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Symbiotic Nitrogen Fixation

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INTRODUCTION

Biosphere nitrogen is subjected to rapid turnover, and because it is eventually lost as nitrogen into the atmosphere, its maintenance requires continuous replenishment with reduced nitrogen from atmospheric nitrogen. Biological reduction of nitrogen to ammonia can be performed only by some prokaryotes and is a highly oxygen-sensitive process. The most efficient nitrogen fixers establish a symbiosis with higher plants in which the energy for nitrogen fixation and, in general, the oxygen protection system are provided by the plant partner. In two groups of symbiotic interactions, the prokaryotic partners are soil bacteria (rhizobia in legume symbioses and *Frankia* bacteria in actinorhizal symbioses), whereas in the case of symbiosis of *Gunnera*, nitrogen is fixed by the cyanobacterium *Nostoc*. In *Gunnera*, the symbionts reside in already existing stem glands, whereas in legumes and actinorhizal plants, new organs, the root nodules, are formed by the plant upon infection with the symbiont. In all three systems, the prokaryotes fix nitrogen inside the cells of their host, but they are separated from the plant cytoplasm by membranes derived from the plant plasmalemma (Figure 1).

Because research on legume symbiosis is the most advanced of these three symbiotic systems, in this article we concentrate mainly on this system. The interaction of rhizobia and legumes begins with signal exchange and recognition of the symbiotic partners, followed by attachment of the rhizobia to the plant root hairs. The root hair deforms, and the bacteria invade the plant by a newly formed infection thread growing through it. Simultaneously, cortical cells are mitotically activated, giving rise to the nodule primordium. Infection threads grow toward the primordium, and the bacteria are then released into the cytoplasm of the host cells, surrounded by a plant-derived peribacteroid membrane (PBM). The nodule primordium thereupon develops into a mature nodule, while the bacteria differentiate into their endosymbiotic form, which is known as the bacteroid (Figure 1A). Bacteroids, together with the surrounding PBMs, are called symbiosomes. At this stage, bacteria synthesize nitrogenase, which catalyzes the reduction of nitrogen. The product of nitrogen fixation, ammonia, is then exported to the plant.

All of the steps of nodule development involve the expression of nodule-specific plant genes, the so-called nodulin genes (van Kammen, 1984). The early nodulin genes encode products that are expressed before the onset of nitrogen fixation and are involved in infection and nodule development. The products of the late nodulin genes are involved in the interaction with the endosymbiont and in the metabolic specialization of the nodule (Nap and Bisseling, 1990).

In the first part of this review, we describe the early steps of the interaction between rhizobia and legumes that result in the formation of a nitrogen-fixing nodule. We focus on the role of specific lipooligosaccharides secreted by rhizobia in the induction of these early steps. In the second part, we describe nodule functioning and compare actinorhizal and legume nodules.

EARLY EVENTS OF NODULATION

Nod Factor Structure and Synthesis

The *Rhizobium* signal molecules that play a key role in the induction of the initial stages of nodulation are lipochitooligosaccharides known as Nod factors. The bacterial genes involved in Nod factor synthesis are the *nod* (nodulation) genes. These genes are not expressed in free-living bacteria, with the exception of *nodD*, which is expressed constitutively. NodD has the ability to bind to specific flavonoids secreted by the roots of the host plant (Goethals et al., 1992); upon flavonoid binding, it becomes a transcriptional activator of the other *nod* genes (Fisher and Long, 1992), which encode enzymes involved in the synthesis of Nod factors.

The structure of the major Nod factor of *Rhizobium meliloti* was determined in 1990 (Lerouge et al., 1990), and since then the structures of the Nod factors of most other rhizobia have been determined. (For detailed information on Nod factor structure and biosynthesis, see Fisher and Long, 1992; Spaink, 1992; Dénarié and Cullimore, 1993; Carlson et al., 1995.) In general, Nod factors consist of a backbone of three to five β-1,4–linked *N*-acetylglucosamines bearing a fatty acid on the nonreducing sugar residue (Figure 2). In addition, the factors can have various substitutions on both the reducing and nonreducing terminal sugar residues.

Genetic and molecular analyses have shown that the synthesis of the Nod factor backbone is catalyzed by the products of the *nodA*, *nodB*, and *nodC* loci. NodC has homology with

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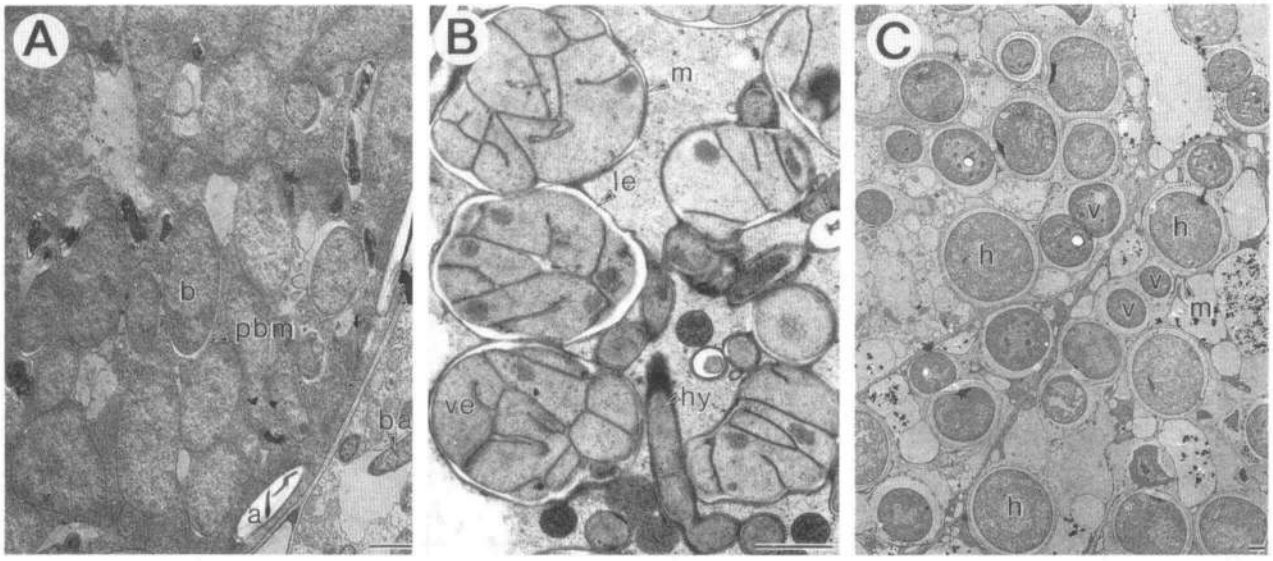


Figure 1. Nitrogen-Fixing Endosymbionts in Three Different Symbioses.

(A) Intracellular rhizobia in a nodule formed on clover by *Rhizobium trifolii*. This magnification of the region of the clover indeterminate nodule shows the transition of the prefixation zone to the interzone. In the right cell (prefixation zone), intracellular bacteria (ba) have not yet differentiated into their nitrogen-fixing form. The left cell (interzone) contains amyloplasts (a) and differentiated nitrogen-fixing bacteroids (b). In both cells, intracellular bacteria are surrounded by a plant-derived peribacteroid membrane (pbm).

(B) Intracellular *Frankia* in a nodule formed on *Alnus serrulata*. Vegetative hyphae (hy) and nitrogen-fixing septate vesicles (ve) can be seen. Vesicles are surrounded by a lipid envelope (le) that provides oxygen protection of the nitrogen fixation process. Both hyphae and vesicles are surrounded by the invaginated plasma membrane (m) of the host cell.

(C) Intracellular *Nostoc* in stem gland cells of *Gunnera*. Vegetative *Nostoc* cells (v) and nitrogen-fixing heterocysts (h) are surrounded by the invaginated plasma membrane (m) of the host.

Bars = 1 μm.

chitin synthases and therefore is the enzyme that most likely catalyzes the synthesis of the chitin oligomer (Geremia et al., 1994). The latter is further modified by the action of NodB, which de–*N*-acetylates the terminal nonreducing end of the molecule (John et al., 1993). At this position, NodA finally transfers a fatty acid from an acyl carrier protein (Röhrig et al., 1994). The backbone is further modified by the action of other Nod proteins that synthesize or add various substituents. These substitutions determine host specificity as well as the biological activity of the molecules. For example, in *R. meliloti*, the *nodH* and *nodPQ* genes are the major host range determinants (Roche et al., 1991). NodPQ proteins have been shown to represent enzymes that generate active forms of sulfate, and NodH is homologous to sulfotransferases. Therefore, these enzymes are probably directly involved in catalyzing the sulfation of *R. meliloti* Nod factors (Roche et al., 1991; Fisher and Long, 1992).

In general, rhizobia have the ability to interact with only a limited number of host plants. However, some rhizobia, for example, *Rhizobium* NGR234, have a more promiscuous nature. This *Rhizobium*, which can nodulate various tropical legumes, excretes 18 different Nod factors (Price et al., 1992). The production of this variety of factors is thought to be the basis for its broad host range (Price et al., 1992). In contrast, most rhizobia produce only a few different Nod factors.

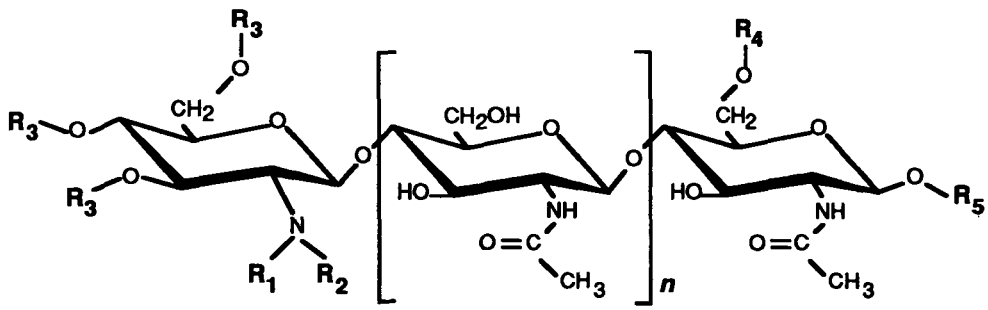
Interaction with the Root Epidermis

When rhizobia colonize legume roots, they induce deformation and curling of root hairs and the expression of several plant genes in the epidermis. In several systems, it has been shown that purified Nod factors induce the deformation of the root hairs at concentrations as low as 10^{-12} M (Lerouge et al., 1990; Spaink et al., 1991; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Mergaert et al., 1993; Heidstra et al., 1994), but in general curling is not observed (Relic et al., 1993). Purified Nod factors can also induce the expression of certain plant genes (Horvath et al., 1993; Journet et al., 1994; Cook et al., 1995).

For *Vicia sativa* (vetch), a fast semiquantitative root hair deformation assay has been developed that allows the root hair deformation process to be characterized in detail. In this plant, root hair deformation is induced only in a small zone of the root, encompassing young root hairs that have almost reached their mature size (Heidstra et al., 1994). Deformation starts with

swelling of the root hair tips, which is already apparent within 1 hr after Nod factors are added. Subsequently, new tip growth is initiated at the swollen tips, resulting in clearly deformed hairs within 3 hr. Incubation with Nod factors for ~10 min is required to set the deformation process in motion (Heidstra et al., 1994); after this, even if the Nod factors are removed, the deformation process continues. These morphological changes are preceded by a depolarization of the plasma membrane (Ehrhardt et al., 1992), changes in the flux of calcium, proton efflux, rearrangements of the actin filaments (Allen et al., 1994), and increased cytoplasmic streaming (Heidstra et al., 1994). These changes occur within 5 to 30 min after Nod factors are added and may be part of a series of events that leads eventually to root hair deformation.

Several plant genes whose expression is activated in the epidermis during nodulation have been cloned and used to study the mode of action of Nod factors. The early nodulins *ENOD5* (Scheres et al., 1990b) and *ENOD12* (Scheres et al., 1990a), which encode proline-rich proteins, and *Mtrip1* (Cook et al., 1995), which encodes a peroxidase, represent such genes. The latter gene is expressed in the root pericycle of uninoculated roots, and all three genes are induced in the epidermis within a few hours after application of Nod factors (Horvath et al., 1993; Journet et al., 1994; Cook et al., 1995).



Species	R_1	R_2	R_3	R_4	R_5	*n*	References
Rhizobium meliloti	-H	-C16:2 (2,9)[a] or -C16:3 (2,4,9)	-$COCH_3$ (O-6)[b] or -H	-SO_3H	-H	1,2,3	Lerouge et al. (1990) Schultze et al. (1992)
Rhizobium leguminosarum bv *viciae*	-H	-C18:4 (2,4,6,11) or -C18:1 (11)	-$COCH_3$ (O-6)	-H or -$COCH_3$[c]	-H	2,3	Spaink et al. (1991) Firmin et al. (1993)
Bradyrhizobium japonicum	-H	-C18:1 (9), -C18:1 (9,Me), -C16:1 (9), or -C16:0	-$COCH_3$ (O-6) or -H	2-*O*-Methylfucosyl group	-H	3	Sanjuan et al. (1992) Carlson et al. (1993)
Bradyrhizobium elkanii	-H or Me	-C18:1	-$COCH_3$ (O-6), -H, or Cb[d, e]	2-*O*-Methylfucosyl or fucosyl group	-H or Gro[f]	2,3	Carlson et al. (1993)
Rhizobium sp strain NGR234	Me	-C18:1 or -C16:0	Cb (0-3 and/or 0-4)[b] or -H	Sulfated or acetylated 2-*O*-methylfucosyl group	-H	3	Price et al. (1992)
Azorhizobium caulinodans strain ORS571	Me	-C18:1 or -C18:0	Cb (0-6) or -H	D-Arabinosyl or -H	-H	2,3	Mergaert et al. (1993)
Rhizobium fredii	-H	-C18:1	-H	Fucosyl or 2-*O*-methylfucosyl group	-H	1,2,3	Bec-Ferté et al. (1994)
Rhizobium tropici	Me	-C18:1	-H	-SO_3H	-H	3	Poupot et al. (1993)

Figure 2. Structure of Nod Factors of Different Rhizobia.

The number of the *N*-acetylglucosamine residues can vary between three and five. The substitutions at positions R_1, R_2, R_3, R_4, and R_5 among the different rhizobia are indicated.

[a] The numbers in parentheses indicate the positions of the double bonds in the fatty acids.

[b] O-n indicates the position of the substitution on the *N*-acylglucosamine residue.

[c] This substitution is present only in Nod factors of *R. leguminosarum* bv *viciae* strain TOM.

[d] Cb indicates carbamyl group.

[e] The position of the carbamyl group could be 0-3, 0-4, or 0-6.

[f] Gro indicates glyceryl group.

Mtrip1 is not expressed during other steps of nodulation, whereas *ENOD12* and *ENOD5* are also expressed during infection and nodule development (see later discussion).

The induction of *ENOD12* and *Mtrip1* expression occurs in a relatively broad zone of the root, starting just above the root tip, where root hairs have not yet emerged, and extending to the region containing mature root hairs (Pichon et al., 1992; Cook et al., 1995). Cytological studies have shown that Nod factors elicit the expression of these genes in all epidermal cells (Journet et al., 1994; Cook et al., 1995) and that a direct contact between Nod factors and epidermal cells is required (Journet et al., 1994). Thus, it is likely that within the susceptible zone, root hair–containing cells as well as the other epidermal cells recognize Nod factors. However, the response is restricted to the epidermis, because *ENOD12* and *Mtrip1* are not even induced in the hypodermal cell layer.

The root hair deformation assay and tests of their ability to induce early nodulin genes have been used to analyze the structural requirements of Nod factors to elicit epidermal responses. When roots are treated with Nod factors, molecules containing three or fewer sugars are found in the growth medium and on the root. These molecules are probably generated by chitinases secreted by the plant, and they are at least 1000-fold less active in a deformation assay than the Nod factors with four to five glucosamine residues (Heidstra et al., 1994; Staehelin et al., 1994b). Therefore, the length of the sugar backbone plays an important role in recognition by the plant. Furthermore, the fatty acyl group present at the nonreducing end is required for recognition, because chitin molecules neither cause deformation (Heidstra et al., 1994) nor induce *ENOD12* expression (Journet et al., 1994). However, the structure of the fatty acyl group is not very important, because molecules with different fatty acid substitutions have similar activities.

In contrast, substitutions at the reducing terminal sugar can have a dramatic effect on recognition. For example, Nod factors of *R. meliloti* bear a sulfate group on this sugar residue. This sulfate moiety is important for induction of root hair deformation as well as for the elicitation of *ENOD12* expression in the *R. meliloti* host, alfalfa. Desulfation of the *R. meliloti* factors reduces their activity at least 1000-fold on alfalfa (Journet et al., 1994), whereas they attain the ability to deform root hairs of the nonhost vetch (Roche et al., 1991). Vetch can form nodules with *Rhizobium leguminosarum* bv *viciae*, which produces Nod factors that lack a substitution at the reducing terminal sugar residue. Hence, the sulfate substitution is a major host specificity determinant.

Thus, very low concentrations of Nod factors induce several responses in the root epidermis. If a receptor is involved in the elicitation of these responses, it must recognize the length of the Nod factors as well as the substitutions at the reducing end. Because the presence of a fatty acyl moiety is essential but its structure is not important, it is likely that this part of the molecule is not recognized by a receptor. Instead, the fatty acyl group might play a role in "docking" the Nod factors in the membranes and, in that way, facilitate binding to a putative receptor.

Infection

After attachment of rhizobia to the root hair tips, the tips curl tightly and bacteria become entrapped in the curls. A local hydrolysis of the plant cell wall takes place in the curled region (Callaham and Torrey, 1981; Van Spronsen et al., 1994), and the plasma membrane invaginates and new plant cell wall material is deposited (for reviews, see Bauer, 1981; Newcomb, 1981; Brewin, 1991; Kijne, 1992). This results in the formation of a tubular structure, the so-called infection thread, by which the bacteria enter the plant.

The ultrastructure of the wall of the infection thread is very similar to that of the normal plant cell wall, but the incorporation of certain nodulins may endow it with unique properties. The proline-rich early nodulins ENOD5 and ENOD12 are candidates for components of the infection thread wall, because cortical cells containing an infection thread express the corresponding genes (Scheres et al., 1990a, 1990b). The bacteria in the infection thread are surrounded by a matrix that seems to consist of compounds secreted by both the plant and the bacteria. For example, a 95-kD glycoprotein normally present in the intercellular spaces of the root cortex is localized in the infection thread matrix (Rae et al., 1992).

Concomitant with infection thread formation, cortical cells are mitotically reactivated, forming the nodule primordium (see later discussion). Infection threads grow toward this primordium and, once there, release bacteria into the cytoplasm. In those legumes that form indeterminate nodules, such as alfalfa and pea (see Nodule Functioning), nodule primordia arise from inner cortical cells. Hence, in the formation of this nodule type, the infection threads must traverse the outer cortex before they reach these cells. Before infection thread penetration, the outer cortical cells undergo morphological changes. The nuclei move to the center of the cells, and the microtubules and the cytoplasm rearrange to form a radially oriented conical structure, the cytoplasmic bridge, that resembles a preprophase band (Kijne, 1992). The infection threads traverse the cortical cells through the radially aligned cytoplasmic bridges, which are therefore called preinfection threads (Van Brussel et al., 1992).

Although the preinfection thread–forming outer cortical cells never divide, the induced morphological changes are reminiscent of those seen in cells entering the cell cycle. In situ hybridization experiments (Yang et al., 1994) showed that narrow rows of outer cortical cells express the S phase–specific histone *H4* gene (Figure 3A). However, a mitotic cyclin gene specifically expressed during the G2-to-M phase transition is not activated. Hence, the cells that form the preinfection thread reenter the cell cycle and most likely become arrested in the G2 phase, whereas the inner cortical cells progress all the way through the cell cycle and form the primordia (Figure 3A). This shows that part of the infection process is derived from a general process, namely, cell cycling. In some way, rhizobia have modified it and now exploit it for a completely different purpose, the infection process.

Purified Nod factors induce preinfection thread formation, but infection threads are not formed (Van Brussel et al., 1992).

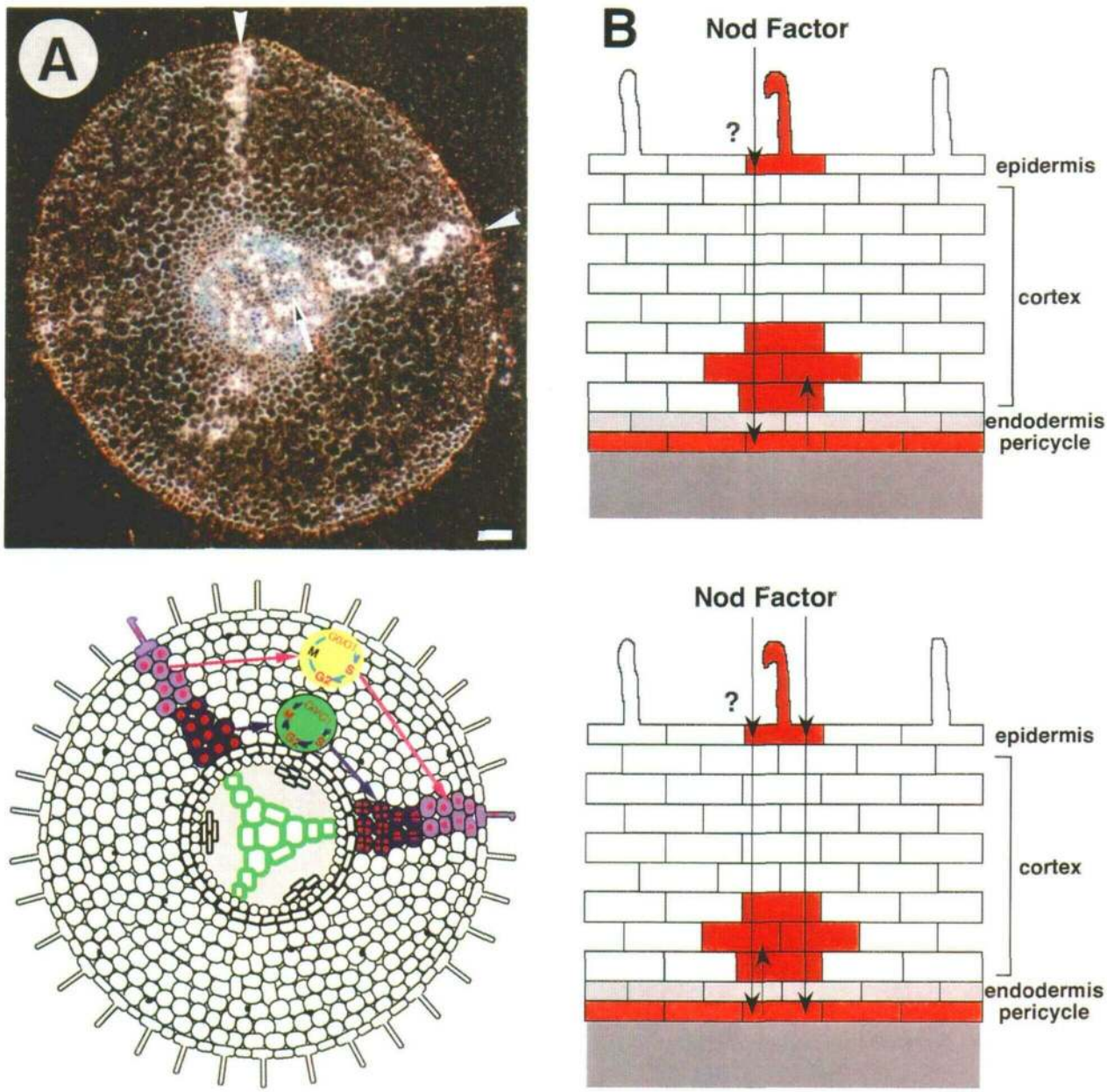


Figure 3. Events in the Cortex during Induction of an Indeterminate Nodule.

(A) Activation of the cortex after inoculation with rhizobia. The top panel is a dark-field micrograph of a cross-section of a pea root 1 day after inoculation with *R. leguminosarum* bv *viciae* that was hybridized with a histone *H4* gene probe. *H4* transcripts are localized in narrow rows of cortical cells in front of the infection sites, which are indicated by arrowheads. The silver grains represent the hybridization signal. Note that the infection sites are opposite to the protoxylem poles (arrow). Bar = 50 μm. The bottom panel is a schematic drawing showing the reactivation of cortical cells in pea roots after inoculation with rhizobia or application of Nod factors. The outer cortical cells, shown in lavender, reenter the cell cycle, proceeding from the G0/G1 phase to the S phase, and finally becoming arrested in G2, as indicated in the cell cycle in the yellow circle. In contrast, inner cortical cells, shown in purple, progress all the way through the cell cycle, as indicated by the cell cycle in the green circle, dividing and forming the nodule primordia. The activated cells are opposite the protoxylem poles of the root, which are shown in green. **(B)** Mode of action of Nod factors shown in a schematic depiction of a longitudinal section of a legume root. Application of Nod factors leads to root hair deformation, followed by an activation of the pericycle, due either to the action of Nod factors themselves or to that of second messengers (indicated by ?) generated in the epidermal cells. *ENOD40* expression in the pericycle cells may cause a change in the cytokinin/auxin ratio that results in cortical cell divisions (top panel). Alternatively (bottom panel), *ENOD40* expression in the pericycle may lead to susceptibility of the cortical cells, which then divide due to the action of either Nod factors or the second messengers generated in the epidermis. Cells shown in red are those that are sensitive to the action, direct or indirect, of Nod factors. In the bottom panel, the right arrow should point to the activated cortical cells instead of to the pericycle.

Thus, bacteria seem to be required for the formation of infection threads. It has been shown that pretreatment of clover root hairs with lipopolysaccharides of *R. trifolii* can improve the efficiency of infection thread induction by this strain, whereas pretreatment with lipopolysaccharides from a noninfectious *Rhizobium* strain leads to an increase in aborted infections (Dazzo et al., 1991). Furthermore, mutations in rhizobial exopolysaccharide biosynthesis can render the bacteria unable to induce infection threads (Dylan et al., 1986; Niehaus et al., 1993). Thus, interaction with bacterial surface compounds seems to play an important role in infection thread formation.

Cortical Cell Divisions

During mitotic reactivation of root cortical cells by rhizobia, genes that control the progression through the cell cycle, such as *cdc2* and mitotic cyclins, are induced (Yang et al., 1994). In addition, several nodulin genes are expressed, allowing a distinction to be made between nodule primordia and root or shoot meristems. Examples of such nodulin genes are *ENOD12* (Scheres et al., 1990a), *Gm93* (Kouchi and Hata, 1993), *ENOD40* (Kouchi and Hata, 1993; Yang et al., 1993; Asad et al., 1994; Matvienko et al., 1994), and *MtPRP4* (Wilson et al., 1994). These genes are activated in all cells of the primordia. Furthermore, *ENOD40* is also induced in the region of the pericycle opposite to the dividing cortical cells (Kouchi and Hata, 1993; Yang et al., 1993; Asad et al., 1994; Matvienko et al., 1994). Another early nodulin gene, *ENOD5* (Scheres et al., 1990b), is transcribed only in primordial cells that contain rhizobia.

Nod factors are sufficient for mitotic reactivation of the cortical cells (Spaink et al., 1991; Truchet et al., 1991; Relic et al., 1993). The early nodulins *ENOD12* and *ENOD40* are activated in such primordia (Vijn et al., 1993). In some host plants, purified Nod factors even induce nodule formation (Truchet et al., 1991; Mergaert et al., 1993; Stokkermans and Peters, 1994).

Interestingly, only certain cortical cells are susceptible to Nod factors. In tropical legumes, such as soybean, it is the outer cortical cells that are mitotically activated. In temperate legumes, such as pea, vetch, and alfalfa, it is the inner cortical cells, and especially those located opposite protoxylem poles, that divide (Kijne, 1992). The mechanism that controls the susceptibility of cortical cells is unknown. It has been postulated for decades that the susceptibility of cortical cells is conferred by an arrest in the G2 phase (Wipf and Cooper, 1938; Verma, 1992). However, use of cell phase–specific genes as probes in in situ hybridization experiments shows that this is not the case (Yang et al., 1994). Instead, susceptible cortical cells are, like other cortical cells, arrested in G0/G1.

The pattern of responding cortical cells provides some hints about a possible mechanism. Figure 3A shows that only narrow rows of cortical cells are activated to express the histone *H4* gene by rhizobia. At this time, the infection thread tips—the site where Nod factors are released—are still in the epidermis, indicating that Nod factors act at a distance. These rows of susceptible cells are located opposite protoxylem poles. More than 20 years ago, Libbenga et al. (1973) found that an alcohol extract of the stele could induce cell divisions in explants of the pea root cortex in the presence of auxin and cytokinin. A substance responsible for this activity, the so-called stele factor, has since been isolated and is thought to be released from the protoxylem poles. Such a compound might confer susceptibility to the cortical cells located opposite the protoxylem poles (Smit et al., 1993).

Which Nod factors can induce mitotic reactivation of cortical cells depends on the host plant. Rhizobia that induce cell divisions in the inner cortical layers, such as *R. leguminosarum* bv *viciae* and *R. meliloti*, produce Nod factors with highly unsaturated fatty acyl groups (Figure 2), whereas rhizobia that mitotically reactivate outer cortical cells, such as *Bradyrhizobium japonicum*, generally contain a C18:1 acyl group. The highly unsaturated fatty acyl group appears to be important for inducing cell divisions in the inner cortex. For example, only those *R. leguminosarum* bv *viciae* factors containing a C18:4 acyl group cause the formation of nodule primordia in vetch (Van Brussel et al., 1992). Whether the highly unsaturated fatty acyl moiety is recognized by a specific receptor and whether it is required for transport to the inner layers, for example, are unknown.

To unravel the mechanism by which Nod factors elicit cortical cell divisions, studies with compounds that can mimic their mitogenic activity have been performed. Two lines of evidence strongly suggest that Nod factors cause a change in the auxin/cytokinin balance. Both cytokinin (Cooper and Long, 1994) and compounds that block polar auxin transport (Hirsch et al., 1989) induce the formation of nodule-like structures in which early nodulin genes are expressed. Because some early nodulin genes are activated before cortical cells divide, an interesting question is whether such nodulins are involved in changing the phytohormone balance. The early nodulin gene *ENOD40*, which is induced by Nod factors in root pericycle as well as in dividing cortical cells, has a phytohormone effect when introduced into the nonlegume tobacco. This effect was examined in a protoplast assay in which the correlation between efficiency of cell division and auxin concentration was monitored. Tobacco protoplasts expressing a legume *ENOD40* gene under the control of the cauliflower mosaic virus 35S promoter divide efficiently at high auxin concentration, whereas in control protoplasts, this level of auxin suppresses their ability to divide (T. Bisseling and R. Walden, unpublished data).

Because *ENOD40* is sufficient to cause a phytohormone-like effect in tobacco and because induction of *ENOD40* expression in the pericycle precedes the first cortical cell divisions (T. Bisseling, unpublished data), we hypothesize that *ENOD40* expression in the pericycle of legume roots can cause the cytokinin/auxin ratio of the cortical cells to change, resulting in cell division (Figure 3B). In this model, mitotic reactivation would be induced in an indirect manner—that is, cortical cells would not themselves interact with the Nod factors. Alternatively, *ENOD40* expression in the pericycle might cause a

change in the cortical cells that renders them susceptible to Nod factors (or to second messengers generated in the epidermis), resulting in their mitotic reactivation (Figure 3B).

The role of *ENOD40* in altering phytohormone balance is not yet clear. *ENOD40* cDNA clones have been isolated from different legumes, and only in the soybean cDNAs could a long open reading frame be found (Kouchi and Hata, 1993; Yang et al., 1993; Crespi et al., 1994; Matvienko et al., 1994). Therefore, it has been postulated that this gene is active on the RNA level (Crespi et al., 1994; Matvienko et al., 1994).

Nod Factor Perception and Signal Transduction

As we have discussed, Nod factors induce responses in three different tissues of the root, namely, epidermis, cortex, and pericycle. Because Nod factors play a pivotal role in the early steps of nodulation, major efforts are being directed toward unraveling their mode of action. Nod factors are active at low concentrations, and their biological activity on a particular host is controlled by the presence of certain substitutions on the factor. These data suggest that Nod factors are recognized by a receptor in the host plant. However, it is unclear whether Nod factors interact directly with all three responding tissues or whether their interaction with epidermal cells results in the generation of second messengers that, after diffusion or transport, trigger responses in the inner tissues (Figure 3B).

The induction of certain host responses requires Nod factors with a very specific structure, whereas the demands for the induction of other responses are less stringent. For instance, for the induction of alfalfa root hair deformation, neither the structure of the fatty acid nor the presence of the *O*-acetyl group at the nonreducing end is important. On the other hand, the induction of infection thread formation in the same alfalfa root hairs requires a very specific structure. *R. meliloti* strains producing Nod factors that either are non-*O*-acetylated at the nonreducing end or do not contain the appropriate C16 unsaturated fatty acid initiate markedly fewer infection threads (Ardourel et al., 1994). A double mutant secreting Nod factors lacking the *O*-acetyl group and containing an inappropriate fatty acid has completely lost the ability to induce infection threads. This led Ardourel et al. (1994) to postulate that at least two different Nod factor receptors are present in the epidermis: a "signaling receptor" involved in the induction of root hair deformation, and an "uptake receptor" that is activated only by molecules with a very specific structure and that initiates the infection process.

The existence of distinct signaling and uptake receptors is supported by studies on the pea gene *sym2*, which controls nodulation. *sym2* originates from the wild pea variety Afghanistan. Afghanistan peas and cultivated peas carrying an introgressed *sym2* region nodulate only after inoculation with an *R. leguminosarum* bv *viciae* strain carrying an additional *nod* gene, namely, *nodX*. NodX catalyses the *O*-acetylation of *R. leguminosarum* bv *viciae* Nod factors (see Figure 2) at the reducing end (Firmin et al., 1993). *R. leguminosarum* bv *viciae* lacking *nodX* induces root hair deformation, but the ability to induce infection thread formation is strongly reduced. Therefore, Sym2 is a good candidate for an uptake receptor that interacts with NodX-modified Nod factors (T. Bisseling, unpublished data).

A biochemical approach to isolate a Nod factor receptor is feasible because large quantities of purified Nod factors, as well as chemically synthesized ones (Nicolaou et al., 1992), are available. A first report (Bono et al., 1995) on Nod factor binding proteins has revealed the occurrence of a binding protein that is present predominantly in the 3000*g* fraction of root extracts from alfalfa. However, the affinity of this binding protein for its ligand is lower than the concentration at which Nod factors are active. Furthermore, it binds to sulfated and nonsulfated factors in a similar way, whereas factors lacking the sulfate group are barely active on alfalfa. Therefore, it is unlikely that this protein is the Nod factor receptor. The availability of labeled Nod factors also creates the possibility of clarifying whether lectins play a role in binding of Nod factors, as has been postulated in the past (Long and Ehrhardt, 1989).

At present, genetic approaches to unravel Nod factor perception are restricted to legumes such as pea and soybean. Unfortunately, these species are recalcitrant to molecular genetic strategies leading to gene cloning. To study the mode of action of Nod factors, it might therefore be essential to develop new legume model systems (Barker et al., 1990; Handberg and Stougaard, 1992) or to explore the potential of nonlegume systems such as Arabidopsis. The latter may at first seem illogical, but a few observations show that Nod factors are recognized by nonlegumes. For example, expression of rhizobial *nod* genes in tobacco affects the development of these plants (Schmidt et al., 1993). Furthermore, a mutated carrot cell line that has lost the ability to form somatic embryos can be rescued by Nod factors (De Jong et al., 1993), and Nod factors trigger the alkalinization of the medium by tomato suspension-cultured cells (Staehelin et al., 1994a). Consequently, Nod factor receptors may be present in nonlegumes, a conclusion supported by the existence of a nonlegume, *Parasponia*, that can be nodulated by rhizobia (Marvel et al., 1987).

The availability of a root hair deformation assay and knowledge of some of the plant genes that are activated by the Nod factors, together with methods to inject root hairs (Allen et al., 1994), should make it possible to unravel the signal transduction cascades that are activated after Nod factor perception. These tools have been developed only recently, and therefore our understanding of Nod factor signal transduction is still in its infancy. Additional studies are required to determine the relevance of Nod factor–induced changes such as ion fluxes, membrane depolarization, and rearrangements of the actin filaments in the signal transduction pathways (Allen et al., 1994). Furthermore, it has now become possible to study whether, in addition to Nod factors, other rhizobial compounds are involved in facilitating Nod factor–induced responses. A

candidate is the NodO protein (Sutton et al., 1994). *nodO*, which is present in *R. leguminosarum* bv *viciae*, encodes a secreted protein that is not involved in Nod factor biosynthesis. When added to lipid bilayers, the purified protein can form channels that allow the movement of monovalent ions. Therefore, it has been suggested that NodO may amplify the Nod factor–induced responses by integration into the plant plasmamembrane (Sutton et al., 1994).

From Nodule Development to Nodule Functioning

As described previously, research on early stages of nodulation has emphasized the developmental steps of these processes. The achievements of rhizobial genetics allowed the dissection of the early stages and facilitated the characterization of the rhizobial signal molecules, the Nod factors, which play a key role in all early nodulation processes. The availability of purified Nod factors has now made the system accessible for biochemical approaches that should yield insight into the structure and distribution of Nod factor receptors and their signal transduction pathways.

Regarding the final steps of nodule formation, however, the proteins involved in nodule nitrogen, carbon, and oxygen metabolism have been studied on a biochemical level for decades, whereas research on the developmental aspects of the final steps of nodule formation is still in its infancy. Although several bacterial genes have been identified that, when mutated, cause a block in relatively late steps in nodule development, it has not been possible to identify the factors directly affecting differentation. Most mutants show pleiotropic effects or display host plant–dependent symbiotic phenotypes (see, for example, Gray et al., 1991; Hotter and Scott, 1991; Rossbach and Hennecke, 1991). The function of potential regulatory factors in nodule development, for example, in bacteroid differentiation, cannot be assessed because the technology for targeting these compounds to their sites of activity is not available. Thus, questions regarding signal exchange and developmental switches during the later steps of nodule formation have been difficult to address. For these reasons, in the following sections the major emphasis is on the biochemistry of nodule formation; developmental aspects are mentioned only briefly.

NODULE FUNCTIONING

Symbiotic nitrogen fixation takes place in specialized bacterial cells, in bacteroids in legume nodules, and in *Frankia* vesicles in actinorhiza. The bacterial enzyme nitrogenase catalyzes the following reaction:

$$N_2 + 8H^+ + 8e^- + 16\text{Mg-ATP} \rightarrow 2NH_3 + H_2 + 16\text{Mg-ADP} + 16P_i \quad (1)$$

Nitrogenase consists of two components, the homodimeric Fe protein, encoded by *nifH*, and the tetrameric molybdenum–iron (MoFe) protein, encoded by *nifD* and *nifK*, which contains the MoFe cofactor. Hydrogen evolution is part of the nitrogenase mechanism; in the absence of other reducible substrates, the total electron flux through nitrogenase is funneled into hydrogen production (Hadfield and Bulen, 1969). Crystallographic structure analyses of the Fe protein (Georgiadis et al., 1992) and MoFe proteins of free-living nitrogen-fixing bacteria have shown structural similarities with other electron transfer systems, including hydrogenases and the photosynthetic reaction center (Kim and Rees, 1992; Kim et al., 1993; see von Wettstein et al., 1995, this issue).

In symbiosis, ammonium, the product of nitrogen fixation, is exported to and assimilated in the plant, which in turn supplies the bacteria with carbon sources to provide the energy for the nitrogenase reaction. The structure of a mature nodule has developed to meet the requirements set by this nutrient exchange between both symbiotic partners.

Nodule primordia differentiate into nitrogen-fixing nodules when bacteria have been released from the infection threads into the infected cells. Two types of legume nodules can be distinguished by their growth pattern—indeterminate and determinate nodules (Newcomb, 1981). Both types of nodule are characterized by peripheral vascular bundles and a central tissue containing infected and uninfected cells. In indeterminate nodules, a developmental gradient from the distal persistent meristem to the proximal senescence zone is present in which the central tissue is divided into specific zones (Figures 1A and 4; Vasse et al., 1990). The meristem is followed by the prefixation zone, where infection of the cells takes place. In the interzone, bacterial nitrogen fixation is induced, and it proceeds throughout the nitrogen fixation zone. In the senescence zone, bacteroids are degraded by the plant. In determinate nodules, the nodule meristem ceases to divide at an early stage of development. As a result, all of the cells of the central tissue are at a similar stage of development at any given time. Actinorhizal nodules display an indeterminate growth pattern, but in contrast to legume nodules, they represent coralloid structures composed of several modified lateral roots without root caps (lobes). Actinorhizal nodule lobes contain a central vascular bundle as well as infected and uninfected cells in the cortex (reviewed by Berry and Sunell, 1990).

Metabolite Exchange between Plant Cells and Intracellular Bacteria: The PBM as Interface

Root nodules provide the proper environment to allow efficient nitrogen fixation by the microsymbiont. Part of this specialization is the occurrence of plant-derived membranes that in all cases surround the "intracellular" microsymbiont (Figure 1). In legume nodules, these membranes are called peribacteroid membranes (PBMs; Figure 1A). They form the interface between the symbiotic partners across which signals and metabolites are exchanged and prevent a defense response by

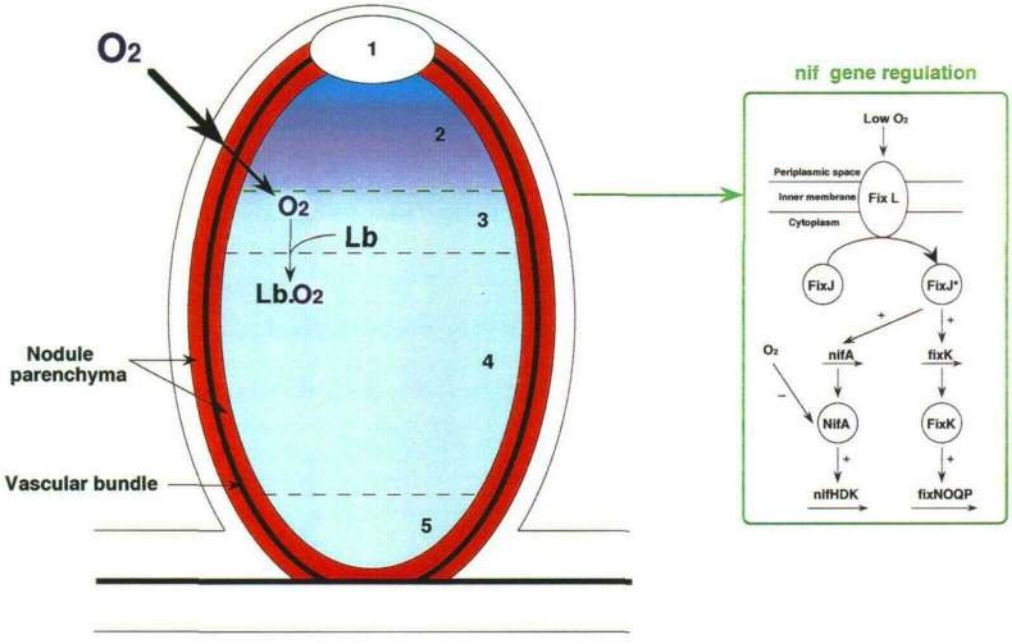


Figure 4. Oxygen Regulation in Indeterminate Legume Nodules.

Indeterminate legume nodules consist of five distinct regions: 1, nodule meristem; 2, prefixation zone; 3, interzone; 4, nitrogen fixation zone; and 5, senescence zone. An oxygen barrier is present in the nodule parenchyma surrounding the nodule vascular bundle (shown in red) that reduces oxygen access to the central tissue of the nodule. However, because this oxygen barrier is interrupted in the meristem, an oxygen gradient forms that extends from the distal to the proximal end of the nodule (shown by blue shading). In the first cell layer of the interzone (shown by the dashed green line), the low oxygen concentration leads to the events described in the green box. Low oxygen concentrations activate the bacterial transmembrane oxygen sensor protein FixL, which in turn phosphorylates and thereby activates the transcriptional activator FixJ. The activated FixJ protein (FixJ*) induces transcription of *nifA* and *fixK*, and the protein products of these genes induce the transcription of different genes encoding proteins involved in the process of nitrogen fixation. As an additional level of control, the NifA protein itself is oxygen sensitive. Leghemoglobin (*lb*) genes are expressed in the prefixation zone, the interzone, and the fixation zone. Leghemoglobin proteins transport oxygen to sites of respiration, thus enabling ATP production in a low-oxygen environment.

the plant against the "intracellular" bacteria (Nap and Bisseling, 1990; Verma, 1992; Werner, 1992).

Upon release from the infection thread, bacteria become internalized in legume nodules by a process resembling endocytosis (Basset et al., 1977). In actinorhizal nodules, however, *Frankia* hyphae penetrate the cell wall of cortical cells and start branching, while the plasma membrane invaginates and cell wall material is deposited around the growing hyphae. Thus, *Frankia* is not released into the plant cytoplasm and stays surrounded by encapsulating cell wall material throughout the symbiosis (Berry and Sunell, 1990). Subsequently, the endosymbionts multiply, enlarge, and eventually occupy most of the volume of the infected cell. During this process, growth of the microsymbiont and the surrounding membrane is synchronized by an unknown mechanism. This process of endosymbiont internalization and propagation requires massive membrane synthesis—in the case of legume nodules, 30 times the amount of plasma membrane synthesis (Verma, 1992).

The membrane surrounding the microsymbiont is derived from the host plasma membrane. The PBM of legume nodules has phospholipid (Perotto et al., 1995) and protein composition that are different from those of the plasma membrane (Verma, 1992) and that (presumably) endow it with specialized functions. The PBM contains several nodulins and may also contain a rhizobial protein (Fortin et al., 1985; Miao et al., 1992). Within the peribacteroid space between the bacteroids and the PBM, several proteins are present that are also found in vacuoles, for example, α-mannosidase II (Kinnback et al., 1987; Mellor and Werner, 1987), proteases (Mellor et al., 1984), and protease inhibitor (Garbers et al., 1988; Manen et al., 1991). Thus, the PBM may have adapted some properties of the tonoplast membrane (Mellor and Werner, 1987). Indeed, it has been proposed that the symbiosome (the PBM with enclosed bacteroids) has properties of a lytic compartment continuously being neutralized by ammonia exported by the bacteroids (Kannenberg and Brewin, 1989). According to this hypothesis, one would expect that the lack of bacterial nitrogen fixation

would lead to bacteroid degradation. In fact, there is evidence for premature bacteroid degradation of nonfixing *Rhizobium* mutants (for example, see Hirsch and Smith, 1987).

The extensive membrane biosynthesis in infected cells, together with the possibility to manipulate gene expression in root nodules without affecting other parts of the plant, has made the PBM an ideal system to study membrane biogenesis in plants. By using an antisense strategy in combination with nodule-specific promoters, it has been possible to show that homologs of the Ypt1 protein (Schmitt et al., 1986), which controls membrane biosynthesis in yeast, are involved in PBM biosynthesis in soybean nodules (Cheon et al., 1993). In nodules expressing antisense RNA of such a homolog, the number of bacteroids per cell was reduced and the infected cells did not expand.

Because the PBM constitutes the interface between bacteroids and host plants, it plays an important role in controlling the exchange of metabolites. These include ammonium, the product of nitrogen fixation, and heme, the prosthetic group of the oxygen transport protein leghemoglobin, which are exported by the bacteroids to the host cytoplasm (O'Gara and Shanmugan, 1976; Nadler and Avissar, 1977), as well as carbon sources and probably also assimilated ammonium, which are supplied by the host to the bacteroids (De Bruijn et al., 1989; Werner, 1992). Which proteins are involved in the transport of these compounds is largely unclear. Bacteroids express a dicarboxylic acid uptake system, isolated bacteroids take up dicarboxylic acids, and mutants in this uptake are symbiotically ineffective (Ronson et al., 1987; Werner, 1992), all of which indicates that dicarboxylic acids are likely to be the carbon source supplied by the plant to the intracellular bacteria. It has been suggested that nodulin-26 transports the dicarboxylic acids to the bacteroids (Ouyang et al., 1991). However, its low substrate specificity in vitro indicates that it is more likely to form a pore responsible for the uptake of ions or small metabolites in general (Weaver et al., 1994).

After division, the intracellular bacteria differentiate into bacteroids. Because both plant (Häser et al., 1992) and bacterial (Glazebrook et al., 1993) mutants have been identified that are specifically defective in bacteroid differentiation, this process may be independent of internalization of bacteria by the infected cells. Bacterial mutants specifically defective in the release of bacteria from the infection thread are known as well (De Maagd et al., 1989). Bacterial *nod* genes are expressed in the distal part of the prefixation zone (Figure 4; Schlaman et al., 1991), indicating that Nod factors may play a role in signal exchange within the nodule. However, because bacterial release and bacteroid development can be impaired in bacterial strains with functional *nod* genes, other bacterial and/or plant signals must also play a role in these steps of development.

Metabolite Exchange between Nodule and Plant: Nitrogen Transport

In the context of the whole plant, the root nodule functions as a nitrogen source and a carbon sink. In fact, it has been suggested that legume nodules evolved from carbon storage organs (Joshi et al., 1993). The carbon source transported from the leaves to the nodules is sucrose (Hawker, 1985), which is introduced into nodule metabolism through degradation by sucrose synthase. This enzyme is present at high levels in both legumes and actinorhizal nodules (Thummler and Verma, 1987; M. van Ghelue, A. Ribeiro, A. Akkermans, B. Solheim, A. van Kammen, T. Bisseling, and K. Pawlowski, unpublished observations). The form in which nitrogen is transported depends on the plant: temperate legumes, which generally form indeterminate nodules, export amides, whereas tropical legumes, which form determinate nodules, export ureides. Actinorhizal plants export mostly amides, with the exceptions of *Alnus* sp and *Casuarina equisetifolia*, which are citrulline exporters (Schubert, 1986; Sellstedt and Atkins, 1991). In all cases, ammonium is exported by the microsymbiont as the first product of nitrogen fixation and is assimilated in the cytoplasm of nodule cells via the glutamine synthetase (GS)/glutamate synthase pathway (Schubert, 1986; see Lam et al., 1995, this issue). Subsequently, glutamate is metabolized into nitrogen transport forms. The products of several late nodulin genes play a role in this metabolism.

In ureide-producing determinate legume nodules, the assimilation of ammonium by GS and the biosynthesis of ureides are spatially separated to some extent: whereas GS is expressed in both infected and uninfected cells of soybean nodules (Miao et al., 1991), uricase (nodulin-35), a key enzyme in purine oxidation that catalyzes the oxidation of uric acid to allantoin, has been found in peroxisomes of uninfected cells only (Hanks et al., 1981; Nguyen et al., 1985). Allantoinase, which catalyzes the next step in purine oxidation, has also been localized to uninfected cells (Hanks et al., 1981). The uninfected cells of determinate nodules also seem to be involved in the transport of fixed nitrogen. These cells constitute a more or less continuous network throughout the whole central tissue that facilitates the transport of assimilated ammonium to the nodule vascular bundle (Selker, 1988). An elaborate tubular endoplasmic reticulum system that is appressed to the peroxisomes, where ureides are produced, and continues through plasmodesmata connects all uninfected cells (Newcomb et al., 1985). In indeterminate nodules, by contrast, no specialized function has been assigned to the uninfected cells in the central tissue. Instead, efficient transport of fixed nitrogen is achieved by the presence of transfer cells in the pericycle of the nodule vascular bundles (Pate et al., 1969).

Oxygen Protection of Bacterial Nitrogen Fixation

Nitrogenase is highly oxygen sensitive because one of its components, the MoFe cofactor, is irreversibly denatured by oxygen (Shaw and Brill, 1977). On the other hand, the large amount of energy required for this reaction has to be generated by oxidative processes; thus, there is a high demand for oxygen in nodules. Different strategies are used in different symbiotic interactions to cope with this paradox. In legume nodules, a low oxygen tension in the central part of the nodule is achieved

by a combination of a high metabolic activity of the microsymbiont and an oxygen diffusion barrier in the periphery of the nodule, that is, in the nodule parenchyma (Figure 4; Witty et al., 1986). Because oxygen diffuses $\sim 10^4$ times faster through air than through water, it is generally assumed that oxygen diffusion in nodules occurs via the intercellular spaces. The nodule parenchyma contains very few and small intercellular spaces, and this morphology is thought to be responsible for the block in oxygen diffusion (Witty et al., 1986). In the nodule parenchyma, nodulin genes such as *ENOD2* are expressed whose protein products might contribute to the construction of the oxygen barrier (Van de Wiel et al., 1990). In the infected cells of the central part of the nodule, high levels of the oxygen carrier protein leghemoglobin facilitate oxygen diffusion. In this way, the microsymbiont is provided with sufficient oxygen to generate energy within a low overall oxygen concentration (Figure 4; Appleby, 1984).

In contrast to *Rhizobium*, *Frankia* bacteria can form specialized vesicles in which nitrogenase is protected from oxygen (Figure 1B; Benson and Silvester, 1993). However, vesicle formation during symbiosis does not take place in all *Frankia*–root interactions (Benson and Silvester, 1993) and does not always seem to provide full oxygen protection of nitrogenase (Tjepkema, 1983; Kleemann et al., 1994). In these cases, an oxygen diffusion barrier is established around groups of infected cells by lignification of the walls of adjacent uninfected cells (Berg and McDowell, 1988; Zeng et al., 1989). In addition, the oxygen transport protein hemoglobin, the equivalent of leghemoglobin, is found in the infected cells (Fleming et al., 1987; Tjepkema and Asa, 1987; Jacobsen-Lyon et al., 1995).

As in actinorhizal symbioses, in the *Nostoc–Gunnera* symbiosis, oxygen protection of nitrogen fixation is achieved by the formation of a specialized compartment containing nitrogenase: *Nostoc* forms heterocysts that are protected from oxygen by a glycolipid cell wall (Figure 1C; Bergman et al., 1992).

Gene Regulation in Nodules

To obtain nitrogen-fixing root nodules, several genes of both symbionts are specifically induced or repressed during nodule development. The use of reporter genes as well as in situ hybridization studies has provided detailed insights into the spatial and temporal regulation of such genes in indeterminate nodules. In such nodules, major, sudden developmental changes occur at the transition of the prefixation zone to the interzone: starch is deposited in the plastids of the infected cells, and the bacteroid morphology alters (Figures 1A and 4; Vasse et al., 1990). These events are accompanied by changes in bacterial gene expression: transcription of bacterial *nif* genes, which encode enzymes involved in the nitrogen fixation process, is induced, whereas expression of the bacterial outer membrane protein gene *ropA* is dramatically reduced (Yang et al., 1991; De Maagd et al., 1994).

All of these events, together with dramatic changes in plant gene expression (see later discussion), take place within a single cell layer. What plant factor causes this rapid change in bacterial differentiation? To answer this question, rhizobial *nif* gene regulation has been studied extensively and has generally been found to be induced by microaerobic conditions (reviewed in Merrick, 1992; Fischer, 1994). The regulation of *nif* gene expression in *R. meliloti* is described here (see Figure 4) because it can be correlated to morphological changes observed in an indeterminate nodule.

Transcription of *R. meliloti* nitrogen fixation (*nif/fix*) genes is controlled either by the transcriptional activator NifA together with the sigma factor RpoN (Gussin et al., 1986; Morrett and Buck, 1989) or, for some genes, by the transcriptional activator FixK. NifA activity is under oxygen control at two levels: the NifA protein itself is oxygen sensitive (Krey et al., 1992), and its transcription, together with that of *fixK*, is induced under microaerobic conditions by the transcriptional activator FixJ (David et al., 1988). FixJ is part of a two-component system that includes the oxygen-sensing hemoprotein FixL. FixJ is activated by FixL by phosphorylation upon microaerobiosis (see Figure 4; David et al., 1988; Gilles-Gonzalez et al., 1991; Da Re et al., 1994). It is the activated FixJ protein that in turn induces the transcription of *nifA* and *fixK* (Batut et al., 1989).

Although microaerobic conditions are essential for rhizobial *nif* gene transcription in symbiosis, it has long been debated whether the reduction of oxygen concentration is the sole regulatory factor for the induction of *nif* gene expression in the interzone. Recent results (Soupène et al., 1995) have shown that *R. meliloti nif* gene expression in plants can be modified by changing the external oxygen concentration: in nodules immersed in agar, *nif* gene expression is extended to a younger part of the nodule and now also occurs in the prefixation zone. This effect is controlled by the FixLJ system, because the same result is obtained by nodulation with a strain carrying a constitutively active mutant form of FixJ (FixJ*; see Figure 4). Thus, oxygen concentration seems to be a major factor in controlling symbiotic *nif* gene transcription during symbiosis. In contrast, *ropA* expression is not under oxygen control in free-living bacteria, and *ropA* repression can even be uncoupled from *nif* gene induction in the same cell layer. In mutant nodules induced by a *Rhizobium* strain whose host range had been manipulated, *ropA* mRNA distribution was equal to that in wild-type nodules, whereas bacteroid differentiation and *nif* gene induction did not take place (De Maagd et al., 1994). Therefore, further analyses are required to determine the other regulatory factors responsible for the changes in bacterial gene expression in the first cell layer of the interzone.

The expression of several plant genes is also controlled at the transition of the prefixation zone to the interzone as well as in other zones of the central tissue (Scheres et al., 1990a, 1990b; Yang et al., 1991; Kardailsky et al., 1993; Matvienko et al., 1994). However, the expression of these genes seems not to be controlled by the oxygen tension (Govers et al., 1986) but rather to be under developmental control. To analyze the regulators of plant nodulin gene expression, the expression of nodulin promoter–β-glucuronidase fusions has been studied in heterologous legumes (Forde et al., 1990; Szabados et al., 1990; Brears et al., 1991).

The most extensive studies have been performed on the leghemoglobin genes. So far, promoter analysis of these genes has led to the identification of a so-called organ-specific *cis*-acting element (OSE; Ramlov et al., 1993), also called the nodule-infected cell-specific element (NICE; Szczyglowski et al., 1994), which has also been found in the promoter of the nodule-specific hemoglobin gene of the actinorhizal plant *Casuarina glauca* (Jacobsen-Lyon et al., 1995). A *C. glauca* hemoglobin promoter–β-glucuronidase fusion is expressed in the infected cells of *Rhizobium*-induced nodules from *Lotus corniculatus* (Jacobsen-Lyon et al., 1995), which implies that similar regulatory factors are involved in both legume and actinorhizal systems. However, the corresponding transcription factors that bind to these promoter elements have yet to be identified.

CONCLUDING REMARKS

Symbioses between higher plants and nitrogen-fixing microorganisms provide a niche in which the prokaryote can fix nitrogen in a very efficient manner. A comparison of the development and functioning of the three different nitrogen-fixing symbioses has provided and continues to provide insight into how both common and unique strategies have evolved to solve problems imposed by various requirements of nitrogen fixation. For instance, in all systems the plant copes with intracellular bacteria by enclosing them in a plasmalemma-derived membrane, whereas protection of the enzyme nitrogenase against oxygen is achieved in diverse manners.

An intriguing aspect of the nitrogen-fixing symbioses is their host specificity, whose strictness varies in the different systems. In the *Gunnera–Nostoc* system, only a single plant genus can establish the interaction, whereas rhizobia can interact with most members of the legume family. *Frankia* bacteria are the most promiscuous microsymbionts, because they can establish a symbiosis with plants belonging to different families; however, recent molecular phylogenetic studies have shown that these families are actually rather closely related (Chase et al., 1993; Maggia and Bousquet, 1994).

Host specificity provides a serious restraint in the application of symbiotic nitrogen fixation in agriculture, because most major crops are unable to establish such a symbiosis. Therefore, it is not surprising that since the development of plant genetic engineering techniques, an important goal has been to transfer the ability to form a nitrogen-fixing symbiosis to important crops, such as rice. However, molecular genetic research has shown that a relatively high number of specific host functions are involved in forming a nitrogen-fixing organ. Therefore, it has seemed impossible to achieve this aim with the methodology available.

The possibility of reaching this goal has become newly invigorated as a result of research indicating that mechanisms controlling nodule development might be derived from processes common to all plants. For example, Nod factors might be recognized by receptors that are also present in nonlegumes; preinfection thread formation appears to involve a mechanism derived from the cell cycle machinery; and several plant proteins that were thought to function exclusively in nodules appear to have nonsymbiotic counterparts, as has been described for soybean nodulin-26 (Miao and Verma, 1993) and *Casuarina* hemoglobin (Jacobsen-Lyon et al., 1995). Furthermore, actinorhizal nodules and nodules induced by rhizobia on the nonlegume *Parasponia* closely resemble lateral roots (Hirsch, 1992). Thus, the processes modified in the nodule developmental programs are common to all higher plants. Studies of how these common processes have been altered might therefore provide new means to design strategies by which nonlegume plants can be given the ability to establish a symbiosis with a nitrogen-fixing microbe.

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