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Spaink; H.P.

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THE MOLECULAR BASIS OF INFECTION AND NODULATION BY RHIZOBIA: The Ins and Outs of Sympathogenesis

Herman P. Spaink
Clusius Laboratory, Institute of Molecular Plant Sciences, Leiden University, Leiden, The Netherlands

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ABSTRACT

Bacteria belonging to the genera *Rhizobium, Bradyrhizobium,* and *Azorhizobium*, collectively known as rhizobia, penetrate the roots (or adventitious roots) of their leguminous host plants via tubular structures, the infection threads. During infection of the host plant they trigger the formation of a new organ, the root nodule, in which a differentiated form of rhizobia, the bacteroid, fixes nitrogen into ammonia, which can then be used by the plant. This review presents an update of the recent literature on the molecular biology of the infection and nodulation of plants by rhizobia, with special emphasis on results pertinent to other plant-microbe interactions. Particular attention is given to determinants of host specificity such as flavonoid and lipo-chitin oligosaccharide signal molecules.

INTRODUCTION

Bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively known as rhizobia, penetrate the roots (or adventitious roots)

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of their leguminous host plants via tubular structures, the infection threads. During infection of the host plant they trigger the formation of a new organ, the root nodule, in which a differentiated form of rhizobia, the bacteroid, fixes nitrogen into ammonia, which can then be used by the plant. This symbiosis can be very host specific; cross-inoculation groups of bacterial species are defined by host range. Examples of cross-inoculation groups include *R. leguminosarum* biovar *viciae* with peas, vetches, lentils, and sweet peas as hosts; *R. leguminosarum* biovar *trifolii* with clovers as hosts; *R. meliloti* with alfalfa and sweet clovers as hosts; or *B. japonicum* with soybean and a few other (sub)tropical leguminous plant species as hosts. By contrast, other rhizobial strains have a very broad host range of infection and nodulation. For example, *Rhizobium* strain NGR234 can nodulate at least 70 genera of legumes as well as the nonlegume *Parasponia andersonii* (111).

This review presents an update of the recent literature on infection and nodulation of plants by rhizobia, with special emphasis on results pertinent to other plant-microbe interactions. In previous reviews the properties of rhizobia were compared to those of other plant-invasive microorganisms (35, 43, 90, 154). Arguments were advanced that in the early stages of the rhizobium-plant interaction, before the host plant derives any benefit from the symbiosis, rhizobia can act as parasites or potential plant pathogens. Only in later stages of the infection process is it clear that the interaction is symbiotic, not strictly parasitic, which prompted Djordjevic et al (35) to state that rhizobia are "the refined parasites of legumes." The conclusion that this is a highly coevolved relationship is supported by the existence of certain rhizobitoxine-producing strains of *Bradyrhizobium* that are pathogenic on their host and may represent ancestral forms not yet "refined" to a symbiotic interaction. Nontoxin-producing rhizobia also represent a potential pathogenic threat to a host plant until the nodule is formed and beneficial nitrogen fixation is insured. Hence, to avoid nonsymbiotic infections, the plant likely evolved intricately regulated mechanisms to assess the infection process at various stages and to activate resistance strategies if an infection is not moving toward symbiosis. An interesting parallel evolutionary issue regards the benefit of symbiosis to the bacteria, at least in terms of reproduction. Once the bacteroids are formed in the nodule, the rhizobia are effectively locked into this state since there is no evidence that the bacteroids dedifferentiate to free-living bacteria and emerge from a senescent nodule. This apparent dilemma that follows infection of root hairs by rhizobia has been described by Kijne et al (77) as the "rhizobium trap." Given the potential risks of engaging in symbiosis, the selection pressure for symbiosis to evolve was strong. The reason why this form of "sympathogenesis" has evolved exclusively in the family of the leguminous plants remains mysterious.

This review describes the mechanisms by which rhizobia and host plants regulate each other's gene expression. I also discuss how the linkage between the mechanisms for plant defense and those for normal plant (organ) development complicates study of the specific mechanisms involved in regulating symbiosis. Constraints of space prevent my dealing with the stages of the symbiosis after the release of the bacteria from the infection threads (for reviews, see References 50, 74, 158, 159).

REVIEW OF REVIEWS

The molecular basis of the infection and nodulation process has received widespread attention as a model system to study host-specific infection and organogenesis. Recent progress in molecular and genetic technology and understanding of signal transduction have fostered rapid advances in the fundamental study of plant responses to bacterial signals. Numerous research papers have appeared over the past 10 years on the rhizobium-plant interaction and the reported data have been regularly and widely reviewed. Recent reviews have focused on the following:

1. The genetics of the rhizobial genes involved in infection and nodulation that are inducible by plant signal molecules. Subjects covered include the transcriptional regulation of these genes (called *nod*, *nol*, or *noe* genes (Figure 1) (58, 79, 126, 131) and the biochemical function of their translation products (19, 32, 51, 58, 128, 131).
2. The exchange of molecular signals between rhizobia and plants leading to root nodule formation, particularly the plant flavonoids, which induce the transcription of the *nod*, *nol*, and *noe* genes, and the rhizobial lipo-chitin oligosaccharides (LCOs), which can induce nodule organogenesis. Evidence that these signal molecules are the basis of the host specificity of nodulation and, at least in part, infection (17, 31, 32, 37, 42, 43, 51, 54, 58, 86, 86, 90, 128, 131, 135, 141, 154a) has attracted great attention.
3. Factors other than LCOs that also are thought to be involved in the infection process include extracellular polysaccharides (EPS) (85), lipopolysaccharides (LPS) (74), cyclic β-glucans (16), and capsular polysaccharides (CPS) (15).
4. Factors involved in rhizobial competition for infection and nodulation (148), including those involved in the attachment of rhizobia to root hairs (130).
5. The response of plants to rhizobial infection and nodulation signals, especially nodulins, i.e. plant genes differentially expressed during infection and nodulation (18, 52, 64, 158, 160).

FUNCTION OF RHIZOBIAL GENES REGULATED BY PLANT SIGNALS

Regulation of the nod, nol, *and* noe *Genes*

Flavonoids or isoflavonoids excreted by the plant induce transcription of the rhizobial *nod*, *nol*, or *noe* genes (Figure 1). The promoter of the (iso)flavonoid-inducible operons usually, but not always (14), contains a consensus sequence called the *nod* box. In all cases, the induction process requires the gene *nodD*, which is a member of the *LysR* family of transcriptional regulators. Multiple allelic forms of the *nodD* gene are present in different rhizobia. In *B. japonicum* gene regulation by isoflavonoids also involves a set of genes of the two-component regulatory family, *nodV* (sensor) and *nodW* (regulator) (122). The possibility of cross talk between other members of this family, *nwsA* and *nwsB*, has been demonstrated (59). In addition, (iso)flavonoids also have been shown to exert negative regulation on *nodD*-mediated expression. Repressors were found in *R. meliloti*, called *nolR* (79), and in *B. japonicum*, called *nolA* (40). In the later stages of symbiosis, transcription of the *nod* and *nol* genes is turned off by an as yet unknown mechanism that does not involve the reported capacity of some (iso)flavonoids to inhibit *nod* or *nol* gene expression. For a more detailed overview of *nod* and *nol* gene transcriptional regulation the reader is referred to several recent, detailed reviews (51, 58, 79, 126).

Biosynthesis and Secretion of LCO

STRUCTURES OF LCO In response to induction by flavonoids, rhizobia produce LCO signals that induce various symbiosis-related responses (see below). As indicated in Figure 2, LCOs produced by all rhizobia consist of an oligosaccharide backbone of β-1,4-linked *N*-acetyl-D-glucosamine, varying in length between 3 and 5 sugar units. A fatty acid group, of variable structure, is attached to the nitrogen of the nonreducing sugar moiety. A special α,β-unsaturated fatty acid moiety can be present in LCOs produced by *R. meliloti* and *R. leguminosarum* biovars *viciae* and *trifolii* (Figure 3) (87, 128a, 134, 136). This polyunsaturated fatty acyl group is not present in the LCOs of other rhizobial species where the fatty acyl moieties consist of classes commonly found in cell membrane phospholipids. Other substitutions on the chitin backbone are rhizobial strain specific. *O*-linked substitutions include sulphate (*R*5), acetyl (*R*4 or *R*5), carbamoyl (*R*2–4), glycerol (*R*6), and sugar moieties (*R*5) such as arabinose, fucose, or various derivatives of fucose (11, 20, 49, 86, 91, 97, 106, 107, 121). An *N*-linked methyl group (*R*2) also occurs in the LCOs of several species (20, 91, 97, 106, 107).

BACKBONE SYNTHESIS Proteins encoded by the rhizobial *nod* and *nol* genes play a crucial role in the biosynthesis of the LCOs (Figure 1). The NodA,

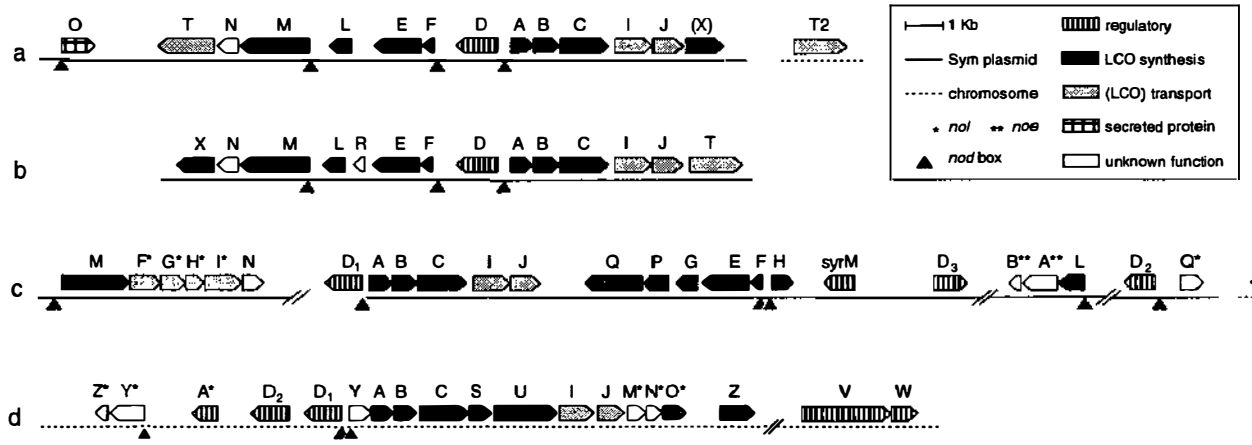

Figure 1 Genetic map and proposed role of *nod*, *nol*, and *noe* genes of rhizobia. Arrows depict the open reading frames of the genes of (*a*) *R. leguminosarum* biovar *viciae* (strain 248, except *nodX*, which is present in strain TOM), (*b*) *R. leguminosarum* biovar *trifolii* (strain ANU843), (*c*) *R. meliloti* (strains 2011 and AK41), and (*d*) *B. japonicum* (strain USDA110). [Data are from References 128, and 131, except those for *noeAB* (3; J Dénarié, personal communication), *nodT2* (116), and the genes of *B. japonicum* (40, 41, 92).] Other rhizobial genes include *nolB*, *nolE*, *nolJ*, *nolT*, *nolU*, *nolV*, *nolW*, and *nolX* of *R. fredii*, which have been indicated to determine cultivar specificity (14, 96), *nodK* of *B. elkanii* (homologous to *nodY*) (39), *nolC* of *R. leguminosarum* biovar *phaseoli* and *nolP*, and *nolK* of *A. caulinodans* (57).

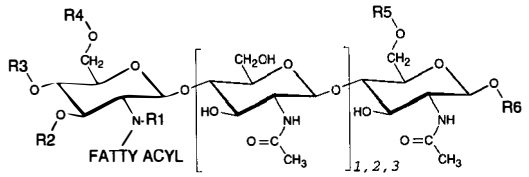

Figure 2 General structure of lipo-chitin oligosaccharides (LCOs). The nature of possible substituents (indicated by *R*) is described in the text. In the absence of host-specific modifications, *R*1–6 stand for hydrogen groups.

NodB, and NodC proteins are sufficient to produce a basic LCO structure. Recent results indicate that the NodC and NodB proteins function as a chitin synthase and a chitin deacetylase, respectively, providing a key intermediate in the synthesis of LCO, called the NodBC metabolite (Figure 4) (56, 71, 73, 139). The NodA protein has been implicated in the addition of the fatty acyl moiety to the NodBC metabolites (4, 118, 139).

LCO-MODIFYING ENZYMES The acetyl transferase NodL (12) and the methyltransferase NodS (53a) are responsible for the presence of an *O*-acetyl and *N*-methyl moiety on the nonreducing terminal saccharide, respectively. Both appear to use the NodBC metabolite as a preferred substrate (Figure 4) (53; GV Bloemberg, personal communication). The NodH protein, which acts as a sulphotransferase responsible for the sulphate moiety on the reducing terminal sugar can use chitin oligomers as substrates (4). However, NodH has been indicated to prefer the complete LCO molecules as a substrate (128b) over chitin oligosaccharides and NodBC metabolites. In this respect, the NodH protein is different from the NodS and NodL proteins.

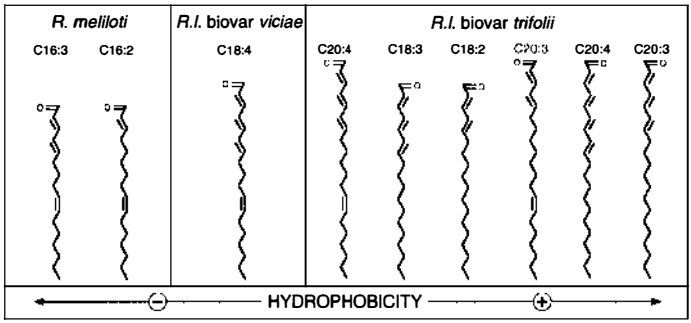

Figure 3 Chemical structures of polyunsaturated fatty acyl moieties of LCOs of *R. meliloti* and *R. leguminosarum* biovars *viciae* and *trifolii*.

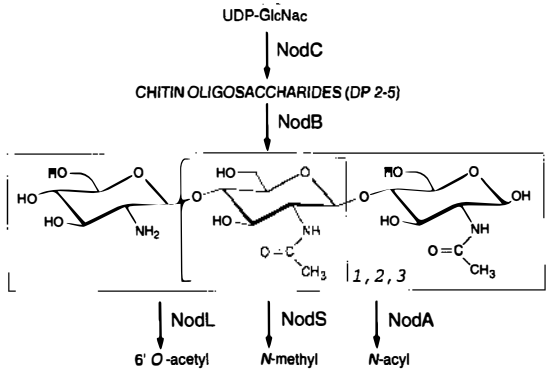

Figure 4 Model for the biosynthesis of lipo-chitin oligosaccharides.

ENZYMES INVOLVED IN THE SYNTHESIS OF BUILDING BLOCKS The NodF and NodE proteins probably function as an acyl carrier protein and a β-ketoacyl-synthase, respectively, and are required for the biosynthesis of the special polyunsaturated fatty acid moiety shown in Figure 3 (30, 55, 115). These fatty acyl moieties are incorporated into LCO by means of the putative acyltransferase NodA and can also be found as substituents of the phospholipids (54, 55). The NodP and NodQ proteins, encoded by members of a family of three sets of genes in *R. meliloti*, function together as a sulphurylase and a kinase, leading to the production of the sulphate donor 3′-phosphoadenosine 5′-phosphosulphate (PAPS), which is the substrate for NodH (4, 129). The NodM protein is a glucosamine synthase homologous to the chromosomally encoded glucosamine synthase (GlmS) counterpart (5, 94).

OTHER ENZYMES Synthesis of the LCOs and various host-specific substituents requires other enzymes as well. Some are encoded by genes that also are required for other nonsymbiotic functions, such as fatty acid biosynthesis. Other functions could be encoded by *nod* or *nol* genes that are involved in the biosynthesis of LCO, but whose biochemical function is still unclear (19, 58).

SECRETION OF LCO Little is known about the mechanisms of secretion of LCOs. Sequence homologies with genes of ATP-dependent transport systems suggest that the NodI protein (which contains an ATP-binding cassette), NodJ (which is probably a dimer and integrated in the cytoplasmic membrane), and NodT protein (which is integrated in the outer membrane and contains a fatty acyl substituent) may be involved in transport of LCOs (43, 58, 156). Some experimental results point to the involvement of these gene products in secre-

tion of LCOs in *R. leguminosarum* biovar *trifolii* (95). Recent results (HP Spaink, AHM Wijfjes & BJJ Lugtenberg, submitted) show that NodI and NodJ are involved in the efficiency of LCO secretion but are not required for secretion of LCOs in *R. leguminosarum* biovars *viciae* and *trifolii*. It also has been suggested that the genes *nolF* and *nolGHI* may play a role in LCO secretion, based on their sequence homology with putative membrane fusion proteins and efflux pump proteins, respectively (119).

Other Functions

The gene *nodO* from *R. leguminosarum* biovar *viciae*, which also is positively regulated by *nodD* and flavonoids, encodes an excreted calcium-binding protein (27, 43–45). The NodO protein forms ion channels in membranes that would allow the movement of monovalent cations across the membrane (147). It was suggested that NodO plays a role in nodulation signaling by forming specific channels in the plasma membrane of the host plants. Other rhizobia also excrete proteins after flavonoid induction (82); however, the genes encoding these proteins and their possible functions are not known. Other flavonoid-inducible phenotypes whose the regulatory mechanisms have not yet been documented include: modulation of cell-associated polysaccharides (114); the accumulation of diglycosyl diacylglycerol membrane glycolipids (99); indole-3-acetic acid production (108); and a leucine-responsive regulatory protein (102). Rhizobial genes, encoding putative outer membrane proteins, are down-regulated in the plant, but the signals responsible have not been identified. Interestingly, one gene, *ropA*, is very similar to a gene of the animal cell-invading pathogen *Brucella abortus* (28, 48).

DETERMINANTS OF GUEST-HOST RECOGNITION

Flavonoids

Flavonoids may not seem to be likely candidates to function as host-specific signals to *Rhizobium*. First, flavonoids are not unique to the roots of leguminous plants, but are present in several organs of a wide range of plant species (93). Second, several leguminous plants exude flavonoid compounds that also activate rhizobia that are unable to nodulate these plants. Nevertheless, there is strong evidence that recognition of specific flavonoids by rhizobia is an important basis of host specificity (67, 79, 131, 138). The host specificity of this induction process is determined by the regulatory bacterial NodD protein, which presumably interacts directly with the flavonoids. Recognition of flavonoids is complex on several grounds. (*a*) All rhizobia recognize broad spectra of (iso)flavonoids as inducers or antiinducers. This ability may reflect an adaptation to the range of compounds produced by different species of host

plant. Some bacterial species have single *nodD* genes that recognize some structural aspects of flavonoids or have multiple copies of the *nodD* gene with different, more-defined, flavonoid-specificities (79). (*b*) In the latter case, there may be a differential response towards certain plant signals, allowing qualitative response of bacterial signal production to plant signals during the symbiotic process (29). (*c*) After induction by bacterial signals the plant modulates the expression of the genes involved in (iso)flavonoid biosynthesis (see below).

The Lipo-Chitin Oligosaccharides

LCOs are major mediators of host specificity in nodule induction. Different substituents of the LCOs account for their host-specific characteristics. The NodH-determined sulphate moiety of the LCOs from *R. meliloti* is required to induce responses in host plants and to prevent activity on individual nonhost plants (3, 87, 117). The NodE-determined polyunsaturated fatty acyl moieties of the *R. leguminosarum* biovars *viciae* and *trifolii* and *R. meliloti* (Figure 3) are also important effectors or determinants of host specificity (137, 150). For *R. leguminosarum* biovars *viciae* and *trifolii*, the difference in the hydrophobicity of polyunsaturated fatty acyl moieties (Figure 3) determines the difference in their respective host ranges, *Vicia* or *Trifolium* (134). With respect to their occurrence in the LCOs of various rhizobia (Figure 2), the polyunsaturated fatty acyl moieties seem to play a role only in the initiation of indeterminate root nodule primordia (Figure 5). A role of the specialized fatty acids in targeting of LCO to the inner cortex could be proposed on the basis of these observations. The LCOs from bacteria that associate mainly with plants that form determinate root nodules often contain an NodS-determined *N*-linked methyl group at the nonreducing saccharide or an additional 6-linked saccharide moiety. The presence of the *2-O*-methylfucose moiety of the LCO from *B. japonicum* has been linked to the *nodZ* gene, which is not flavonoid inducible (140). However, the LCOs of broad host range bacteria such as *R. tropicii*, which nodulate a broad range of determinate nodule-forming plants species, seem not to contain an additional saccharide moiety (106). Furthermore, the presence of the *2-O*-methylfucose moiety is apparently not essential to induce nodules on the hosts of *B. japonicum* (NK Peters, personal communication). Thus the role of additional saccharide substituents is unclear.

In addition to a role in nodule formation, LCOs also determine the specificity of the infection process. Since externally added LCOs from homologous rhizobia allow the infection of *Vigna* and *Glycine* plants by heterologous rhizobia, Relić et al (111) characterized LCO as "a key to the legume door."

Bacterial Exopolysaccharides

The exopolysaccharides (EPSs) have also been suggested to play a role in host specificity of the infection process (35). The observation that nodule invasion

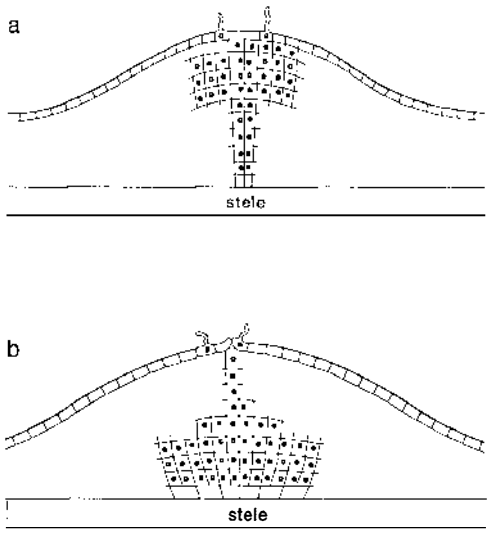

Figure 5 Schematic description of the induction of early stages of determinate (*A*) or indeterminate (*B*) nodule formation by LCOs. This figure is based upon microscopic studies of LCO-induced roots of *Vicia sativa* (indeterminate nodules), *Glycine soja* (145), *Lotus preslii* (91), and *Phaseolus vulgaris* (determinate nodules). [For further description of the development of indeterminate and determinate nodules the reader is referred to references (17, 18, 52).] In some plant species, such as *Acacia*, the induction of determinate primordia (*A*) lead to the formation of indeterminate type nodules (IM López-Lara, personal communication).

by EPS mutants could be restored by exogenously added EPS from the guest bacteria but not by EPS from incompatible rhizobia (7, 38) supports this proposed role. LPSs or CPSs, such as the recently described polysaccharide that resembles group II K antigens of *E. coli*, may also be determinants of host specificity during the infection process (15, 74, 113).

Plant Determinants

Plant lectins are the only known example of plant factors that determine host specificity. Diaz et al (33) constructed transgenic white clover plants containing the pea lectin gene *psl*, which could be nodulated by the heterologous pea-nodulating bacteria. Although their results initially pointed to a function of pea lectin as a receptor of LCO, more recent findings raise questions about this function. A more likely explanation is that the pea lectin facilitates infection by the rhizobia and thereby makes the transgenic plants susceptible to the heterologous nodulation signals (78). Various lectins might also play a role during the late stages of symbiosis (8, 75, 76). Plant chitinases, which can specifically degrade LCOs to their disaccharidic inactive forms (144), also

have been suggested to play a role in specificity. However, a general role in autoregulation of the nodule induction process is more likely (63, 144). One possible explanation for the role of LCO hydrophobicity in host specificity could lie in the difference in fatty acid composition in the host plants. However, Bloemberg & Thomas-Oates (personal communication) demonstrated that such differences are not detectable by gas chromatographic analysis of total fatty acyl extracts of the roots of host plants.

REACTION OF PLANTS TO RHIZOBIAL INFECTION

Root Hair Curling

After rhizobia attach to the tips of emerging root hairs in the host plant, the infection process is initiated by curling of the root hairs. Rhizobia are thus trapped inside so-called shepherd's crook structures, and infection thread formation is initiated within the crook (76, 77). External application of LCOs, at concentrations between 10^{-8} and 10^{-12} M, elicits responses such as depolarization of membrane potential (46), modulation of proton and calcium ion fluxes (2), or curling, branching, and swelling (20, 63, 87, 91, 105, 112, 136) of root hairs of the respective host plants. These responses are probably all related to the process of root hair curling. The earliest detectable response to LCO is induction of membrane depolarization, which occurs within 10 min (46). On *Vicia sativa*, 5–10 min interaction between LCO and the root is sufficient to induce root hair deformation that is visible within 1 h (63). After 3 h, most root hairs in a small susceptible zone of the roots can be deformed. LCOs apparently do not need to contain host-specific substituents for the induction of these responses. For instance, root hair deformation on *V. sativa* can be induced by LCOs derived from the compatible *R. leguminosarum* biovar *viciae* but also by the LCOs from *B. japonicum* (20) or *R. loti* (91). By contrast, the sulphate group of the LCOs from *R. meliloti*, which is important for curling of root hairs in *Medicago sativa* (87), substantially decreases the capacity of the LCO to induce root hair deformation on *V. sativa* (32, 63, 144). LCOs can induce the formation of shepherd crook's curling on *Macroptilium atropurpureum* (112). Induction of this response appears not to be dependent on host-specific substituents of the LCO, although it takes 10–100 times higher concentrations of LCOs from heterologous rhizobia to achieve the same degree of curling as that given by the LCOs from *Rhizobium* strain NGR234. On *V. sativa* plants, shepherd crook's structures cannot be induced by purified LCOs. For this plant, the physical presence of rhizobia attached to root hairs is required in addition to the LCOs.

Little is known about the genetic basis of root hair deformation. Krause et al (81) recently identified a gene encoding a lipid transfer-like protein, the

expression of which increased in root hairs treated with LCOs or rhizobia. It was speculated that this gene may have a role in the transport of the LCOs.

The Infection Thread

Rhizobial infection proceeds in many host plants via the formation of infection threads (17, 76). The mechanisms underlying the growth of these tubular structures through the root hair cell and subsequently through the root cortical tissue are poorly understood. Cellular changes include nuclear movements mediated by microtubuli, the breakdown of cell wall material, the formation of new cell walls, and the production of an infection thread matrix. The matrix contains the bacteria as well as plant-derived glycoproteins, as was shown for *Pisum sativum* (74). Evidence indicates that infection thread formation is a result of activation of the cell cycle in cells of the outer cortex. This activation was suggested by the host-specific induction of preinfection thread structures in the outer cortex of *Vicia* roots by LCOs (151). These preinfection thread structures are characterized by the formation of so-called cytoplasmic bridges (6); they are radially aligned, giving the impression of cytoplasmic threads crossing the outer cortex. The formation of these structures, which are indistinguishable from those observed after infection with bacteria from *R. leguminosarum* biovar *viciae*, always precedes the formation of infection threads (6, 151, 155). The formation of cytoplasmic bridges in vacuolated cells is preceded by cell polarization; the nucleus moves to the center of the cell just as in cells that are about to divide. Yang et al (164) showed that cells of the preinfection threads have indeed entered the cell cycle but do not divide as a result of arrest in the G2 phase. The process of preinfection thread formation is accompanied by local cell wall modifications that are probably related to the induction of tip growth. However, complete cell wall degradation was not observed in the absence of rhizobia. Based on these observations, Van Spronsen et al (155) proposed a two-step process of cell wall degradation for infection thread formation: local cell wall modification by plant enzymes induced by LCO, followed by complete cell wall degradation in the presence of rhizobia. Given the developmental similarities between infection thread growth and polar tip growth, these findings suggest that other related processes such as pollen tube growth may be directed by parallel signal transduction pathways. Interestingly, a genetic locus has been described in *Arabidopsis* that is involved in both root hair and pollen tube expansion (125).

At the molecular genetic level, LCO signals produce some of the same responses as those observed during the process of rhizobial infection, including the induction of nodulin gene expression, e.g. *enod12* from which the expression in time and place is strongly correlated with the early steps in the symbiosis. *Pisum sativum* and *Medicago sativa* appear to contain two copies of this nodulin gene, *enod12a* and *enod12b* (9, 52, 68, 124). In *M. sativa*, the two

copies of *enod12* appear to be differentially regulated: *enod12a* expression in roots is associated with the infection process, whereas *enod12B* expression is related to root nodule organogenesis (9). Transgenic *M. varia* plants have been constructed that contain gene fusions of the single *enod12* gene of *M. truncatula* with the reporter gene *uidA*. These fusions provide a valuable molecular marker for studying LCO signal transduction in the plant (104). The results obtained with this system show that rhizobial infections as well as purified LCOs elicit *enod12* expression in the epidermal cells in the zone of emerging and maturing root hairs within 3 h of inoculation (72). Infection studies of these transgenic reporter plants with *R. meliloti exoA* mutants indicate that the transcription of the *enod12* gene is activated at a very low level in noninfected regions of *Rhizobium*-elicited nodules (84, 103). This conclusion is consistent with the results of Hirsch et al (65), who did not detect *enod12* expression in *M. sativa* nodules induced by bacterial infection mutants. By contrast, expression studies of *M. truncatula enod12* in another genotype of *M. varia*, which can develop nodules in the absence of rhizobia (Nar^+) (149), show that *enod12* is expressed in spontaneous nodules and that its expression can therefore be uncoupled from the infection process (103). Although the expression of the *enod12* gene is closely correlated with the infection process in *Rhizobium*-induced nodules, the *enod12* gene apparently is not required for infection and nodulation of *Medicago* plants (24).

The Root Nodule

At micromolar concentrations, externally applied purified LCOs elicit the formation of nodule primordia in the cortex that are indistinguishable from the nodule primordia in the first stage of normal nodule organogenesis. Furthermore, as in plants infected by rhizobia, the primordia are only induced at certain positions in the plant root, i.e. where young root hairs emerge, opposite (or almost opposite) the protoxylem poles of the central cylinder. This holds true for indeterminate nodule-forming plants such as *Vicia sativa* (136) and *Medicago sativa* 150), as well as for determinate nodule-forming plants such as *Glycine soja* (145), *Lotus preslii* (91), and *Phaseolus vulgaris* (IM Lopez-Lara, personal communication) (Figure 5). In *Medicago* and *Glycine soja*, the nodule primordia can develop into full-grown nodules with the anatomical and histological features of genuine rhizobia-induced nodules. This development has not been observed in other plants such as *Vicia*, rather nodules stop developing at a stage at which small outgrowths are externally visible on the roots. Induction of root nodule primordia on *Vicia* by LCO is strongly inhibited by ethylene (153) in agreement with its inhibitory effect on nodulation by *Rhizobium* (83). Furthermore, root nodulation by *Rhizobium* is reduced in *Vicia* roots treated with LCOs, a phenotype called JAN (jamming of nodulation) (AAN van Brussel, personal communication). These observations cannot yet be

linked with findings that rhizobial infection strongly autoregulates the number of root nodules (18). Caetano-Anollés & Gresshof (18) showed that organized nodular structures trigger the feedback regulatory mechanisms even in the absence of bacterial infection. By contrast, nodules formed spontaneously on *Medicago* plants with a Nar^+ phenotype apparently have a different autoregulatory mechanism or lack a key controlling element in the process (103).

Little is known about the chemical signals determining the position of root nodulation. The gradient hypothesis offers one explanation for the local reaction of individual inner cortical cells to rhizobial signals; this postulates that variations in concentration of a signal determines that only certain cortical cells respond (88). A hormone from the central stele, which stimulated cell division in pea root explants at nanomolar concentrations, has been purified and shown to be uridine. The strong correlation between the activity of this root signal and autoregulation of nodulation suggests that uridine plays an essential role in initiating root nodule formation (G Smit, C de Koster, J Schripsema, et al, submitted). Microtargeting experiments of signal molecules confirmed the important role of uridine; these results showed that induction of root cortical cell divisions by chitin oligosaccharides was dependent on the cointroduction of uridine (133). 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) (52, 64). Furthermore, mutant strains of *R. meliloti* that are unable to produce LCO can be restored to their nodulation phenotype by introducing the *tzs* gene from *Agrobacterium*, which results in the production of *trans*-zeatin (22). These results suggest that nodule initiation is regulated by common plant hormonal mechanisms.

At the genetic level, the induction of root nodule primordia coincides with the activation of various genes involved in cell cycle regulation (164). Savouré et al (123) showed that the cognate LCOs also have host-specific responses on gene expression in suspension cultures of *Medicago microcallus*. At nanomolar concentrations, increased expression of histone H3-1, the *cdc2* homologue from *Schizosaccharomyces pombe*, and a cyclin-encoding gene demonstrated the host-specific activation of the cell cycle. Stimulation of the cell cycle also was indicated by enhanced thymidine incorporation, elevated numbers of S-phase cells, and increased kinase activity of *cdc2*-related complexes.

Several (nodulin) genes that are differentially expressed during the early stages of nodule formation have been reported (34, 52, 80, 161, 165). The nodulin gene *enod40*, a good marker for nodule primordium formation (80, 165), is activated early after treatment of roots with wild-type compatible rhizobia, purified LCOs, TIBA, or *Rhizobium* LCO-strains containing a zeatin gene from *Agrobacterium* (K Pavlowski, H Franssen, T Bisseling, personal communication). However, expression of *enod40* was detectable in the cells

of the protoxylem poles bordering the nodule primordia before it could be detected in the cells of the nodule primordium itself. The function of the *enod40* transcript is unknown. Because no significant open reading frames were found in *enod40* cDNAs of several plant species, it was suggested that *enod40* encodes a nontranslatable RNA (23). The introduction into *M. varia* of constructs that overexpress *enod40* resulted in overactive cell proliferation of the transformed explants. Introduction of the antisense *enod40* construct resulted in an arrest of growth of the transformed explants (23). These results indicate that *enod40* plays an important role in plant development.

Responses Related To Plant Defense

There are several reports on the induction of phenotypes by wild-type or mutant rhizobia that could be related to a defense response. The induced responses include: phytoalexin accumulation (143), chitinase and peroxidase enzyme activity (120, 143, 157), de novo (iso)flavonoid production and excretion (25, 109, 127, 152, 163), expression of genes of the (iso)flavonoid pathway (36, 47, 60, 89, 110), accumulation of intercellular matrix material (101), secondary cell wall modifications (101), and the hypersensitive response, as defined by the observation of local cell death (157). The suggestion that the observed reactions, except for the latter phenotype, serve as a defense response should be treated with caution. Alternatively, these phenotypes may merely reflect the fact that, in plants, regulation of several (presumed) defense-related mechanisms is integrated with regulation of normal developmental processes, and vice versa (1, 13, 66, 135). However, since induction of plant defense responses is not necessarily correlated with the induction of the hypersensitive response (69), all the observed phenotypes might well have a function in defense against (sym)pathogens.

Differences in the type of rhizobial strain or mutants, plant species, and the experimental conditions used in most test systems make it difficult to draw general conclusions from published results. In some cases, the rhizobial mutants that were used have not been characterized at a molecular level and thus the genetic, molecular, or biochemical bases of their actions cannot be determined. Induction of the reported phenotypes, however, can be linked to a condition or a specific bacterial trait in the following results:

1. Activation of the hypersensitive response as a result of abortive infections, even in *Medicago* plants infected by the compatible wild-type *R. meliloti* bacteria (157).
2. Triggering of some of the defense-related responses described above by EPS-defective mutants of *R. meliloti* and *B. japonicum* and LPS-defective mutants of *R. leguminosarum* that can nodulate the host plant but not invade the nodules (98, 100, 101). Exogenously supplied EPS forms appear to be

able to restore the defect in nodule invasion of EPS-defective mutants (7, 38). A low molecular weight form of the EPS of *R. meliloti* has been shown to be responsible for the complementing effect (7, 85). These EPSs might be the equivalent of known "silencers" of a defense response (70).

3. Induction of flavonoid synthesis genes in the host plant. These genes include those encoding phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) (110). The observed induction of PAL and CHS gene expression in *Vicia* by wild-type *R. leguminosarum* biovar *viciae* is correlated with the production of new flavonoids that induce transcription of the *nod* genes (109). These responses are condition dependent since they are only detectable in roots that are not shielded from light, and are probably dependent on the presence of ethylene (152, 153). It was also shown that other rhizobia induce the (local) synthesis of (iso)flavonoids in the host plants, but the role of ethylene was not tested (25, 36, 47, 89, 127). CHS gene expression was higher for rhizobial mutants that were unable to infect nodules than it was in normal wild-type nodulation (60, 163). These results suggest that induction of nodule primordia, combined with the absence of nodule invasion, triggers a regulatory mechanism involving flavonoids, although its function is not clear.

The resemblance of LCOs to other reported elicitors of defense responses makes them obvious candidates as elicitors responsible for the above-mentioned responses (10, 21, 142). A positive correlation with LCO production has indeed been demonstrated in the induction of the (iso)flavonoid biosynthesis pathway. Interestingly, the structural requirements of LCOs to induce flavonoid biosynthesis in *Vicia* plants are identical to the requirements to induce nodule primordia. Possible functions of the inducible flavonoids include:

1. Modulation of *nod* gene expression to enforce "better" LCO production (higher or lower levels, or structurally different molecules) by the rhizobia. Such a continuous feed-back mechanism could enable the plant to differentially regulate rhizobial signal production at different stages of the infection and nodulation process. These different stages could have different requirements with respect to quantity or quality of LCOs.
2. Induction of a direct hormonal effect by acting as auxin transport inhibitors. This could lead to the stimulation or inhibition of further nodule initiation or nodule outgrowth. An inhibitory effect would seem to contrast with the nodule-inducing capacity of (externally supplied) auxin transport inhibitors such as TIBA.
3. Provision of a defense mechanism against co-invading microorganisms, which are not potentially beneficial. Toxicity in inducible flavonoids may be higher than in the constitutively expressed flavonoids. The effect of

ethylene would be consistent with its synergistic function in triggering plant defense mechanisms (21, 162).

4. Finally, a similar mechanism also may play a role in the infection of plants by other microorganisms. One example is the interaction between roots of *Medicago* and mycorrhizal fungi, which is also accompanied by differential expression of (iso)flavonoid pathway genes (61, 62).

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