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

Spaink, H.P.; Lugtenberg, E.J.J.

Plant Molecular Biology **26**: 1413–1422, 1994.

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

Herman P. Spaink* and Ben J.J. Lugtenberg
*Institute of Molecular Plant Sciences, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands (*author for correspondence)*

Received 1 April 1994; accepted in revised form 26 April 1994

Key words: plant-microbe interactions, nod metabolites, *nod* genes, oligosaccharins

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 east 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 alkalinization 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

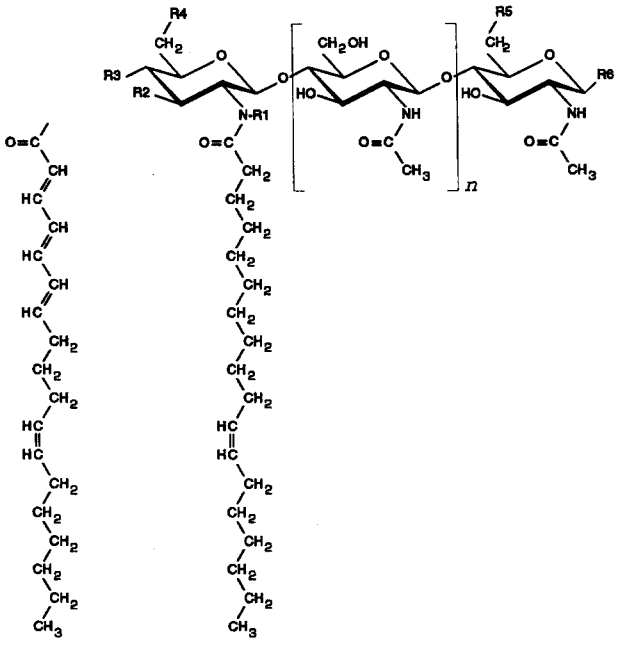

Fig. 1. Chemical structures of the rhizobial LCOs. The length of the chitin oligosaccharide backbone varies between 3 and 5 sugar units. The attached fatty acid shown is *cis*-vaccenic acid. At the left is indicated the highly unsaturated ($C_{18:4}$) moiety produced by *R. leguminosarum* biovar. *viciae* 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 rhizobia[1].

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

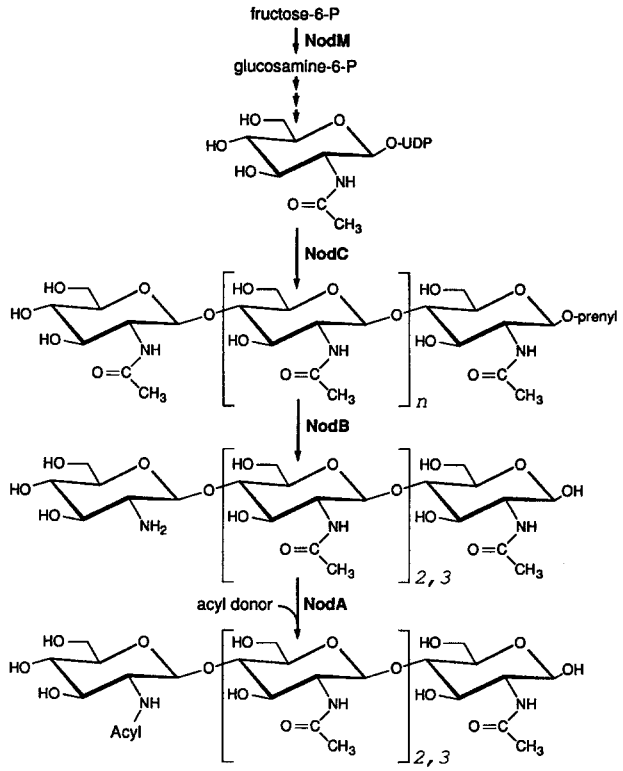

Fig. 2. Model for the functions for the NodA, NodB, and NodC proteins in the synthesis of rhizobial LCOs based upon data described in references [3, 13, 14, 18, 32, 33, 63]. The function of the NodM protein is postulated on basis of the results of Baev *et al.* [5] and Marie *et al.* [37]. The occurence 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	*nodF* and *nodE*	[21, 29, 58, 60]
Sulphate (R5)	*nodP*, *nodQ* and *nodH*	[2, 46, 55, 56]
O-acetyl (R4)	*nodL*	[10, 60]
N-methyl (R1)	*nodS*	[28]
O-acetyl (R5)	*nodX*	[25]
2-*O*-methylfucose (R5)	*nodZ*	[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 fullfil 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. Spaink, 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 occurence 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.

Acknowledgements

We thank Drs Jan Kijne (Leiden University), Gerrit Smit (Leiden University), Bert van Duijn (Leiden University) and Christof Sautter (ETH, Zürich) for communicating unpublished results. We are grateful to Drs Jane Thomas-Oates (Utrecht University) and Helmi Schlaman (Leiden University) for critically reading the manuscript. Herman Spaink was supported by the Royal Netherlands Academy of Arts and Sciences.

References

1. Aldington S, Fry SC: Oligosaccharins. Adv Bot Res 19: 1–101 (1993).
2. Atkinson EM, Ehrhardt DW, Long SR: *In vitro* activity of the *nodH* gene product from *Rhizobium meliloti*. In: Sixth International Symposium on Molecular Plant-Microbe Interactions, Program and Abstracts, abstract 45. University of Washington, Seattle, WA (1992).
3. Atkinson EM, Long SR: Homology of *Rhizobium meliloti* NodC to polysaccharide polymerizing enzymes. Mol Plant-Microbe Interact 5: 439–442 (1992).
4. Ayers AR, Ebel J, Finelli F, Berger N, Albersheim P: Host-pathogen interactions. IX. Quantitative assays of elicitor activity and characterization of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. Plant Physiol 57: 751–759 (1976).
5. Baev N, Endre G, Petrovics G, Banfalvi Z, Kondorosi A: Six nodulation genes of nod box locus 4 in *Rhizobium meliloti* are involved in nodulation signal production: nodM codes for *D*-glucosamine synthetase. Mol Gen Genet 228: 113–124 (1991).
6. Bakhuizen R: The plant cytoskeleton in the *Rhizobium*-legume symbiosis. Ph.D. thesis, Leiden University, Netherlands (1988).
7. Barber MS, Bertram RE, Ride JP: Chitin oligosaccharides elicit lignification in wounded wheat leaves. Physiol Mol Plant Path 34: 3–12 (1989).
8. Bec-Ferté MP, Savagnac A, Pueppke SG, Promé JC: Nod factors from *Rhizobium fredii* USDA257. In: Palacios R, Mora J, Newton WE (eds) New Horizons in Nitrogen Fixation, pp. 157–158. Kluwer Academic Publishers, Dordrecht (1993).
9. Benhamou N, Asselin A: Attempted localization of a sub-

strate for chitinases in plant cells reveals abundant *N*-acetyl-D-glucosamine residues in secondary walls. Biol Cell 67: 341–350 (1989).
10. Bloemberg GV, Thomas-Oates JE, Lugtenberg BJJ, Spaink HP: Nodulation protein NodL of *Rhizobium leguminosarum* *O*-acetylates lipo-oligosaccharides, chitin fragments and *N*-acetylglucosamine *in vitro*. Mol Microbiol 11: 793–804 (1994).
11. Bowles DJ: Defense-related proteins in higher plants. Ann Rev Biochem 59:873–907 (1990).
12. Bulawa CE, Wasco W: Chitin and nodulation. Nature 353: 710 (1991).
13. Bulawa CE: CSD2,CSD3 and CSD4, genes required for chitin synthesis in *Saccharomyces cerevisiae*: the CSD2 gene product is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. Mol Cell Biol 12: 1764–1776 (1992).
14. Bulawa CE: Genetics and molecular biology of chitin synthesis in fungi. Annu Rev Microbiol 47: 505–534 (1993).
15. Carlson RW, Sanjuan J, Bhat R, Glushka J, Spaink HP, Wijfjes HM, van Brussel AN, Stokkermans TJW, Peters K, Stacey G: The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and type II strains of *Bradyrhizobium japonicum*. J Biol Chem 268: 18372–18381 (1993).
16. Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K: Plant chitinases. Plant J 3: 31–40 (1993).
17. Davis KR, Darvill AG, Albersheim P, Dell A: Host-pathogen interactions. XXIX. Oligogalacturonides released from sodium polypectate by endopolygalacturonic acid lyase are elicitors of phytoalexins in soybean. Plant Physiol 80: 568–577 (1986).
18. Debellé F, Rosenberg C, Dénarié J: The *Rhizobium, Bradyrhizobium* and *Azorhizobium* NodC proteins are homologous to yeast chitin synthases. Mol Plant-Microbe Interact 3: 317–326 (1992).
19. de Jong AJ, Cordewener J, Lo Schiavo F, Terzi M, Vandekerckhove J, Van Kammen A, de Vries S: A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4: 425–433 (1992).
20. de Jong AJ, Heidstra R, Spaink HP, Hartog MV, Hendriks T, Lo Schiavo F, Terzi M, Bisseling T, Van Kammen A, de Vries S: A plant somatic embryo mutant is rescued by rhizobial lipo-oligosaccharides. Plant Cell 5: 615–620 (1993).
21. Demont N, Debellé F, Aurelle H, Dénarié J, Promé JC: Role of the *Rhizobium meliloti* nodF and nodE genes in the biosynthesis of lipo-oligosaccharidic nodulation factors. J Biol Chem 268: 20134–20142 (1993).
22. Dénarié J, Cullimore J: Lipo-oligosaccharide nodulation factors: a new class of signalling molecules mediating recognition and morphogenesis. Cell 74: 951–954 (1993).
23. Deverall BJ, Deakin AL: Genetic tests of the basis of wheat cultivar selectivity in symptom elicitation by preparations from rust pathogens. Physiol Mol Plant Path 30: 225–232 (1987).
24. Ehrhardt DW, Atkinson EM, Long SR: Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science 256: 998–1000 (1992).
25. Firmin JL, Wilson KE, Carlson RW, Davies AE, Downie JA: Resistance to nodulation of cv. Afghanistan peas is overcome by *nodX*, which mediates an *O*-acetylation of the *Rhizobium leguminosarum* lipo-oligosaccharide nodulation factor. Mol Microbiol 10: 351–360 (1993).
26. Fisher RF, Long SR: *Rhizobium*-plant signal exchange. Nature 357: 655–660 (1992).
27. Fry SC, Aldington S, Hetherington PR, Aitken J: Oligosaccharides as signals and substrates in the plant cell wall. Plant Physiol 103: 1–5 (1993).
28. Geelen D, Mergaert P, Geremia RA, Goormachtig S, Van Montagu M, Holsters M: Identification of *nodSUIJ* genes in locus *1* of *Azorhizobium caulinodans*: evidence that *nodS* encodes a methyltransferase involved in Nod factor modification. Mol Microbiol 9: 145–154 (1993).
29. Geiger O, Spaink HP, Kennedy EP: Isolation of *Rhizobium leguminosarum* NodF nodulation protein: NodF carries a 4′-phosphopantetheine prosthetic group. J Bact 173: 2872–2878 (1991).
30. Heidstra R, Geurts R, Franssen H, Spaink HP, Van Kammen A, Bisseling T: A semi-quantitative root hair deformation assay to study the activity and fate of Nod factors. Plant Physiol 105: 787–797 (1994).
31. Horvath B, Heidstra R, Lados M, Moerman M, Spaink HP, Promé J-C, Van Kammen A, Bisseling T: Induction of pea early nodulin expression by Nod factors of *Rhizobium*. Plant J 4: 727–733 (1993).
32. John M, Röhrig H, Schmidt J, Wieneke U, Schell J: *Rhizobium* NodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. Proc Natl Acad Sci USA 90: 625–629 (1993).
33. Kafetzopoulos D, Thireos G, Vournakis JN, Bouriotis V: The primary structure of a fungal chitin deacetylase reveals the function for two bacterial gene products Proc Natl Acad Sci USA 90: 8005–8008 (1993).
34. Kouchi H, Hata S: Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol Gen Genet 238: 106–119 (1993).
35. Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé JC, Dénarié J: Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344: 781–784 (1990).
36. Libbenga KR, Van Iren F, Bogers RJ, Schraag-Lamers MF: The role of hormones and gradients in the initiation of cortex proliferation and nodule formation in *Pisum sativum* L. Planta 114: 19–39 (1973).
37. Marie C, Barny M-A, Downie JA: *Rhizobium leguminosarum* has two glucosamine synthases, GlmS and NodM,

required for nodulation and development of nitrogen fixing nodules. Mol Microbiol 6: 843–851 (1992).
38. Mergaert P, Van Montagu M, Promé J-C, Holsters M: Three unusual modifications, a *D*-arabinosyl, a *N*-methyl, and a carbamoyl group, are present on the Nod factors of *Azorhizobium caulinodans* strain ORS571. Proc Natl Acad Sci USA 90: 1551–1555 (1993).
39. Mohnen D, Eberhard S, Marfà V, Doubrava N, Toubart P, Gollin DJ, Gruber TA, Nuri W, Albersheim P, Darvill AG: The control of root, vegetative shoot and flower morphogenesis in tobacco thin cell-layer explants (TCLs). Development 108: 191–201 (1990).
40. Pichon M, Journet EP, Dedieu A, de Billy F, Truchet G, Barker DG: *Rhizobium meliloti* elicits transient expression of the early nodulin gene *ENOD*12 in the differentiating root epidermis of transgenic alfalfa. Plant Cell 40: 1199–1211 (1992).
41. Poupot R, Martinez-Romero E, Promé J-C: Nodulation factors from *Rhizobium tropici* are sulphated or non-sulphated chitopentasaccharides containing an *N*-methyl-*N*-acylglucosaminyl terminus. Biochemistry 32: 10430–10435 (1993).
42. Price NPJ, Relić B, Talmont F, Lewin A, Promé D, Pueppke SG, Maillet F, Dénarié J, Promé J-C, Broughton WJ: Broad-host-range *Rhizobium* species strain NGR234 secretes a family of carbamoylated, and fucosylated, nodulation signals that are *O*-acetylated or sulphated Mol Microbiol 6: 3575–3584 (1992).
43. Recourt K: Flavonoids in the early *Rhizobium*-legume interaction. Ph.D. thesis, Leiden University, Netherlands (1991).
44. Recourt K, Schripsema J, Kijne JW, Van Brussel AAN, Lugtenberg BJJ: Inoculation of *Vicia sativa* subsp. *nigra* roots with *R. leguminosarum* biovar. *viciae* results in release of *nod* gene activating flavanones and chalcones. Plant Mol Biol 16: 841–852 (1991).
45. Relic B, Talmont F, Kopcinska J, Golinowski W, Promé J-C, Broughton WJ: Biological activity of *Rhizobium* sp. NGR234 Nod-factors on *Macroptilium atropurpureum*. Mol Plant-Microbe Interact 6: 764–774 (1993).
46. Roche P, Debellé F, Maillet F, Lerouge P, Faucher C, Truchet G, Dénarié J, Promé JC: Molecular basis of symbiotic host specificity in *Rhizobium meliloti*: *nodH* and *nodPQ* genes encode the sulphation of lipo-oligosaccharide signals. Cell 67: 1131–1143 (1991).
47. Rosa F, Sargent TD, Rebbert ML, Michaels GS, Jamrich M, Grunz H, Jonas E, Winkles JA, Dawid IB: Accumulation and decay of DG42 gene products follow a gradient pattern during *Xenopus* embryogenesis. Devel Biol 129: 114–123 (1988).
48. Ryan CA: Oligosaccharide signals: From plant defense to parasite offense. Proc Natl Acad Sci USA 91: 1–2 (1994).
49. Sandal NN, Marcker KA: Some nodulin and Nod proteins show similarity to specific animal proteins. In: Gresshoff PM, Roth LE, Stacey G, Newton WE (eds) Nitrogen Fixation: Achievements and Objectives, pp. 687–692. Chapman and Hall, New York (1990).
50. Sanjuan J, Carlson RW, Spaink HP, Bhat UR, Barbour WM, Glushka J, Stacey G: A 2-*O*-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. Proc Natl Acad Sci USA 89: 8789–8793 (1992).
51. Savouré A, Magyar Z, Pierre M, Brown S, Schultze M, Dudits D, Kondorosi A, Kondorosi E: Activation of the cell cycle machinery and the isoflavonoid biosynthesis pathway by active *Rhizobium meliloti* Nod signal molecules in *Medicago* microcallus suspensions. EMBO J 13: 1093–1102 (1994).
52. Scheres B, Van de Wiel C, Zalensky A, Horvath B, Spaink HP, Van Eck H, Zwartkruis F, Wolters A-M, Gloudemans T, Van Kammen A, Bisseling T: The ENOD12 gene product is involved in the infection process during the pea-*Rhizobium* interaction. Cell 60: 281–294 (1990).
53. Schmidt J, Röhrig H, John M, Wieneke U, Stacey G, Koncz C, Schell J: Alteration of plant growth and development by *Rhizobium nodA* and *nodB* genes involved in the synthesis of oligosaccharide signal molecules. Plant J 4: 651–658 (1993).
54. Schultze M, Quiclet-Sire B, Kondorosi E, Virelizier H, Glushka JN, Endre G, Géro SD, Kondorosi A: *Rhizobium meliloti* produces a family of sulphated lipo-oligosaccharides exhibiting different degrees of plant host specificity. Proc Natl Acad Sci USA 89: 192–196 (1992).
55. Schwedock J, Long SR: ATP sulphurylase activity of the *nodP* and *nodQ* gene products of *Rhizobium meliloti*. Nature 348: 644–647 (1990).
56. Schwedock J, Long SR: *Rhizobium meliloti* genes involved in sulphate activation: The two copies of *nodPQ* and a new locus *saa*. Genetics 132: 899–909 (1992).
57. Sharp JK, McNeil M, Albersheim P: The primary structures of one elicitor-active and seven elicitor-inactive hexa(β-*D*-glucopyranosyl)-*D*-glucitols isolated from the mycelian walls of *Phytophthora megasperma* f.sp. *glycinea*. J Biol Chem 259: 11321–11336 (1984).
58. Shearman CA, Rossen L, Johnston AWB, Downie JA: The *Rhizobium leguminosarum* nodulation gene *nodF* encodes a polypeptide similar to acyl-carrier protein and is regulated by *nodD* plus a factor in pea root exudate. EMBO J 5: 647–652 (1986).
59. Smit G, Lugtenberg BJJ, Kijne JW: Isolation of nodulation factor from the stele of the roots of *Pisum sativum*. In: Halick RB (ed) Molecular Biology of Plant Cell Growth and Development, Program and Abstracts, abstract 1427. Department of Biochemistry, Tucson, AR (1991).
60. Spaink HP, Sheeley DM, Van Brussel AAN, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJJ: A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354: 125–130 (1991).

61. Spaink HP: Rhizobial lipo-oligosaccharides: answers and questions. Plant Mol Biol 20: 977–986 (1992).
62. Spaink HP, Wijfjes AHM, Van Vliet, TB, Kijne JW, Lugtenberg BJJ: Rhizobial lipo-oligosaccharide signals and their role in plant morphogenesis: are analogous lipophilic chitin derivatives produced by the plant? Aust J Plant Physiol 20: 381–392 (1993).
63. Spaink HP, Wijfjes AHM, Van der Drift KMGM, Haverkamp J, Thomas-Oates JE, Lugtenberg BJJ: Structural identification of metabolites produced by the NodB and NodC proteins of *Rhizobium leguminosarum*. Mol Microbiol 13: 821–831 (1994).
64. Staehelin C, Granado J, Müller J, Wiemken A, Mellor RB, Felix G, Regenass M, Broughton WJ, Boller T: Perception of *Rhizobium* nodulation factors by tomato cells and inactivation by root chitinases. Proc Natl Acad Sci USA 91: 2196–2200 (1994).
65. Stacey G, Luka S, Sanjuan J, Banfalvi Z, Nieuwkoop AJ, Chun JY, Forsberg LS, Carlson R: *nodZ*, a unique host-specific nodulation gene, is involved in the fucosylation of the lipooligosaccharide nodulation signal of *Bradyrhizobium japonicum*. J Bact 176: 620–633 (1994).
66. 66. Truchet G, Barker DG, Camut S, de Billy F, Vasse J, Huguet T: Alfalfa nodulation in the absence of *Rhizobium*. Mol Gen Genet 219: 65–68 (1989).
67. Truchet G, Roche P, Lerouge P, Vasse J, Camut S, De Billy F, Promé J-C, Dénarié J: Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351: 670–673 (1991).
68. Van Brussel AAN, Recourt K, Pees E, Spaink HP, Tak T, Wijffelman CA, Kijne JW, Lugtenberg BJJ: A biovar-specific signal of *Rhizobium leguminosarum* bv. viciae induces increased nodulation gene-inducing activity in root exudate of *Vicia sativa* subsp. *nigra*. J Bact 172: 5394–5401 (1990).
69. Van Brussel AAN, Bakhuizen R, Van Spronsen P, Spaink HP, Tak T, Lugtenberg BJJ, Kijne J: Induction of pre-infection thread structures in the host plant by lipo-oligosaccharides of *Rhizobium*. Science 257: 70–72 (1992).
70. Velupillai P, Harn DA: Oligosaccharide-specific induction of interleukin 10 production by $B220^+$ cells from schistosome-infected mice: a mechanism for regulation of $CD4^+$ T-cell subsets. Proc Natl Acad Sci USA 91: 18–22 (1994).
71. Vijn I, das Neves L, Van Kammen A, Franssen H, Bisseling T: Nod factors and nodulation in plants. Science 260: 1764–1765 (1993).
72. Wagner GP, Lo J, Laine R, Almeder M: Chitin in the epidermal cuticle of a vertebrate (*Paralipophrys trigloides*, Blenniidae, Teleostei). Experientia 49: 317–319 (1993).
73. Wagner GP: Evolution and multi-functionality of the chitin system. In: Molecular Approaches to Ecology and Evolution. Birkhäuser Verlag, Germany, in press.
74. Yang W-C, Katinakis P, Hendriks P, Smolders A, de Vries F, Spee J, Van Kammen A, Bisseling T, Franssen H: Characterization of *GmENOD40*, a gene showing novel patterns of cell-specific expression during soybean nodule development. Plant J 3: 573–585 (1993).
75. York WS, Darvill AG, Albersheim P: Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. Plant Physiol 75: 295–297 (1984).