

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