**Rhizobium Symbiosis: Nod Factors in Perspective**

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**INTRODUCTION**

*Rhizobium* and its allies (*Azorhizobium*, *Bradyrhizobium*, and *Sinorhizobium*) are Gram-negative bacteria that cause the development of root (and sometimes stem) nodules on plant hosts, which the bacteria inhabit as nitrogen-fixing endosymbionts. The early stages of this process, including gene expression in the bacterium and cell growth, division, and differentiation in the host, are mediated by signal exchange between the eukaryotic host and the prokaryotic symbiont (Figure 1A, left). The plant produces a signal, usually a flavonoid, that induces gene expression in the bacterium; the bacterium subsequently synthesizes a signal that triggers early nodule development on the plant.

The developmental time line for nodulation has been described in several reviews and essays (Sprent, 1988; Truchet et al., 1989; Brewin, 1991; Brewin et al., 1992; Hirsch, 1992; Kijne et al., 1992; Ridge, 1992; Vijn et al., 1993) and is only considered briefly here. Nodules can take on several patterns during development, the form of the nodule being determined by the plant, not the bacterium. One major form is the indeterminate (also called meristematic or cylindrical) type, which develops on alfalfa, clover, and pea roots. A second major type is the spherical or determinate nodule, which is formed by soybean, *Phaseolus*, and *Lotus*. A comparison of these symbiotic nodules with those of nonlegumes is presented elsewhere in this issue (see Pawlowski and Bisseling, 1996).

The twin hallmarks of early nodulation are its developmental complexity and its specificity. The developmental process in the plant involves architectural changes at the cell and organ levels (for example, root hair morphogenesis, cortical cell enlargement, and vascular patterning) as well as internal cellular differentiation that includes cytoplasmic activation, cell division, and new gene expression. Structural and developmental studies of nodule formation remain an important part of the overall *Rhizobium* research picture. Indeed, new views of infection thread formation, cell wall modifications, and intracellular rearrangements in root hairs and elsewhere have recently appeared (Kijne et al., 1992; van Brussel et al., 1992; van Spronsen et al., 1994; DeBoer and Djordjevic, 1995; Ridge, 1995). The exciting tools of video microscopy and image analysis are making it possible to obtain dynamic views of early plant reactions to *Rhizobium* signals (Allen et al., 1994; Sanchez et al., 1996). Thus, in addition to its inherent interest as a model for understanding plant-microbe interactions, the specificity and timing of early nodulation events make the *Rhizobium*-plant symbiosis an attractive model system for general plant cell biology studies.

The specificity of nodulation is likewise remarkable: with one known exception, the *Rhizobium* nodulation habit is restricted to a single plant taxon, the Fabaceae, or legume family. Within this family, individual species, strains, or biovars of bacteria nodulate a restricted set of host plants that are usually but not always related. The signal model (Figure 1) provides an explanation for the species-level pattern of host specificity. But we cannot yet answer the larger mechanistic and evolutionary question: Why only legumes?

Because a short review cannot catalog complete lists of references, even recent ones, the focus of this article is to put selected papers in context: Why is it important for plant biologists to be concerned with bacterial genes, their regulation, and activities? Where do the questions lie in the study of the plant response? What new genetic and cellular methods are needed for their resolution?

**THE NOD FACTOR SIGNAL EXCHANGE MODEL**

Over the years, many molecules have been proposed as candidate host-specific signals that govern the *Rhizobium*-plant symbiosis by mediating bacterial invasion. Those proposed include phytohormones, extracellular polysaccharides, and hydrolytic enzymes (among others). Several independent lines of evidence, with a strong genetic base, have now convinced most researchers that a novel set of molecules, the lipo-oligosaccharide nod factors, is at the heart of the symbiosis (de Bruijn and Downie, 1991; Dénarié et al., 1992; Fisher and Long, 1992; Higashi, 1993; Downie, 1994; Spaink, 1995). The genetic analysis was grounded in the identification of *Rhizobium* nodulation (*nod*) genes, which define the central functions required for plant invasion and host recognition. The *nod* genes can be functionally divided into common *nod* genes, which are widely conserved in *Rhizobium* and its allies, and host-specific *nod* genes, which are required for nodulation of certain plants but not others.

The link between the *nod* genes and the biosynthesis and delivery of the lipo-oligosaccharide signal molecules was confirmed in a number of ways. First, *nod* gene regulation accounts for the production of nod factors as well as for the symbiotic
Regulation of nod Genes

Most of the Rhizobium nodulation genes are transcriptionally silent when the bacteria are grown in culture. Their expression is controlled by a transcriptional activator, NodD, which acts together with inducers from the plant, and also by other bacterial regulators, such as NoIR (reviewed in Kondorosi, 1992; Schlaman et al., 1992). NodD acts on promoter elements that include the conserved "nod box" sequence, which is known to be a site of NodD binding and consequent DNA bending (Fisher and Long, 1993). The molecular basis for NodD activation, although outside the scope of this review, is a subject that continues to present several puzzles, not the least of which is the precise role of the plant inducer in triggering nod gene activation.

Initial characterization of nodD genes showed that the nodD gene products of various bacterial species appeared to activate nod genes differentially in response to the specific mixtures of inducers derived from the roots of each host plant. These inducers include luteolin, methoxylchalcone, naringenin, and daidzein as well as nonflavonoid inducers such as trigonelline and stachydrine (Phillips et al., 1994). The spectrum of secreted flavonoids may change with conditions, which may have ecological and developmental consequences for the free-living rhizobia (Schlaman et al., 1992; Lawson et al., 1995; see Handelsman and Stabb, 1996, in this issue, for a review of plant–microbe interactions in the rhizosphere).

These diverse responses at the level of nod gene induction are mediated in part through the action of specific members of nodD multigene families (Kondorosi, 1992). However, recent studies have provided clues that nod gene regulation involves additional gene regulators and developmental cues, which may have differential effects on Nod factor synthesis, perhaps depending on the host plant or on the subset of nod genes that each induces. For example, Demont et al. (1994) found that Rhizobium meliloti NodD3 controls the production of variant acyl groups (18- to 26-carbon N-acyl groups with omega-1-OH modifications) that were present in Nod factor preparations. Furthermore, in R. meliloti strain 41, Cren et al. (1995) observed that the repressor NoIR has different effects on the expression of the common nod gene operon and on other nod box operons. This suggests that the synthesis of the Nod factor core and the modifying side groups can be differentially regulated.

Additional regulators seem to operate in Bradyrhizobium japonicum. For example, nodW is a novel nod gene regulator that resembles a two-component response regulator; its ex-
pression is more important for successful colonization of some plant hosts than for others and, in its absence, the B. japonicum nod genes are not fully expressed (Stacey et al., 1994). Thus, the NodW circuit adds an additional layer of regulation to the NodD-inducer circuit for nod gene control.

The symbiosis gene cluster in B. japonicum also includes a negative nod regulator, nolA, and at least one gene required for signal synthesis, nodZ, that is not controlled by NodD (Dockendorff et al., 1994; Sanjuan et al., 1994; Stacey et al., 1994). Thus, there is evidence from at least two different symbiont–host pairs that nod gene regulation can be differentially tuned in response to specific signals. It is important to determine whether these additional levels of regulation correlate with developmentally sensitive steps in signaling, with extended host range, or with other decision points in the symbiosis.

The elucidation of the structures of the various bacterial Nod factors (see below) now allows an evaluation of how both plant and bacterial signals contribute to specificity. For example, one recent study used combined analyses of Nod factor structure and nod gene regulation to add a new iteration to our understanding of host range (Figure 1B). Cardenas et al. (1995) showed that among the Nod factors produced by Rhizobium etli were molecules strikingly similar to some produced by Rhizobium loti (Lopez-Lara et al., 1995), yet the two bacterial species were not capable of cross-inoculating each other’s host plants (Phaseolus and Lotus, respectively). Why?

The answer appears to lie in the initial plant induction of the Rhizobium nod genes. If R. etli and R. loti were engineered to produce their Nod factors constitutively, that is, without dependence on plant inducers, cross-inoculation could be achieved. It can be inferred from these observations that the initial signal from the plant root is only capable of inducing Nod factor synthesis in the appropriate bacterial symbiont (Cardenas et al., 1995). Thus, colonization specificity is in some cases directed by the plant signal.

**Nod Genes and Nod Factors**

**Nod Factor Structures**

The functions of the Rhizobium nod genes can be studied in free living cells, if their expression is induced either by providing transcriptional inducers such as flavonoids or by driving the expression of constitutive forms of NodD. This strategy has revealed that nod genes are required for the synthesis of Nod factors. The structures of many Nod factors have been determined. They can be most generally described as lipo-oligosaccharides and as lipo-chito-oligosaccharides. The species of bacteria for which Nod factor structures have been determined and their corresponding host plants are shown in Table 1.

Several caveats should be mentioned here. First, most rhizobia produce mixtures of lipo-oligosaccharide molecules. This is particularly true for broad host range symbionts, such as strain NGR 234, in which the production of diverse Nod factors is controlled by a number of nod loci that are dispersed within a symbiosis plasmid regulation region (Price et al., 1992; Fellay et al., 1995). However, even those rhizobia with a more restricted host range can produce a variety of Nod factor structures (Table 1).

A second important point is that structural analyses are not complete for all Nod factor molecules. In some cases, for example, the initial assignment of an adduct to a particular residue can be made (usually through mass spectrometric analysis), whereas an exact positional assignment awaits further derivatization and spectroscopic analysis (see Figure 2, legend). The chemistry of Nod factor separations continues to be refined and debated. Initial thin layer chromatography (TLC) methods used to define Nod factors may not resolve all chemical species (Spaink et al., 1995), but new methods are emerging (Price and Carlson, 1995). Finally, most factors studied to date were isolated by using protocols developed for the R. meliloti Nod factors (Lerouge et al., 1990), and it is possible that important signal molecules with very different properties may have escaped identification.

**Genotype Effects**

The most favored working model holds that the universal features of Nod factors—the oligosaccharide backbone, and the N-acyl bonds—are constructed by the enzymes encoded in the common nodulation genes, nodA, nodB, and nodC. The side groups that confer specificity on the different bacterial signals are determined by host-specific nod genes (Carlson et al., 1994; see Table 2). However, not all nod genes encode enzymes. For example, the NodO protein of R. leguminosarum by viciae is a calcium binding hemolysin–like protein. NodO, which creates ion channels in membranes, appears to be capable of substituting acyl structures in Nod factors (Downie and Surin, 1990; Sutton et al., 1994). A short review precludes a complete catalog of the nod genes and descriptions of their functions; instead, this section focuses on a few recent highlights and poses the ongoing questions.

Two lines of evidence, structural correlation and direct enzymatic assay, connect nod genes to lipo-oligosaccharide Nod factor synthesis. The first approach has shown that the actual structure of the Nod factor varies according to nod genotype. This has been demonstrated for a number of features, including the 6-O-sulfate in R. meliloti and R. etli Nod factors, the methylfluorese addition in B. japonicum, and the N-methyl and carbamoyl modifications in Azorhizobium caulinodans and Rhizobium NGR 234 (Figure 2; reviewed in Carlson et al., 1993, 1994; see also Geelen et al., 1995; Jabbourit et al., 1995; Folch-Mallol et al., 1996).
The oligomeric backbone of GlcNAc can vary in length from three to six total residues. The backbone is drawn here, by convention, with the reducing end at the right and nonreducing end at the left. Substituents have been defined on the basis of mass spectroscopy, gas chromatography of derivatized fragments, and NMR. For simplicity, only the 3-O-substitution on the nonreducing end residue is shown (*), but either the three or the four position may be carrying the carbamoyl substituent. Nod factors are named by a convention, including the initial letters of the species name, the number of GlcNAc residues in the backbone, the length and unsaturation number of the acyl chain, and the other substituents (Dénarié et al., 1992). A four-residue *R.* meliloti Nod factor, bearing a 16-carbon fatty acyl moiety with two unsaturations, and a sulfate, is thus designated NodRmtVI(C16:2, S). The positions of the unsaturations are sometimes indicated by standard lipid nomenclature conventions. Methylfucose, 2-O-methylfucose; acetyl-methylfucose, 4-O-acetyl, 2-O-methylfucose; sulfo-methylfucose, 3-O-sulfo-2-O-methylfucose.

There is some uncertainty concerning the enzymatic synthesis of the Nod factor fatty acyl moieties (Figure 2). Demont et al. (1993) and Spanik et al. (1991) have reported that nodF and nodE genotypes determine the spectrum of acyl structures found in the secreted Nod factors of *R.* meliloti and *R.* l. viciae. Because the nodE genotype also determines host range, these data imply a major role for the acyl group in host recognition (reviewed in Spanik, 1995). NodF is homologous to acyl carrier proteins, and NodE is homologous to condensing enzymes (Carlson et al., 1994). One possibility, then, is that these proteins form an apparatus for the synthesis of specialized acyl structures, which are then transferred by the nodA-encoded acyltransferase to the GlcNAc backbone (John et al., 1993; Atkinson et al., 1994; Röhrig et al., 1994).

However, reports on the role of nodE in *R.* leguminosarum bv trifolii are less clear. It is known that nodE is also important for host range in this biovar. Indeed, Bloemberg et al. (1995) and Spanik et al. (1995) found that the TLC and HPLC profiles of Nod factors were different in *Rhzobium* strains expressing the nodE gene of *R.* l. viciae or of *R.* l. trifolii. However, these TLC and HPLC analyses were not capable of distinguishing between Nod factor profiles of nodE + and nodE – strains of *R.* l. trifolii. Further spectroscopic analyses did reveal subtle differences between Nod factors from nodE + and nodE – strains that were ascribed to changes in the degree of saturation of the fatty acyl moieties. A parallel study by Philip-Hollingsworth et al. (1995) also compared Nod factors produced by wild-type and nodE – mutant *R.* l. trifolii. However, these authors reported that there were no nodE-related effects on lipo-oligosaccharide structures.

One difference between these two studies that may help to reconcile the results is the experimental strategy used to drive the expression of the nodE genes. Less abundant Nod factor species were more likely to be observed in the experiments reported by Spanik et al. (1995) in which a multicopy nodD gene construct was used. This contrasts with the more physiological single-nodD construct used by Philip-Hollingsworth et al. (1995). What both studies do show, however, is that multiple molecules are present in the Nod factor mix and that the host-specific nodE-determined factors are at
best a small proportion of the total. We are not sure what the functions (if any) of the remaining components in the mixture may be.

Some of the puzzles and inconsistencies described above may relate to the effects of nod gene products on other products of biosynthetic pathways in the bacterial cell. For example, Geiger et al. (1994) found nodE-dependent lipids in the phospholipid pool. Furthermore, genes encoding enzymes that direct glucosamine synthesis and sulfate activation exist in the pholipid pool. Furthermore, genes encoding enzymes that may relate to the effects of nod gene products on other products of biosynthetic pathways in the bacterial cell. For example, may be.

**Table 1. Bacterial-Host Systems with Defined Nod Factors**

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Host Plants</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizobium meliloti</em></td>
<td>Alfalfa</td>
<td>Lerouge et al. (1990)</td>
</tr>
<tr>
<td></td>
<td><em>Medicago truncatula</em></td>
<td></td>
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<tr>
<td></td>
<td><em>Melilotus albus</em></td>
<td></td>
</tr>
<tr>
<td><em>R. leguminosarum</em> by <em>viciae</em></td>
<td><em>Pisum</em> (Pea)</td>
<td>Bloomberg et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>Vetch (Vicia)</td>
<td>Philip-Hollingsworth et al. (1995)</td>
</tr>
<tr>
<td><em>R. leguminosarum</em> by <em>trifoli</em></td>
<td><em>Trifolium</em> (Clover)</td>
<td>Spank et al. (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cardenas et al. (1995)</td>
</tr>
<tr>
<td><em>R. etli</em></td>
<td><em>Phaseolus</em></td>
<td>Price et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>18 genera, including <em>Vigna</em>, <em>Macroptilium</em>, <em>Parasponia</em></td>
<td></td>
</tr>
<tr>
<td><em>R. etli</em> NGR 234 Broad host range;</td>
<td><em>Lotus</em></td>
<td>Lopez-Lara et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>18 genera, including <em>Vigna</em>, <em>Macroptilium</em>, <em>Parasponia</em></td>
<td></td>
</tr>
<tr>
<td><em>R. fredii</em></td>
<td><em>Glycine</em> (Soybean) (max)</td>
<td>Bec-Forté et al. (1994)</td>
</tr>
<tr>
<td><em>Bradyrhizobium japonicum</em></td>
<td><em>G. soja</em></td>
<td>Carlson et al. (1993)</td>
</tr>
<tr>
<td></td>
<td><em>Sesbania</em></td>
<td>Mergaert et al. (1993)</td>
</tr>
<tr>
<td>Azorhizobium cauliformans</td>
<td></td>
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</tbody>
</table>

* Comprehensive reviews by Dénarié et al. (1992), Dénarié and Cullimore (1993), Carlson et al. (1994), Relic et al. (1994), Schultze et al. (1994), Spank and Lugtenberg (1994), and Spank (1995) provide further references to the original literature and to other reviews.
  
  * Some nod factors are fully defined, with all positions of adducts determined; others have approximate structures. See the original literature for details on each structure.
  
  * Differences concerning the identity of nod factor structures and the interpretation of spectra have been reported. See the primary literature for details.
  
  * ParaJose is the only nonlegume host plant known to form symbiotic nodules with *Rhizobium*.
  
  * The type I and type II strains of *B. japonicum* have been renamed *B. japonicum* and *B. elkanii*, respectively.

**Table 2. A Brief Guide to Nodulation Genes**

<table>
<thead>
<tr>
<th>Gene Names</th>
<th>Occurrence</th>
<th>Proposed Role</th>
</tr>
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<tbody>
<tr>
<td>nodABC</td>
<td>Common</td>
<td>Synthesis of Nod factor backbone; deacetylation; acylation</td>
</tr>
<tr>
<td>nodU</td>
<td>Common?</td>
<td>Export of signals</td>
</tr>
<tr>
<td>nodT</td>
<td>Varies</td>
<td>Nod factor export</td>
</tr>
<tr>
<td>nodFE</td>
<td>Present in all species but have effect on host range</td>
<td>Synthesis of specialized acyl moiety</td>
</tr>
<tr>
<td>Most other nod genes</td>
<td>Varies</td>
<td>Synthesis of precursors; modification of backbone on reducing or nonreducing residues</td>
</tr>
</tbody>
</table>

nod cluster. Homologs of these genes are also found elsewhere in the bacterial genome (Carlson et al., 1994). These observations suggest that in addition to the primary Nod factors, symbiosis requires other bacterial molecules (Hirsch, 1992). It seems important, then, to be alert not only for the primary Nod factor signals but also for bacterial molecules that modulate the plant response or act in some other way to tune the sensitivity of the plant to the bacterial signal. Ultimately, to generate a complete picture of how the bacterium signals its plant partner, we need to know how the synthesis of highly specific nodulation factors and possible secondary signals may be coordinated with the synthesis of housekeeping metabolites such as amino acids, surface structures, and membrane components.

**Nod Factor Enzymes: Answering Questions and Providing Experimental Tools**

Resolution of the Nod factor biosynthetic pathway and confirmation of Nod factor structures will be achieved through further enzymatic studies. Some important steps, such as sulfation, 6-O-acetylation, 2-N-methylation, and 2-N-deacetylation, have now been demonstrated in vitro (reviewed in Carlson et al. 1994; Spank, 1995; see also Bourdineaud et al., 1995; Ehrhardt et al., 1995; Geelen et al., 1995; Mergaert et al., 1995; and Schultze et al., 1995). Moreover, the nodC gene product, which is hypothesized to be the polymerase for the backbone, may be located in the inner bacterial membrane (Barney and Downie, 1993; Carlson et al., 1994). This protein has been shown to catalyze the synthesis of GlcNAc oligomers in vitro (Kamst et al., 1995; Mergaert et al., 1995).
Another highlight from recent biochemical approaches is the in vitro synthesis of active Nod factors. This achievement is important for future efforts to identify plant receptors for Nod factors: to study ligand–receptor interactions, it is important to be able to synthesize, modify, and label ligands. The total variety of lipo-oligosaccharide structures (Rohrig et al., 1995).

The in vitro synthesis of active Nod factors: to study ligand–receptor interactions, it is important to be able to synthesize, modify, and label ligands. The total variety of lipo-oligosaccharide structures (Rohrig et al., 1995). Alternatively, a galactosyl β-1,4-transferase has been used to join tailored glucosamine residues to the nonreducing end of chitin oligomers (Atkinson et al., 1994). Radioactive labeling of Nod factors has been performed both by chemical synthesis (Nicolaou et al., 1992; Bourdineaud et al., 1995) and by using purified enzymes. The side group modifications, such as sulfates, are a particularly promising target for enzymatic modification. For example, sulfation by the R. meliloti NodH enzyme has been used to generate 35S-labeled Nod factors in vitro (Bono et al., 1995; Ehrhardt et al., 1995; Schultze et al., 1995). The use of enzymes in the synthesis of radiolabeled ligands has the advantage of creating labeled molecules that have the exact structure and activity of the native signal molecules.

RECOGNITION

The diverse responses of plants to Nod factors and the precise nature of side groups needed for activity suggest that molecular recognition is accomplished by one or more plant receptors that interact with the bacterial signals. What are the precise structural requirements for Nod factor specificity? The picture is getting more complicated, as shown in two recent reports. Stokkermans et al. (1995), using both synthetic and Bradyrhizobium-produced Nod factors, found complex relationships between backbone and side group configurations in nodulation of the Glycine host. For example, GlcNAc tetramers were active without side group modifications on the reducing end residue, but pentameric molecules were inactive unless they carried a side group modification at the reducing end GlcNAc residue.

The second study, by Arndourel et al. (1994), made use not of pure Nod factors but of R. meliloti cells with mutations in the nodL or nodFE genes, or both. The products of these genes direct O-acetylation on the nonreducing residue and the structure of the acyl tail, respectively. Although nodF− or nodL− mutants could stimulate nodule primoridia, infection thread formation was inefficient. Double nodF nodL− mutants were completely defective at entry and infection thread formation, but they were still able to trigger cell divisions in the plant. On the basis of these experiments, Arndourel et al. (1994) presented a model in which two distinct receptors are involved in the plant response (see also Hirsch, 1992). One, a signaling receptor, is able to interact with non-host-specific Nod factors, thereby triggering cell division. The second, a stringent entry receptor, must interact with specifically tailored molecules (i.e., the host-specific Nod factors) before formation of infection threads and cell invasion can proceed.

This model is appealing because it provides an explanation of the current complexities of Nod factor signaling as well as providing room for evolutionary speculation. For example, the low stringency signaling receptor could be more evolutionarily ancient, with the high stringency entry receptor evolving later, perhaps concurrent with the development of the root hair invasion habit. However, there are some weaknesses in this model. It does not fully explain how one would obtain single-gene recessive Nod− plant mutants that lack both the root hair curling and cell division phenotypes. Also, one would predict on the basis of this model that interactions between Rhizobium species and nonhost plants (e.g., R. meliloti placed on pea roots) may induce new cell divisions in roots. These possibilities are testable.

Alternatively, it is possible that the different effects of Nod factors on host and nonhost plants are due to different binding affinities of the factors, combined with complex behavior by receptor(s). The receptor-capping model described by Hirsch (1992) provides one possible scenario for how receptors may engage in different levels of interaction. Promoting plant cell division could be possible, for example, at a low threshold of receptor activity, whereas bacterial entry may require a higher level of the same activity.

Clearly, identifying one or more plant receptors for specific Nod factors is important. In a situation distinct from certain plant–pathogen systems discussed in other reviews in this issue (see, for example, Bent, 1996), Rhizobium researchers have a ligand but no cloned host genes corresponding to Nod− plant mutations. How does one find a receptor? The first principles are clear: a receptor can only be defined as such if it satisfies both of two general criteria. First, it must bind the ligand, and when variant ligands and inhibitors are defined, the receptor should bind these with an affinity proportional to their activity. Second, the receptor must exhibit functional activity, either in vitro (ligand-dependent dimerization, phosphorylation, etc.) or in a reconstituted cellular or artificial membrane system (for example, ligand-stimulated events in a naïve cell or liposome). Alternatively, the receptor should have activity in vivo, such as a genetic function for its encoding DNA (for example, complementation of a mutant or conferral of a novel specificity in a heterologous background).

In the absence of functional activity, a protein that simply binds a Nod factor ligand cannot be considered a receptor, although it automatically becomes a promising candidate for functional studies. Binding proteins that are not true receptors may nonetheless be significant for the plant symbiotic response. For example, Nod factor binding proteins such as those described by Bono et al. (1995) may be involved in Nod factor processing or breakdown (Staehelin et al., 1995), delivery to the plant membrane, or regulatory sequestration.

The classic legume seed lectins have also attracted interest as possible receptors. Although they are unlikely to provide...
the sugar binding specificity necessary for specific binding of nod factors, genes for pea lectin were reported to confer novel bacterial nodulation properties on clover plants that had been transformed with a pea lectin gene (see, e.g., van Eijden et al., 1995). Finally, novel lectins from species such as Dolichos and soybean may prove to be promising candidates to test for interaction with the bacterial signals (M. Etzler and G. Stacey, personal communication). The ultimate tests for necessity and sufficiency of nod factors, and for putative receptors, will require genetic analyses in the host plant. In particular, plant mutants in the putative receptor genes should be sought to provide confirmation of in vivo function.

PLANT RESPONSES TO NOD FACTORS

Assays for nod gene–encoded exudates, and subsequently for purified nod factors, revealed that these bacterial signals can cause multiple nodulation-like responses in plant hosts in the absence of the producing bacteria. These responses include altered root hair growth, initiation of cell division, and the expression of a class of nodule-specific plant genes, the early nodulins, or ENODs (reviewed in Hirsch, 1992; Kjøne et al., 1992; Dénaré and Cullimore, 1993; Schultze et al., 1994; Spaink, 1995). Most recent work relates to three enduring research themes: first, the production of new or upregulated transcripts (ENODs) in the plant during early nodulation; second, the involvement of plant hormones; and third, the pattern of cell division and cell rearrangement early in the nodulation response. In addition, two new areas are emerging: the genetic analysis of the host plant and the cell biology of early plant responses to nod factors.

Nodulins

The overall pattern of new gene expression in developing nodules, revealed originally through antibody and mRNA analyses, has more recently been explored at the cellular level, both by in situ hybridization and by promoter–β-glucuronidase fusion gene expression (reviewed in Hirsch, 1992; Vijn et al., 1993; de Bruijn et al., 1994; Schultze et al., 1994; see also Horvath et al., 1993; Journet et al., 1994; Pichon et al., 1994; Vijn et al., 1995a). These analyses make it clear that the information carried in the Rhizobium nod factor is sufficient to trigger the plant to mount a complex tissue-specific response at the transcriptional level. However, both the function of these so-called nodulins and the regulatory pathway controlling their induction remain unknown. Several recent studies can be used to illustrate the debates surrounding nodulin function and induction.

What functions do nodulins perform? Many ENODs are proline rich and potentially may be located in the cell wall (Hirsch, 1992; Löbler and Hirsch, 1993; Vijn et al., 1993; Schultze et al., 1994; Wilson et al., 1994), although explicit tests have yet to be made. There may be some surprises, as shown for the ENOD12 gene; this gene was widely studied because its expression pattern suggests that it is part of a relatively early signal transduction cascade (for example, see Horvath et al., 1993; Löbler and Hirsch, 1993; Bauer et al., 1994; Journet et al., 1994). Recent data bring into question the function of ENOD12, however; a Medicago subspecies lacking the ENOD12 gene forms functional nodules (Csansdi et al., 1994). So, although the expression pattern of ENOD12 may prove to be a useful marker for early plant responses, it is not clear whether the locus has a required role in nodulation.

The sequence of at least one newly identified ENOD, rip1, does provide clues to function. Cook et al. (1995) discovered a gene that is expressed in Medicago root hairs, both in response to Rhizobium and to nod factor, that shows high sequence similarity to peroxidases. The rapid but transient expression of this gene in root hairs responding to Rhizobium or nod factors suggests that an oxidative process may occur early in the symbiotic response of the plant. This is especially interesting because of the importance of oxidative processes for cell wall architecture and for defense and developmental responses in plants (see Dangl et al., 1996; Hammond-Kosack and Jones, 1996, in this issue.)

Another interesting early nodulin is ENOD40, for which the gene sequence is broadly conserved in legumes but which does not always include a long open reading frame. One possibility is that ENOD40 is a nontranslated regulatory RNA (Asad et al., 1994; Crespi et al., 1994). However, the identification (Vijn et al., 1995b; van de Sande et al., 1996) of a small conserved open reading frame near the 5' end of ENOD40 transcripts opens the possibility that a small active peptide is encoded by this locus.

Morphogenesis and Molecules

Gene expression in nodule development occurs in the broader context of organogenesis, including cytoplasmic activation and new cell division (van Brussel et al., 1992; Savoure et al., 1994; Stokkermans and Peters, 1994; Yang et al., 1994). However, the pathways and control points for cytoplasmic and cytokinetic activity have not been determined. Because nodulation is developmentally specific yet occurs in response to a facultative signal, nodules provide an experimental opportunity to study cellular events using numerous experimental approaches. For example, cell division and nodule morphogenesis provide an assay system for possible secondary messengers. It has been established that applications of either auxin transport inhibitors or cytokinins cause nodule morphogenesis and nodulin expression (reviewed in Hirsch, 1992). These findings have led to the development of a secondary signal model (Hirsch, 1992; Cooper and Long, 1994; Hirsch and Fang, 1994) in which the initial bacterial signal is transduced by a series of steps that lead to changes in the activities of endogenous hormones.

The predictable patterns of cell division in nodules suggest that nod factors affect root cells according to a specific physical
pattern. Libbenga and others predicted that this pattern might be the result of interactions between incoming bacterial signals and gradients of plant substances emanating from the vascular system (Libbenga and Bogers, 1974). Smit et al. (1995) singled out uridine as a component present in the vascular stele that affects cell growth and division rates in root explants. Whether a gradient of uridine exists in roots at concentrations that would correlate with the tissue culture effect is not known. Finally, several lines of evidence point to the involvement of ethylene in the regulation of early nodulation. It is known that plants downregulate nodulation in response to numerous conditions, and endogenous ethylene, based on inspection of the plant response and on experimental manipulation of ethylene levels, is a candidate to mediate this regulatory process (Peters and Crist-Estes, 1989; van Spronsen et al., 1995; van Workum et al., 1995).

The examination of cell division patterns provides the opportunity to explore the effect of Nod factors on plant development. Cytoplasmic activation, including phragmosome formation, occurs in Rhizobium-stimulated roots. In some plants such as Vicia, phragmosome formation is highly site specific and occurs early in response to Nod factors (van Brussel et al., 1992). In other plants, this behavior is less evident (Yang et al., 1994). Using cyclin, histone H4, and cdk gene expression as markers for cell activation, Yang et al. (1994) found that the cell cycle was activated (by inheritance, passage into G1 and S phases) in a pattern that included both the inner cortical cells at the site of the future nodule primordium and the outer cortical cells that showed phragmosome formation. The outer cells were invaded by the bacteria and did not divide further; the inner cells continued to divide, forming the nodule cortex and nodule meristem. Therefore, it appears that Rhizobium exploits both a direct result of cell division—an increased number of cells that will form the organ on which the bacteria will ultimately reside—and also an indirect consequence of cell division, namely, increased cell wall synthesis and vesicular traffic. These functions could help cause the usually inactive outer cortical cells to be as conducive to infection thread formation as are the tip-growing root hairs of the epidermis (see also discussions in Hirsch, 1992; Kjne et al., 1992; Ridge, 1992; van Brussel et al., 1992; Yang et al., 1994).

**Early Cell Responses**

The earliest cell divisions in nodule development occur between ~12 and 24 hr after bacterial infection, with ENOD expression preceding this by several hours. Early signal transduction events in epidermal or other cells may account for the coordination of the diverse host responses. Little is known of the early events in root hairs, but several recent studies have explored ion movements as one kind of indicator of cellular activity related to signal perception. Ehrhardt et al. (1992) found that alfalfa root hairs displayed a characteristic depolarization of the cytoplasmic membrane in response to the application of R. meliloti Nod factors. Felle et al. (1995) and Kurkdjian (1995) subsequently found that this cell behavior was specific to the correct structure of the Nod factor and was correlated with the developmental stage of the root hair itself. The ionic basis for this behavior is unknown, but Allen et al. (1994), using a vibrating probe electrode, found variable currents of two ions, H+ and Ca2+, outside root hairs that had been exposed to Nod factor.

More recently, Ehrhardt et al. (1996), using ion-specific reporter dyes to monitor internal root hair Ca2+ concentrations, observed a distinctive signal transduction behavior, termed calcium spiking, in alfalfa root hairs exposed to R. meliloti Nod factors. No such response was seen after exposure of root hairs to either chitin oligosaccharide or to Nod factors from R. leguminosarum. Furthermore, a Nod− alfalfa mutant that lacks both root hair curling and cell division also showed no calcium spiking. The pattern of spiking, which had a 1-min periodicity, and the lag time after Nod factor presentation, which averaged ~10 min, suggest that this behavior is not mechanistically related to membrane depolarization. The basis for calcium spiking in plants is not characterized, but in animal cell systems, this behavior is typically associated with inositol-triphosphate signaling. Whether this is the case for calcium spiking in root hairs could be tested in future work.

As with receptors, proof of functional involvement of nodulins, early cell behaviors, and signal transduction components must await analyses of plant mutants affected in nodule development (Caetano-Anollés and Gresshoff, 1991). Plant nodulation mutants that show no response to the bacterial signal or that show response to an altered signal are likely to be defective in a receptor or in early stages of signal transduction. One such candidate is the sym2 mutant of Pisum sativum, which interacts differently with R. leguminosarum Nod factors, depending on whether or not they have a 6-O-acetyl group on the nonreducing residue of the oligosaccharide (see Firmin et al., 1993, and papers cited therein). Tests for early cell autonomous responses such as calcium spiking may help sort mutations into those affecting early or middle parts of the signal transduction pathways. Arabidopsis is not an option for such genetic studies, so research focuses on genetic analysis in crop species such as pea and soybean (Caetano-Anollés and Gresshoff, 1991; Kneen et al., 1994; Landau-Ellis and Gresshoff, 1994; see also Gianinazzi-Pearson, 1996, in this issue) and in diploid legumes with small genomes, such as Lotus japonicus, Melilotus albus, and M. truncatula (Barker et al., 1990; Miller et al., 1991; Handberg and Stougaard, 1992; Hirsch, 1992).

**Nod Factors as Native Plant Growth Regulators?**

**A Cautious View**

Two general scenarios can be envisioned for the evolution of the Rhizobium–plant signaling system: Rhizobium signals could mimic a class of native plant hormones, previously undiscov-
ered, which are active not only in legumes but also in other plants. Alternatively, the *Rhizobium* signals, with their chitin backbone, could have coopted a plant defense response pathway originally responding to chitinaceous or other elicitors (see discussions of this and other considerations in Sprent, 1989; Truchet et al., 1989; Brewin et al., 1992; Fisher and Long, 1992; Hirsch, 1992; Kijne et al., 1992; Spahn, 1995).

The first model, in which *Rhizobium* Nod factors mimic native plant growth regulators, has received recent experimental and theoretical attention (Truchet et al., 1991; Dénarié and Cullimore, 1993; Spahn et al., 1993; Röhrig et al., 1995). This is an interesting, lively, and important issue about which there is at present no consensus within the *Rhizobium* research community.

Proof that a compound acts as a true plant hormone requires that (i) the compound is actually found in plants, and (ii) it is active in causing plant responses at levels that correlate with its abundance. Presently, there is no direct biochemical evidence for the production or existence of lipo-oligosaccharide molecules anywhere in plants other than *Rhizobium*-induced nodules. Nor have there yet been identified any plant homologs of the genes that encode Nod factor—synthesizing enzymes. From these most stringent standpoints, we must conclude that to date, there is no evidence for endogenous Nod factor or other lipo-oligosaccharide molecules in plants.

What of indirect evidence? Several recent studies report that lipo-oligosaccharide molecules can be active in tissue culture systems. Based on these observations, it has been suggested that such molecules may function as plant hormones (De Jong et al., 1993; Dénarié and Cullimore, 1993; Spahn et al., 1993; Röhrig, 1995). The tissue culture data may provide the basis for direct experimental tests of lipo-oligosaccharide activities by pointing to likely places of action. Nevertheless, we should recall the lesson learned from many years of phytohormone studies—effects in tissue culture do not always occur because the applied compound closely imitates the structure of a native regulator. Consider auxin: its structure and mode of action would not likely be deducible from an inspection of the structures and activities of functional analogs such as 2,4-dichlorophenoxyacetic acid and naphthylphthalamic acid.

One study of lipo-oligosaccharide activity exploited the carrot somatic embryogenesis system (De Jong et al., 1993). A wild-type cell line of carrot forms ~150 embryos per 10,000 cells plated; the ts11 variant is defective in somatic embryogenesis, forming only 0.3 embryo per 10,000 cells at 32°C. This low number of embryos regenerating at the restrictive temperature could be elevated, although to only 1 to 2% of wild-type levels (~2.4 embryos per 10,000 cells), by treatment of the cultured ts11 cells with a purified *R. l. vicieae* Nod factor. The variability of the assay was high, and in the experiments reported to date, the putative inactive compounds (such as Nod factors with altered structure) were evaluated in very few trials compared with those for the proposed active compounds. Future work with a larger number of trials would help to substantiate the hypothesized relationship between Nod factors and embryogenesis. For example, this link could be further explored through the use of Nod factors in attempts to rescue zygotic embryo-defective mutants, such as those described for Arabidopsis (reviewed in Jürgens et al., 1994; Meinke, 1995).

A second study utilized a different series of assays to follow the effects of Nod factor–like lipo-oligosaccharides on cultured plant cells. Röhrig et al. (1995) constructed a series of lipo-oligosaccharide molecules by combined enzymatic and chemical modifications of chitin oligomeric backbones (John et al., 1993). This versatile method permitted the construction of Nod factor–like molecules with structures distinct from those synthesized by *Rhizobium*. Among the molecules synthesized by these authors were molecules with both cis- and trans-conformations at the C9 and C11 positions of the N-acyl moiety. The molecules with trans conformation consistently showed elevated activity in a series of assays. This result is striking because such molecules have not been found among naturally occurring *Rhizobium* Nod factors, all of which have the cis conformation at the corresponding position. The active synthetic Nod factors also enhanced tobacco protoplast division rates, independent of auxin/cytokinlin ratios. Nod factors with cis-conformation acyl groups, like chitin oligomers, had slight effects. When the active compounds were presented to protoplasts along with kinetin, expression from a cauliflower mosaic virus 35S partial promoter was stimulated. Moreover, in protoplasts given Nod factor only, transcription from the auxin responsive locus, *AXI*, was increased. Röhrig et al. (1995) propose from these experiments that auxin and lipo-oligosaccharides share a signal transduction pathway and that the observed increase in tobacco protoplast division is caused by a mechanism similar to that triggering nodule formation in legumes. If it is indeed the case that lipo-oligosaccharides imitate auxin through a common mechanism, one may also expect to observe effects of Nod factor–like compounds on morphogenesis or transcription in intact plants or to effect the rescue of some categories of auxin mutant using these compounds.

CONCLUDING REMARKS

One of the most distinctive features of the *Rhizobium*–legume symbiosis is its specificity. Any model for receptor function and evolution must account for the restriction of specific *Rhizobium* species or biovars to particular host plants and must explain in general why the *Rhizobium* symbiosis is restricted to the Fabaceae (Brewin et al., 1992). The suggestion that Nod factors may be related to or function as native and universal plant growth regulators is somewhat difficult to reconcile with their observed biological specificity in symbiosis. If Nod factors represent a broadly based and universal morphogenetic pathway, one would need to explain how this pathway evolved into a beneficial symbiosis in only one plant family that originated ~85 to 90 million years ago. Perhaps, if this general category
of compounds exists and is active in plants, then the native plant compounds have some basic structural difference, as suggested by the data of Röhrig et al. (1995). At present, evolutionary theories have few data on which to build. New experimental details, arising from biochemical, cellular, and genetic studies of the plant's role in early nodulation, will help refine our conceptual framework for how nodulation arose in evolution.

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