Genes and Signals in the *Rhizobium*-Legume Symbiosis[1]

Sharon R. Long*

Department of Biological Sciences and Howard Hughes Medical Institute, Stanford University, Stanford, California 94305

Rhizobium-legume symbiosis begins with two free living organisms, and ends with an intimate cellular co-existence. *Rhizobium* bacteria recognize specific plants, provoke development of a root nodule, and invade the plant tissue. Eventually, the *Rhizobium* cell transfers itself into a host cell, surrounds itself with plant membrane, and arranges a nutrient exchange in which the bacteria brings fixed nitrogen to the plant, receiving in turn the sanctuary and sugars that the plant cell can provide (8,13,17,20). This historical note concerning 25 years of *Rhizobium* plant research will focus mostly on a few stories related to the discovery of *nod* genes and signals. I refer the reader mostly to books and reviews for the details of these and of related research stories that are mentioned more briefly in the latter part of this review.

RHIZOBIUM GENES

The *Rhizobium*-legume symbiosis had attracted serious study ever since Beijerinck's demonstration that bacteria caused nodule formation (13). Considerable progress was made prior to 1975 in studying the biochemistry of nitrogen fixation itself. The mechanism of nodule formation, however, was the subject of a great deal of speculation without much concrete experimental proof. The critical first step turned out to be the identification of *Rhizobium* genes, rather than of plant components. Looking back, we can see that experiments before 1980 had little chance of success, because they analyzed free-living bacteria: In these conditions, most *Rhizobium* symbiosis genes would have been silent, and thus symbiosis-related properties would have been cryptic.

The first *Rhizobium* genes for nitrogen fixation (*nif*) and for nodulation (*nod*) were cloned in the early 1980s by Gary Ruvkun and by myself, respectively, with our colleagues in Fred Ausubel's laboratory (16), and soon many more *nif*, *nod*, and *fix* (symbiotic fixation) genes were found in laboratories worldwide. Allan Downie, Nick Brewin, and Andrew Johnston at the John Innes Institute found that not just genes for nodule formation, but those for host specificity, were tightly clustered with *nif* genes on a transmissible plasmid in *Rhizobium leguminosarum viciae*, and Barry Rolfe, Michael Djordevic, and Roger Innes working in Canberra found a parallel situation in *R. leguminosarum trifolii.* The groups of Jean Dénarié in Toulouse and Adam Kondorosi in Szeged showed that clusters of symbiosis genes in *Rhizobium meliloti* were on incredibly large "megaplasmids," over a million bases in size, an exciting discovery that changed the concept of bacterial genome architecture. By contrast, Hauke Hennecke's group in Zurich and Gary Stacey's group in Tennessee defined *Bradyrhizobium japonicum nod* genes on the chromosome. Bill Broughton and his group showed that *Rhizobium* strain NGR234 had an astonishingly broad host range (over 18 genera, including one non-legume) and with multiple host specificity genes dispersed around a 500-kb plasmid. Since the cloning of *nif* and *nod* in 1980 to 1981, over 30 different research groups have contributed to our present understanding of *Rhizobium* symbiosis genes through physical cloning, chromosomal walking, plasmid identification, site-directed mutagenesis, and many phenotypic studies on diverse plant hosts (4,16,17). The rules of genome organization are different for diverse *Rhizobium*; in some cases, symbiosis genes are clustered, in other cases they are dispersed. In some cases, the genes are on plasmids and can spread at high frequency by conjugation; in others the genes are scattered among many chromosomes and plasmids; and one case of symbiosis island transfer has been shown for *Mesorhizobium loti* (18). With all of this genomic diversity it is no wonder that systematists have had a field day classifying and reclassifying the bacteria (16), sometimes to the bafflement of the molecular biologists studying the genetics of these species.

As the story of *nod* genes and signals has unfolded, described below, comparably deep and interesting stories have emerged in every aspect of the symbiosis, and details can be found in a number of recent reviews. Graham Walker and his colleagues at MIT showed that genes for *Rhizobium* surface polysaccharides are required for invasion, although not for early nodulation or host specificity. Through their work and that in other laboratories, a diverse set of such components (extracellular polysaccharides, lipopolysaccharides, and novel types of surface carbohydrate) are now known to be important, in some cases as signals that undergo processing from the large M_r form (16). Bacterial genes for invasion and bacterial

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* E-mail srl@leland.stanford.edu; fax 650–725–8309.

differentiation have been found by direct and indirect screens (10,16). The bacteria show exquisite physiological adaptation to the low oxygen environment of the nodule, from the production of specialized cytochromes, to the control of nitrogen fixation genes themselves (6,16). Bacteria also may react to and manipulate host respiration in early stages of symbiosis via a novel signal, lumichrome (11). Carbon and nitrogen metabolism in the differentiated nitrogen fixing bacteria shows amazing new adaptations (16) including the production of "rhizopines" that may supply specialized nutrition to sibling bacteria in the environment (16,20).

Are there more symbiosis genes to be found? This is almost certain, especially in light of the elucidation of the first symbiosis plasmid sequence, revealing new secretion systems and novel genes that respond transcriptionally to the host (7). The approaching complete genome sequence of *Rhizobium meliloti* and other species promises to reveal many more bacterial genes required for invasion of and function within the host. The big questions will remain: What do these genes do, and how are they regulated? The case of the *nod* genes shows an example.

PLANT FLAVONOIDS: NEW SIGNALING ROLE FOR A VENERABLE MOLECULAR FAMILY

The identification of the *Rhizobium* nodulation genes and subsequent study of their expression showed that *nod* genes were not expressed in free-living cells. My laboratory at Stanford, Allan Downie and colleagues in the John Innes Institute, Ben Lugtenberg's department at Leiden University, Barry Rolfe at the Australia National University and John Redmond at Macquarrie University in Australia, and several other groups worked to find how these genes were regulated. Our laboratories exploited reporter fusions to show both that the *nod* genes required plant inducers to be transcribed and that NodD appeared to be the transcription activator.

What exactly are the plant compounds that trigger *nod* gene expression? The availability of the *Rhizobium nod-lacZ* reporter fusions allowed bioassay of fractions separated by reverse phase chromatography, followed by spectroscopic analyses to solve the structure of the natural compound. For example, Kent Peters and I determined that the active fraction from alfalfa seed exudate was luteolin, a tetrahydroxyflavone. By comparable approaches and by assay of available compounds the groups in Leiden, Norwich, Canberra, and Melbourne found other plants export either flavones or flavanones, and the team at Agrigenetics found that the soybean inducer was daidzein, an isoflavone. From this and subsequent work, it was found that each legume produces a distinct cocktail of flavonoids and that the quantity and spectrum of compounds may vary with the age and physiological state of the plant (4,12,16). The flavonoid family of compounds, used by *Rhizobium* as a positive signal, is also the source of many legume phytoalexins, which raises some interesting co-evolutionary questions. Our field's view of how flavonoid signals are used physiologically and ecologically may expand, as the plant-microbe field moves in its next era from focus on first-order effects, such as simple transcription activation, to the physiological and ecological context (12).

NOD FACTORS: BACTERIAL CARBOHYDRATES WITH PLANT HORMONE ACTIVITIES

How exactly does *Rhizobium* cause host-specific nodule development? The identification of the *nod* genes and the elucidation of their regulation was a key that unlocked an exciting new room of discoveries: Now, it was possible to trigger symbiotic behaviors by bacteria grown in culture and to use wild-type versus Nod^- strains as controls. Over the period from 1986 to 1990, genetics, cell biology, and biochemistry came together to identify a completely new category of signal: the Nod factor (3,9,15). Contributions of many groups, notably Ton van Brussel and colleagues in Leiden, laid the groundwork showing *Rhizobium* exudates had effects on plants, depending on *Rhizobium nod* gene content and expression. This came to fruition in 1990 with the work in Toulouse by the groups of J. Dénarié, G. Truchet, and J.-C. Promé. Having observed that *R. meliloti* pretreated with flavonoid inducer could cause alfalfa plants to display nodule-like behaviors, they fractionated the *Rhizobium* medium and used careful microscopic bioassay of plant reactions to identify specific active fractions, or "Nod factors." Chemical analysis revealed the active component to be a novel lipo-chito-oligosaccharide, based on a chitin oligomer backbone, and carrying a sulfate at the reducing end of the oligomer. Ben Lugtenberg, Herman Spaink, and colleagues in Leiden and Utrecht next found that in *R. leguminosarum viciae*, host-specific modifications occur in an *N*-acyl group on the non-reducing end residue.

In the decade since 1990, a vigorous international enterprise led to isolation and characterization of Nod factors from many *Rhizobium* species. To give readers an idea of the difficulty and scope of this work, it has involved microbiologists, geneticists, plant cell biologists, physiologists, biochemists, and analytical chemists from Cuernevaca, Geneva, Gent, Gif-sur-Yvette, the John Innes Institute, the University of Georgia, Michigan State, the University of Missouri, Ohio State, Tennessee, and the University of Utrecht with participation by a number of other groups supplying various wild-type and mutant *Rhizobium* strains. The outcome of this work showed that diverse *Rhizobium* all produce Nod factors with a basic similar structure: a chito-oligo backbone with side groups that include novel modified sugars,

acetyl or carbamoyl residues, and modified lipids. Bioassay on plant hosts demonstrated that the side groups provide host specificity for one plant or another.

My laboratory was among several that took a complementary approach: We asked what were the biochemical activities of the *nod* gene products known from previous analysis to be essential for symbiosis? This approach bore fruit the same year as Nod factor identification, in 1990, with the demonstration by Julie Schwedock and myself of the first biochemical function for a *nod* gene enzyme: *nodP* and *nodQ* encoded an enzyme that activated sulfate to its nucleotide form, APS. It was an exciting moment when we all realized that the independent searches for bacterial compounds with Nod factor activity on the one hand, and for functions of bacterial genes found only by phenotype on the other, had led to the same place: a molecule with a sulfate side group. In the past decade, well over a dozen research groups (notably including the list above, and research groups in Köln, Leuven, and elsewhere) contributed to the demonstration that most Nod proteins have enzymatic activities such as polymerases and *N*-acyl transferases (encoded by common *nod* genes), and *O*-sulfonyl, *O*-acetyl, *N*-methyl, and exotic glycosyl transferases (encoded by host specific *nod* genes). These activities are consistent with the synthesis of the lipochitooligosaccharide Nod factors (for details, see 16).

With the conjunction of structural determination, bioassay, molecular genetics, and in vitro biosynthetic proof, the Nod factor hypothesis (that *nod* genes encode the synthetic enzymes for host specific lipooligosaccharides) was solidified by the mid-1990s. A new star in the firmament of biochemical signals had been discovered, and the fact that it was such a new, unexpected chemical species was tremendously exciting within and outside of the plant research community.

But we now know that not all *nod* genes encode enzymes with such activities, a clue that more signal surprises may await us. For example, NodO acts to form ion channels in membranes (4,5). Could the bacteria be sending other signals in the early nodulation stages? Where do the exopolysaccharides fit in? Are loci defined in genome projects important? Our assays to detect plant responses now need to be refined to detect subtle bacterial effects.

BEYOND SIGNALS: CELL RESPONSES, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY

My main narrative has concerned the discovery of bacterial *nod* genes and the elucidation of signals that control these symbiosis genes. This is the tip of the iceberg. Symbiosis researchers have used genetics, molecular biology, cell biology, biochemistry, and physiology to produce a wealth of information about bacterial and plant transcription, cellular organization, and exchange and assimilation of nutrients. A few highlights follow with references to reviews that do better justice to this dynamic field and that point to the primary contributions of many researchers whose names could not all be included here.

In addition to morphogenesis itself, plant transcriptional responses to *Rhizobium* are striking, from the leghemoglobin genes first cloned by Desh Pal Verma and colleagues (20) to the early nodulins, or ENODs, identified by Ton Bisseling and colleagues as Pro-rich sequences with possible cell wall locations. Researchers at many laboratories (a partial list includes Wageningen, Versailles, Toulouse, Texas A&M, Sevilla, Ohio State, Minnesota, Leiden, the John Innes Institute, Gif-sur-Yvette, Cuernevaca, UCLA, Canberra, Bielefeld, and Aarhus) used both in situ hybridization and transgenic plant constructs to demonstrate the dramatic transcriptional response of plant genes to *Rhizobium* signals and to correlate these with the developmental and metabolic changes that characterize symbiotic interactions (2,16). Many of those same research groups and others, including groups in Tennessee, Roskilde, Moscow, Marburg, Dartmouth, and Adelaide, have shown how the symbiosome compartment is constructed by targeting of novel plant proteins that control exchange between the partners, such as the novel ammonium transporter discovered by Udvardi and Day and colleagues (16,19).

Cellular and tissue rearrangements were studied by microscopic, immunochemical, and biochemical analysis: George Truchet in Toulouse, Nick Brewin at the John Innes Institute, Jan Kijne and colleagues in Leiden, Bob Ridge working in Australia and Japan, and Kate VandenBosch and Doug Cook at Texas A&M, among a number of other laboratories, found changes in cytoskeletal architecture, cell wall biochemistry, and oxidative metabolism during infection (5,16). David Ehrhardt and others in my laboratory discovered membrane depolarization and calcium spiking in root hairs, and Hubert Felle in Giessen working with the Kondorosi laboratory at Gif-sur-Yvette, showed that fast ionic changes across the cytoplasmic membrane accompany alfalfa treatment by Nod factors (5). Research on calcium signaling, cytoskeletal dynamics, and other aspects of signal transduction, are now being expanded at the frontiers by colleagues in Cuernevaca, Leiden, University of Massachusetts, the John Innes Institute, Toulouse, Wageningen, and beyond, as many new laboratories join this exciting search.

For all of these details of "what" happens during nodulation, we are still in the dark about the "how." Literally dozens of laboratories, including veterans and newcomers, are now focused on the next set of questions, and the writer of next year's review will have much to say about this fast-moving field. What signaling pathways do the plants use to transduce *Rhizobium* Nod factors into such diverse responses?

What is the fate of the Nod factor in the plants? From Nod factor localization to Nod factor breakdown, from binding proteins that are novel lectins to binding sites on plant cell membranes, new work on how Nod factors interact with plant cells is exciting and dynamic but still with many more questions than answers (5,15). What happens after the Nod factor finds its initial target? Possible downstream events have been inferred from inhibitor and pharmacological studies (5,16). Plant hormones such as auxin and cytokinin are likely to play a role downstream (8), and ethylene appears to have very early effects (1,16). With many events, many correlations, and many components, are these cause or consequence, significant correlations, or minor side effects? To sort this out, our field looks to plant genetics as the key approach to sort out what is centrally important in nodule development (1).

It is thus appropriate to end this bird's eye research overview with one specific and exciting new advance, the cloning by Stougaard and colleagues of the first plant nodulation gene, *Lotus japonicus NIN-1*, encoding a probable transcription factor required for nodule morphogenesis (14). As genes are cloned that correspond to various plant nodulation defects, it will become possible to identify the essential steps in plant recognition of its symbiont and to come to the evolutionary heart of the matter: why legumes? And thus also to the agronomically important corollary, the question asked is can the symbiosis be genetically altered, extended, or improved.

PERSPECTIVE

The path of the past 25 years has led to elucidation of signal exchange primarily through genetic analysis and analytical chemistry. But these experimental approaches would not have been possible without careful and detailed studies of growth, metabolism, and cell and organ structure. As we look forward we should recognize that not all signals will be detected by following gene transcription changes and that the concept of "signal" should be considered in its broadest sense. Consider what we already know: *Rhizobium* evolved not only to detect flavonoids, but to sense oxygen and carbon dioxide, which are molecular gases of central metabolism. On the plant side, we will doubtless find transcription factors and kinases that trigger nodule development, but perhaps we will also find genes that affect basic plant architecture. In a new era of post-genomic study, it is not only exotic chemistry and gene regulation but basic physiology that may provide many useful clues to follow the thread of molecular signaling, between or within organisms, in the complex fabric of plant function.

LITERATURE CITED

1. **Cook DR, Vandenbosch K, De Bruijn, FJ, Huguet T** (1997) Plant Cell **9:** 275–281
2. **Crawford N, Leustek T, Kahn M, Long SR** (2000) *In* BB Buchanan, W Gruissem, RL Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp 786–849
3. **Dénarié J, Debellé F, Promé J** (1996) Annu Rev Biochem **65:** 503–535
4. **Downie JA** (1994) Trends Microbiol **2:** 318–324
5. **Downie JA, Walker SA** (1999) Curr Opin Plant Biol **2:** 483–489
6. **Fischer HM** (1996) Trends Microbiol **4:** 317–320
7. **Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X** (1997) Nature **387:** 394–401
8. **Hirsch AM** (1992) New Phytol **122:** 211–237
9. **Long SR** (1996) Plant Cell **8:** 1885–1898
10. **Oke V, Long SR** (1999) Curr Opin Microbiol **2:** 641–646
11. **Phillips DA, Joseph CM, Yang GP, Martinez-Romero E, Sanborn JR, Volpin H** (1999) Proc Natl Acad Sci USA **96:** 12275–12280
12. **Phillips DA, Streit WR** (1996) *In* G Stacey, NT Keen, eds, Plant Microbe Interactions. Chapman & Hall, New York, pp 236–271
13. **Quispel ANN, editor** (1974) The Biology of Nitrogen Fixation. North-Holland Press, Amsterdam
14. **Schauser L, Roussis A, Stiller J, Stougaard J** (1999) Nature **402:** 191–195
15. **Schultze M, Kondorosi A** (1998) Annu Rev Genet **32:** 33–57
16. **Spaink HP, Kondorosi A, Hooykaas PJJ** (1998) The Rhizobiaceae: Molecular Biology of Model Plant-Associated Bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands
17. **Stacey G, Evans H, Burris R** (1992) Biological Nitrogen Fixation. Chapman & Hall, New York
18. **Sullivan JT, Ronson CW** (1998) Proc Natl Acad Sci USA **95:** 5145–5149
19. **Udvardi MK, Day DA** (1997) Annu Rev Plant Physiol Plant Mol Biol **48:** 493–523
20. **Verma DPS** (1992) Molecular Signals in Plant Microbe Communication. CRC Press, Boca Raton, FL