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Update on Signal Transduction

Signal Transduction in *Rhizobium*-Induced Nodule Formation

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During a deficiency of reduced nitrogen sources, leguminous plants are able to live symbiotically with bacteria of the genera *Rhizobium, Bradyrhizobium,* or *Azorhizobium* (here collectively called rhizobia). The interaction starts with an attachment of the rhizobia to the plant root hairs. The plant enables the bacteria to invade its roots by the formation of tubular structures, the infection threads, that grow through the root hairs into the cortex. Simultaneously, cortical cells are mitotically activated, giving rise to a nodule primordium. Infection threads grow toward the primordium. Upon arrival, the bacteria are released into the cytoplasm of the primordial cells and become surrounded by a plant-derived peribacteroid membrane. The nodule primordium develops into a mature nodule, and the bacteria differentiate into their endosymbiotic form, the bacteroids. These bacteroids are able to fix nitrogen into ammonia, which can subsequently be utilized by the plant.

The establishment of a nodule is thus achieved collaboratively from both participants and it can be anticipated that a flow of signals is being transmitted between plant and bacteria; this *Update* highlights the present knowledge of this communication.

SIGNAL EXCHANGE BETWEEN PLANT AND BACTERIUM

The interaction between rhizobia and legumes occurs in a host-specific way; a particular *Rhizobium* strain nodulates only a limited number of plants. This host-specific aspect of symbiosis was recognized long before any signal molecule had been purified. According to the host plant, different cross-inoculation groups have been defined in which *Rhizobium* strains are classified. Some *Rhizobium* strains can nodulate plant species belonging to a few genera, e.g. *Rhizobium leguminosarum* bv *viciae* can only nodulate plants of the genera *Pisum, Vicia, Lathyrus,* and *Lens,* whereas the closely related *R. leguminosarum* bv *trifolii* nodulates plants of the genus *Trifolium*. On the other hand, there are *Rhizobium* species, such as *Rhizobium* sp. strain NGR234, that have a broad host range; they can nodulate a wide range of plant genera.

In the early 1990s bacterial secreted nodulation (Nod) factors were characterized and shown to play a major role in inducing nodule organogenesis and in determining host specificity (Lerouge et al., 1990). Nod factors from several *Rhizobium* spp. have now been characterized, and they all share a β-1,4-linked *N*-acyl-D-glucosamine backbone of three to six units. The nonreducing terminal sugar moiety is substituted on the C-2 position with a fatty acid, the structure of which is variable. All *Rhizobium* spp. studied so far produce several Nod factors that vary in the length of the glucosamine backbone, in the structure of the fatty acid, and in the decorations on the terminal sugar residues. For example, the majority of *Rhizobium meliloti* Nod factors contain an unsaturated C16 fatty acid but factors with C18 to C26 (ω-1)-hydroxylated fatty acids also are produced. The sugar residue at the reducing end is *O*-sulfated, whereas the sugar residue at the nonreducing end can be acetylated (Lerouge et al., 1990; Roche et al., 1991b; Schultze et al., 1992; Demont et al., 1993).

The bacterial nodulation (*nod*) genes, which are in several rhizobia located on a Sym plasmid, are responsible for Nod factor production and secretion. Genetic and biochemical studies have shown that synthesis of the *N*-acyl-D-glucosamine backbone is catalyzed by NodA, NodB, and NodC. The terminal sugar residues and the acyl chain are changed by the action of several other Nod proteins, which can be species-specific (for review, see Carlson et al., 1994). The *nod* genes are organized into operons and their transcription is regulated by the constitutively expressed *nodD* gene, which is a member of the *lysR* family (Henikoff et al., 1988). The encoded protein acts as a transcriptional activator for the *nod* operons after activation due to binding to plant-secreted signals, mostly (iso) flavonoids (Fig. 1). Most rhizobia possess multiple copies of *nodD*. For example, *R. meliloti* has three *nodD* genes and each protein is involved in the recognition of different signals. An important question is whether the various NodD proteins have a different affinity for the *nod* operons leading to a control of the composition and stoichiometry of the produced Nod factors. When the structure of Nod factors secreted by *R. meliloti* strains containing only one of the three *nodD* genes was studied, it was shown that the different regulatory genes do not influence the length of the chitin backbone or the *O*-sulfate or *O*-acetyl decorations. However, only in the presence of $NodD_3$ are Nod factors harboring a C18 to C26 (ω-1)-hydroxylated fatty acid made (Demont et al., 1994).

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Abbreviation: (X:Y), a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

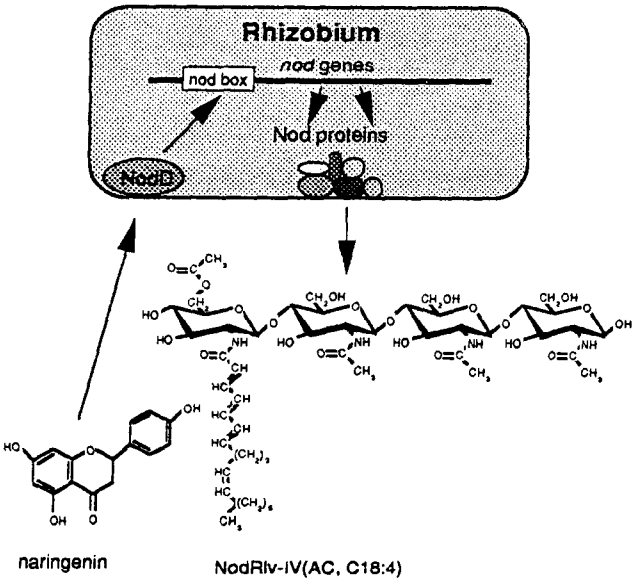

Figure 1. Plant-secreted (iso) flavonoids are recognized by the *Rhizobium* bacteria via an interaction with the transcriptional activator NodD protein. Upon binding of the flavonoid, NodD is activated, leading to transcription of the bacterial *nod* genes. The Nod proteins are involved in the production and secretion of Nod factors. Shown are the flavonoid naringenin and the tetrameric Nod factor harboring a C18:4 fatty acid of *R. leguminosarum* bv *viciae*. The Nod factor nomenclature is based on similarities of all Nod factors. In this nomenclature the species indication (e.g. Rlv for *R. leguminosarum* bv *viciae*) is followed by a roman numeral, referring to the number of glucosamine subunits, and a term in parentheses indicating the length and the degree of unsaturation of the acyl chain (e.g. C18:4) and substitutions on the nonreducing and reducing sugar residues (e.g. Ac for an *O*-acetyl group).

The following two examples illustrate that the decorations of Nod factors play a role in determining the host specificity. Between the closely related biovars *R. leguminosarum* bv *viciae* and *R. leguminosarum* bv *trifolii*, the host specificity is determined by the structure of the acyl chain of the Nod factor. *R. leguminosarum* bv *viciae* produces mainly Nod factors that contain *cis*-vaccenyl (C18:1) or a highly unsaturated C18:4 fatty acid that is determined by *nodE* (Spaink et al., 1991). Furthermore, minor quantities of Nod factors harboring the common fatty acids C16:0, C16:1, or C18:0 are produced. The biovar *trifolii* does not produce Nod factors that contain a C18:4 but instead produce a complex mixture of Nod factors carrying unsaturated fatty acyl groups containing either two, three, or four double bonds (Spaink et al., 1995). An inactive *nodE* gene will, in both cases, lead to the production of Nod factors that are mainly acylated with vaccenic acid (C18:1) and results in a dramatic decrease of nodulation efficiency on their host plants. In contrast, the loss of *nodE* in *R. leguminosarum* bv *trifolii* enables this bacteria to nodulate the nonhost pea (*Pisum sativum*). By introducing the *nodE* gene of *R. leguminosarum* bv *viciae* into this *trifollii nodE*$^-$ mutant, it can nodulate even more host plants of *R. leguminosarum* bv *viciae*, but the nodulation efficiency on its own host range does not improve (Spaink et al., 1989).

For the nodulation of the host plants of *R. meliloti* a sulfate decoration at the reducing end of Nod factors is required. In *R. meliloti* the *nodH*, *nodP*, and *nodQ* genes encode for enzymes involved in this sulfation. *R. meliloti nodH*$^-$ mutants produce Nod factors lacking the sulfate group at the reducing end (Roche et al., 1991a). These mutants lost their ability to nodulate on their host plant alfalfa (*Medicago sativa*). When the *R. meliloti* genes *nodPQ*, *nodFEG*, *nodH*, and *nodD*$_3$ are introduced into *R. leguminosarum* bv *viciae*, this bacterium can induce noninfected nodules on alfalfa. At the same time, it has lost the ability to nodulate on its own host plants. If *nodH* is mutated, the transformed *R. leguminosarum* bv *viciae* behaves again like a wild-type strain (Faucher et al., 1989).

NOD FACTOR-INDUCED RESPONSES

Purified Nod factors are able to induce responses similar to those induced by *Rhizobium*. The first responses are provoked at spatially separated sites: the epidermis, the cortical cells, and the pericycle. This immediately raises the question of whether the observed responses are directly provoked by Nod factors or by Nod factor-induced, diffusible secondary signals. We will discuss the *Rhizobium*-induced responses and the role Nod factors play in more detail, and we will try to answer the question put forward.

INTERACTION WITH THE ROOT EPIDERMIS

Nod factor concentrations in a picomolar range are sufficient for the induction of responses in the epidermis. These low concentrations are indicative for a receptor-mediated activity. The responses include membrane depolarization of epidermal cells (Ehrhardt et al., 1992; Felle et al., 1995; Kurkdjian, 1995), calcium spiking in root hours (Ehrhardt et al., 1996), deformation of root hairs (Heidstra et al., 1994), and induction of gene expression (Journet et al., 1994; Cook et al., 1995) (Fig. 2). These responses occur in a host-specific manner and take place in a narrow zone of the root where young root hairs are present. The earliest response after Nod factor application is the depolarization of the membrane, which can be detected within minutes in trichoblasts as well as in atrichoblasts (Felle et al., 1995). The first detectable morphological change is root hair deformation, for which a 10-min period of Nod factor-root contact is sufficient. Within 3 h the tip of young root hairs, which have almost reached their mature stage, swell and new tip growth is initiated (Heidstra et al., 1994).

Two genes were identified previously in which expression is induced in the root epidermis within 2 to 3 h following the addition of Nod factors. These genes are *ENOD12*, encoding a Pro-rich protein, and the peroxidase encoding *MtRip1* (Journet et al., 1994; Cook et al., 1995). Both genes are expressed in all epidermal cells in a specific zone of the root extending from the root meristem through the area of root hair differentiation. Based on the timing and the site of expression, it is not likely that these genes are involved in root hair deformation, but they might play a role in the next step to occur, the infection. In this view, it has been speculated that peroxidases may contribute to

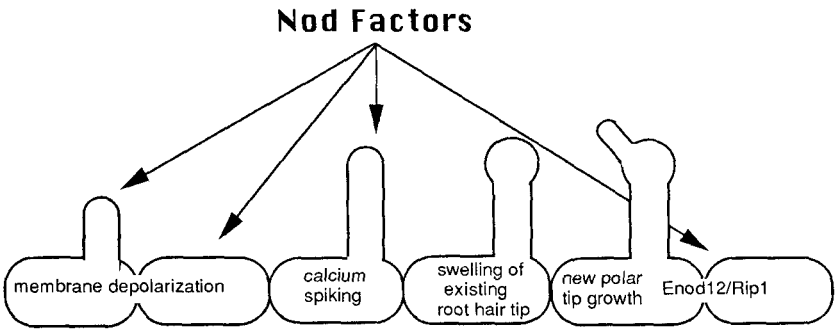

Figure 2. Nod factors induce several responses in a narrow zone of the epidermis. Within minutes membrane depolarizations can be detected in trichoblasts as well as atrichoblasts. Root hairs that are just bulging out are highly sensitive, whereas epidermal cells that are not yet differentiated into root hairs do not respond. The sensitivity of very long root hairs is weak (Kurkdjian, 1995). Deformation is induced within 3 h in a small zone of young root hairs that have almost reached their mature stage. First, the existing root hair tip swells and new tip growth is reinitiated. A 10-min period of Nod factor root contact is sufficient to induce root hair deformation. A similar time period is needed to induce calcium spiking. Expression of Nod factor-induced genes such as *ENOD12* or *RIP1* is detectable within 2 to 3 h in all epidermal cells extending from the root meristem through the area of root hair differentiation.

the infection process by altering root hair wall structure at sites of incipient penetration (Salzwedel and Dazzo, 1993). The expression of *ENOD12* in the epidermis has been hypothesized to be involved in the preparation for infection (Journet et al., 1994).

INTERACTION WITH INNER CELL LAYERS

The formation of a root nodule primordium requires the dedifferentiation of fully differentiated cortical cells. Which root cortical cells divide depends on the type of nodule a particular host plant forms. In general, two types of nodules can be distinguished in leguminous plants: a determinate and an indeterminate form. In indeterminate nodules the cells of the inner cortex are reactivated to divide and a nodule with a persistent apical meristem is formed, e.g. in pea, vetch, and alfalfa. In the determinate nodule type the primordium is formed in the root outer cortex and the formed nodule has a determinate growth pattern. In these determinate nodules mitotic activity ceases during development, and cell expansion, rather than cell division, is responsible for the increase in nodule size, e.g. soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*).

The expression of several nodule-specific genes has been detected in nodule primordia, such as *ENOD12, ENOD40,* and *PrP4* (Scheres et al., 1990a; Yang et al., 1993; Wilson et al., 1994), and expression of these genes has not yet been observed in other primordia. Therefore the nodule primordium is distinguishable from other primordia.

Nod factors are able to trigger mitotical reactivation of cortical cells. It is still unclear whether Nod factors themselves are perceived by these cortical cells or whether a secondary signal generated in the epidermis is involved. Auxin and cytokinin are known to be involved in cell division, and two types of experiments indicate that changes in the phytohormone balance are required for the induction of nodule primordia. Both *Escherichia coli*, carrying the zeatin gene involved in cytokinin synthesis, and compounds that block the polar transport of auxin, such as *N*-(1-naphthyl)phthalamic acid and 2,3,5-triiodobenzoic acid, are able to induce nodule-like structures on alfalfa roots. In these structures nodulin genes are expressed (Hirsch et al., 1989; Cooper and Long, 1994). A good candidate for a convenor between Nod factors and the phytohormones might be the gene product of *ENOD40*. A phytohormone effect of *ENOD40* is detected in protoplasts of the nonlegume tobacco. Tobacco protoplasts that express a soybean *ENOD40* gene divide efficiently at high auxin concentrations, whereas in the control protoplasts these levels of auxin suppress the ability to divide (Van de Sande et al., 1996). Since detailed cytological studies have shown that the expression of *ENOD40* in the pericycle precedes the first cortical cell divisions, it is hypothesized that *ENOD40* may encode a regulator of cell division whose induction upon *Rhizobium* infection starts the process of root cortical cell division leading to nodule formation. Thus, Nod factors may accomplish changes in the phytohormone balance in the root via the expression of *ENOD40*, which then results in cell division.

ENOD40 homologs have been isolated from several legumes as well as from the nonlegume tobacco. The highest homology among these cDNA clones is restricted to two regions, of which the most 5′-located one encodes for a peptide of 10 to 13 amino acids. These peptides appear to be plant growth regulators. In tobacco protoplasts ENDO40 peptides change the response to auxin at concentrations as low as 10^{-12} to 10^{-16} M (Van de Sande et al., 1996).

Irrespective of the position of the primordium in the cortex, dividing cells are always located opposite to protoxylem poles. This observation implies that positional information of the plant is involved in controlling the site where the primordia will be formed. Recently, uridine was identified as a factor of the stele from pea that, in combination with phytohormones, is capable of inducing cell divisions in the inner cortex of pea root explants (Smit et al., 1995). Thus, it can be postulated that the positioning of cortical cell divisions is determined by the interplay of at least two morphogen gradients that are oppositely ori-

ented; one gradient of the stele factor, and the other of the Nod factor itself or a secondary signal generated by the Nod factor, coming from the outside (Fig. 3).

To initiate cell divisions in the inner cortex, which are required to form an indeterminate nodule, Nod factors with a highly unsaturated acyl chain must be used. For instance, on *Vicia sativa* Nod factors of *R. leguminosarum* bv *viciae* should carry a C18:4 acyl chain to induce cell divisions, whereas factors containing a C18:1 group are unable to do so (Spaink et al., 1991). In contrast, Nod factors produced by rhizobia that nodulate on plants that form determinate nodules do not have specific unsaturated bonds in the fatty acid. These factors are mainly acylated by either C16:0 or C18:1 chains. Apparently, highly unsaturated acyl chains in Nod factors are not required for the induction of cell divisions in the outer cortex. Thus, the presence or absence of highly unsaturated acyl chains in Nod factors is correlated with the place in the cortex where cell divisions will be induced. Accordingly, it has been proposed that highly unsaturated acyl chains might contribute to facilitate transport of the Nod factor to the inner cortical cell layers (Spaink et al., 1995).

Strikingly, the *nodO* gene of some *R. leguminosarum* bv *viciae* strains can partially complement Nod factor structure deficiency due to a *nodE* mutation, although NodO is not involved in Nod factor production or secretion (Economou et al., 1994). NodO is a relatively hydrophilic secreted protein, and it has been shown in vitro that the protein forms calcium-regulated monovalent cation channels when added to planar lipid bilayers (Economou et al., 1990; Sutton et al., 1994).

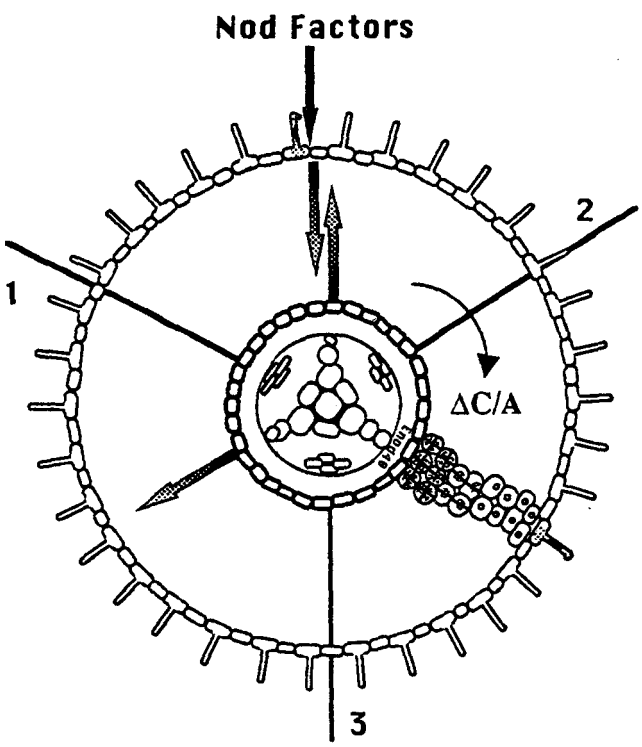

Figure 3. 1, Cell divisions are always induced opposite of protoxylem poles, which implies positional information coming from the stele. 2, Nod factors generate a second signal, and together with the oppositely oriented signal of the stele, it will establish conditions so that cell divisions are possible (3); however, the cytokinin/auxin (ΔC/A) balance most likely has to be changed. The cells of the cortex will enter the cell cycle. For pea it was shown that the outer cortical cells will become arrested in the G2 phase, while the inner cortical cells start to divide. At the same time *ENOD40* expression can be detected in the pericycle as well as in the dividing cells.

The identification of supernodulation mutants in several legumes shows that an autoregulatory mechanism exists that controls the ultimate number of nodules formed. In soybean, the autoregulatory mechanism becomes operational during early nodule ontogeny as soon as a particular number of nodule meristems have been formed (Caetano-Anollés and Gresshoff, 1991). The structure of the signal molecules involved in the control of nodule numbers have not been elucidated yet. Studies of wild-type pea as well as of pea mutants have shown that exogenous ethylene acts as an inhibitor of nodule formation, whereas the number of infections is not affected. This inhibition is overcome by a treatment of the plant roots with silver ions, an inhibitor of ethylene perception (Lee and LaRue, 1992).

INFECTION

In this *Update* we have described Nod factor-induced responses in two spatially separated sites, the epidermis and the cortex. For the successful establishment of a symbiosis, however, rhizobia need to penetrate the legume root, and more precisely, they have to enter the nodule primordium cells. This is achieved by the formation of an inward-growing tubular structure, the infection thread. In plants that form indeterminate nodules, the infection thread has to pass the outer cortical cell layers. Cytological observations show that the growing infection thread is guided toward the primordia via a radially aligned file of cytoplasmic bridges, which is the preinfection thread. These bridges are formed in cells prior to infection thread passage. In these preinfection thread-forming cells, the nucleus has moved to the center and the microtubules and the cytoplasm have been rearranged to form a conical structure, the cytoplasmic bridge, which resembles a preprophase band (Kijne, 1992).

These morphological changes are reminiscent of those seen in cells entering the cell cycle. In situ hybridization experiments, scoring for the expression of the S-phase-specific histone *H4* gene and a G2- to M-phase-expressed mitotic cyclin gene showed that in cells forming the preinfection threads only the histone *H4* gene is reactivated. Therefore, cells that form the preinfection thread re-enter the cell cycle but become arrested in the G2 phase (Yang et al., 1994). These observations indicate that the infection process strongly resembles a general process, namely cell division. Rhizobia, however, have modified it such that it can be applied for a different purpose, the infection process. Purified Nod factors are able to induce the formation of preinfection threads, but they fail in the formation of complete infection threads. Thus bacteria and Nod factors seem to be required for infection thread formation.

Studies with *R. meliloti* mutants showed that structural constraints on Nod factors with respect to initiation of

infection sites are more stringent than for elicitation of root hair deformation or reactivation of cortical cells. *R. meliloti* mutants (*nodL*$^-$ or *nodFE*$^-$) that produce Nod factors with structural deficiencies are able to induce root hair deformation and cortical cell activation as scored by the presence of starch grains, but are affected in their efficiency to initiate infection sites (Ardourel et al., 1994). Based on this, it has been proposed that at the epidermis two types of Nod factor receptors are present–a signaling receptor and an entry receptor. The signaling receptor is less selective in the recognition of Nod factors, and upon activation it triggers the initiation of inner cortical cell divisions and root hair tip growth, leading to root hair deformation. An entry receptor recognizes only Nod factors with the appropriate decorations, leading to the formation of an infection site to allow bacterial ingestion. Only upon triggering of the entry receptor are infection threads formed that will guide the bacteria toward the nodule primordium. The model shown in Figure 4 implies that the main selectivity of the plant about which bacteria is allowed to enter its roots is enclosed in this proposed entry receptor at the epidermis.

To our knowledge, the first Nod factor-binding protein has recently been characterized in alfalfa (Bono et al., 1995). However, this protein has a similar affinity for sulfated and nonsulfated Nod factors and this affinity is lower than the concentration at which Nod factors are active. Therefore, it is unclear whether this protein is a Nod factor receptor.

During the infection process the early nodulin genes *ENOD5* and *ENOD12* are induced. Whereas *ENOD12* expression is detectable in cells containing infection threads and in cells preparing for infection thread passage, *ENOD5* expression is restricted to those cells containing the tip of the growing infection thread. It is therefore probable that the expression of these two genes is differently regulated (Scheres et al., 1990b).

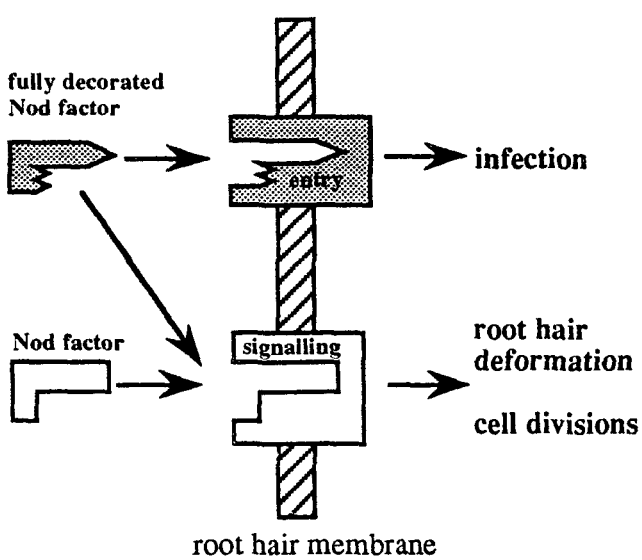

Figure 4. Nod factor recognition at the root epidermis as described by Ardourel et al. (1994). The model implies that there are two Nod factor receptors: a signaling receptor and a entry receptor. The signaling receptor is less selective for Nod factor structure, and upon binding it triggers the initiation of cortical cell divisions as well as root hair deformation. The entry receptor is highly selective and will only recognize Nods factors with the appropriate decorations leading to infection.

CONCLUSIONS AND PERSPECTIVES

During the last decade progress has been made in the molecular dissectioning of the communication between *Rhizobium* bacteria and legumes. The major achievement has been the characterization of signal molecules produced by both partners through which the communication is established. The bacterial signal molecules, the Nod factors, provoke responses in the plant at spatially separated sites. Future research will focus on how Nod factors are perceived and transduced. They are active in a host-specific way and at low concentrations, and therefore, it is likely that a specific receptor(s) exists. Since epidermal cells are the first cells to encounter Nod factors, they should be provided with this receptor(s). It is unclear whether Nod factors are perceived in inner cortical tissue or whether secondary signals, generated in the epidermis, are involved in provoking responses here. To help understand this question, the bacteria-induced plant genes, the *ENOD* genes, are useful markers. The final goal of the symbiotic interaction is to form a new organ, the root nodule, and Nod factors alone are not sufficient to generate a genuine nodule. The presence of *Rhizobium* is needed and the constraints on the bacterium still await elucidation.

Although there are still many unanswered questions with respect to the communication between plant and bacteria, it is already possible to speculate on the nature of the signal transduction cascades. Several lines of evidence show that the activity and therefore the recognition of Nod factors is not restricted to legumes: 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); Nod factors trigger the alkalinization of tomato suspension culture medium (Staehelin et al., 1994) and, like phytohormones, Nod factors stimulate division of tobacco protoplasts (Röhrig et al., 1995). It has recently been shown that a chitin oligosaccharide similar to the Nod factor backbone can be synthesized by the *Xenopus* developmental protein DG42, which shows strong homology with the *Rhizobium NodC*-encoded protein (Semino and Robbins, 1995).

These observations indicate that Nod factors can be considered as (plant) growth regulators and that Nod factor-like molecules normally occur in plants and even in animals. This is supported by the observation that the expression of bacterial *nod* genes in tobacco affects the development of these plants, showing that at least the substrates for the bacteria-encoded enzymes are present (Schmidt et al., 1993). Hence, it seems likely that *Rhizobium* has evolved a genetic pool that enables it to synthesize molecules by which it becomes possible to redirect (plant) developmental programs and to manipulate them for its own benefit. For instance, the formation of an infection site recalls typical cell-tip growth, a process that gives rise to root hairs, trichromes, and the extending pollen tube. During the infection the bacteria trigger the cell-cycle machinery, resulting in cells that

are arrested in the G2 phase, which will form the preinfection thread, and in cells that proceed with the cell cycle, which will lead to the formation of a nodule primordium. The plant genetic pool that is activated in roots by *Rhizobium* comprises genes that are also expressed, albeit at low levels, in other plant tissues.

Thus, these observations indicate that the communication between *Rhizobium* and legumes involves molecules and processes reminiscent of normal plant development. It can be anticipated that the signal transduction pathways involved in this particular symbiotic process are commonly used pathways in plants. As a consequence, studies of the mode of action of, for instance, Nod factors or *ENOD* genes no longer need to be restricted to legumes, and therefore the potential of Arabidopsis might be explored.

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