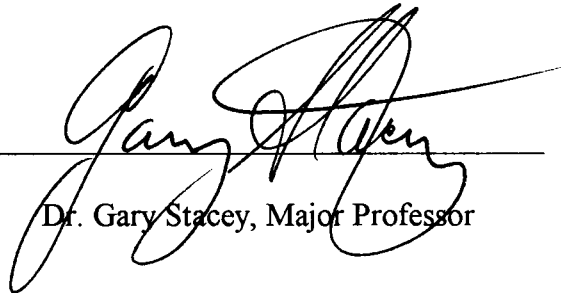


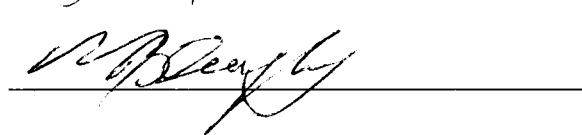
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I am submitting herewith a dissertation written by Minviluz Luna Garcia entitled "Function and Regulation of the *nolA* Gene of *Bradyrhizobium japonicum*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

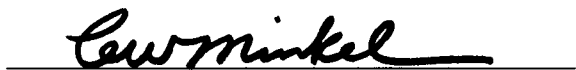


Dr. Gary Stacey, Major Professor

We have read this dissertation and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor and
Dean of The Graduate School

**FUNCTION AND REGULATION OF THE *nolA* GENE OF
*Bradyrhizobium japonicum***

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Minviluz Luna Garcia

December, 1996

This dissertation is dedicated to my grandparents, Elegio and Maria de Luna,
to my parents, Rodolfo and Lydia Garcia, and to my aunt, Linda,
who have all given me invaluable help and
inspiration during my education

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I would like to thank my major professor, Dr. Gary Stacey, for his invaluable guidance during the course of my post-graduate study. I also would like to thank Dr. Jeffrey Becker, Dr. Peter Gresshoff and Dr. Bert Lampson for their suggestions and comments on my Ph.D. work. I would like to express my appreciation to Dr. Neil Quigley for agreeing to serve on my Thesis committee on short notice. I also would like to acknowledge Dr. John Dunlap, Dr. Mike Sadowsky, Dr. John Loh and Josh Cohn for their scientific contributions to this work. My warmest appreciation goes to everybody in the Stacey Laboratory who provided excellent comradeship during my stay at U.T.

I am forever grateful to my grandparents, parents, brothers and sisters for their unfailing love, support and prayers.

model for the transcriptional regulation of NolA_L and NolA_S expression, as well as a putative DNA sequence to which NolA_L binds.

ABSTRACT

Two *Bradyrhizobium japonicum nola* mutants were constructed and used to test the functional role of Nola in nodulation. Contrary to the previous hypothesis that Nola acts as a repressor of *nod* gene transcription, the expression of a *nodD1-lacZ* or *nodY-lacZ* fusion in the *nola* mutant strains was similar to that found in the wild type. However, Nola does appear to act as a transcriptional regulatory protein since it is required for its own expression, as well as that of *nodD2*. Expression of NodD2 from a constitutive promoter led to a significant reduction in *nodC-lacZ* activity. Therefore, the repression of *nod* gene expression by Nola is likely an indirect effect, perhaps mediated by other genes (e.g., *nodD2*) that are regulated by Nola. When inoculated onto soybean (*Glycine max* cultivar Essex) roots, the *nola* mutant strains showed only a slight delay in nodulation as compared to the wild type. However, the mutant strains were grossly defective in nodulation and nitrogen fixation on cowpea (*Vigna unguiculata* cultivar Caloona) plants. Microscopic examination of soybean nodules induced by the *nola* mutant strains showed similar developmental and morphological characteristics to nodules formed by the wild type with only a slight delay in bacteroid maturation. In contrast, cowpea nodules induced by the *nola* mutant strains contained fewer infected cells and bacteroids were not found in a typical symbiosome structure. These results indicate that Nola is a transcriptional activator required for the expression of genes that play a role not only in the early stages of infection, but also during the later stages of bacteroid development and maintenance.

Examination of the *nolA* gene sequence of *Bradyrhizobium japonicum* shows the presence of two putative ATG start codons. Translation for the first ATG (ATG1) would predict a protein (NolA_L) having an N-terminal, helix-turn-helix DNA-binding motif which shares sequence similarity to the DNA-binding domains of the MerR-type regulatory proteins. Translation from the second ATG (ATG2) would give an N-terminally truncated protein (NolA_S) lacking the DNA-binding domain. To investigate the possibility that *nolA* encodes two proteins, plasmids encoding N-terminal or C-terminal *nolA-lacZ* translational fusions were constructed. Expression of *lacZ* fused to NolA_L prior to ATG2 was inducible by soybean seed extract but not by genistein, a normal isoflavone inducer of *nod* gene expression. In contrast, β -galactosidase activity resulting from a C-terminal fusion of *lacZ* to *nolA* was not inducible by soybean seed extract but did require the presence of a functional NolA. These results indicate that *nolA* can be translated from two ATG start codons and that the expression of NolA_L and NolA_S is differentially regulated. Consistent with these results, western blot analysis of *B. japonicum* cell lysates revealed two immunoreactive bands of 29kd and 27 kd that were absent in lysates prepared from a *nolA* mutant strain. Primer extension experiments revealed that *nolA* is transcribed from two promoters, one of which is immediately downstream of a putative NolA_L DNA-binding site. Similarly, *nodD2*, which requires NolA for expression, is also transcribed from a site immediately downstream of a putative NolA_L DNA-binding site. Since only NolA_L would contain a helix-turn-helix DNA-binding domain, these results suggest that *nolA* and *nodD2* require NolA_L for transcription. Based on the above results, I propose a

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CHAPTER I

INTRODUCTION

The biological ability to convert atmospheric nitrogen (N_2) to ammonia is strictly limited to prokaryotes. Diazotrophs such as *Klebsiella*, *Azotobacter* and *Rhodobacter* can fix nitrogen under free-living conditions (reviewed by Hill, 1992). In addition to the free-living diazotrophs, other bacteria can fix nitrogen in close association with plants (e.g., *Azospirillum* spp; Elmerich et al., 1992) or symbiotically within the plant host cells. Bacteria that can fix nitrogen in symbiotic association with leguminous plants belong to three genera, namely *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, and are collectively referred to as rhizobia. These three genera, together with *Agrobacterium* and *Phyllobacterium*, constitute the Rhizobiaceae family of the α subdivision of the protobacteria (Elkan and Bunn, 1992).

The earliest studies on nitrogen fixation have been done on *Klebsiella pneumoniae*. Such studies have elucidated basic concepts of the genetics, biochemistry, and physiology of nitrogen fixation, and have contributed to a general understanding of bacterial gene regulation (Hill, 1992). In contrast to free-living diazotrophs, rhizobial nitrogen fixation is a very complex process that involves a specific recognition between the rhizobium and the plant host cell. During the process, the plant elaborates a new organ and the bacterium goes through a programmed morphogenetic sequence of development. The two organisms differentiate to produce a metabolic cooperation wherein the bacteria

fix dinitrogen to ammonia. The plant assimilates the ammonia into amino acids and, in return, supplies the bacteria with carbohydrate (Werner, 1992). In addition to the obvious agricultural benefits that can be derived from biological nitrogen fixation, the examination of symbiotic nitrogen fixation can also provide insight into the molecular basis for gene regulation, cell-cell recognition, regulation of plant defense mechanism, and cell differentiation.

RHIZOBIAL CHARACTERIZATION AND CLASSIFICATION

Bacterial species belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* are Gram negative soil bacteria that can infect the roots of leguminous plants and establish as nitrogen-fixing symbiosis. The association of rhizobia with the host legume is specific in that certain rhizobial strains can nodulate only a certain subset of legumes (Table 1). In general, *Rhizobium* spp. can nodulate only a few host species (i.e. narrow host range), whereas *Bradyrhizobium* spp. tend to have a broader host range. One exception is *Rhizobium* sp. NGR234 (MPIK3030) which can nodulate over 100 different legume genera (Stanley and Cervantes, 1991; Broughton, personal communication). Species designation within each genus is primarily based on the plant species nodulated. For example, *Rhizobium meliloti* can infect alfalfa, *R. leguminosarum* bv. *viciae* can infect vetch, and *R. leguminosarum* bv. *trifolii* can infect clovers.

In addition to host range, the different rhizobial genera also differ genetically and physiologically. *Rhizobium* spp. are fast growing (2-4 hrs doubling time), produce acidic

Table 1: Nodulation host range of various rhizobial species.

Species	Host plant
<i>Bradyrhizobium japonicum</i>	soybean, siratro, mungbean, pigeon pea Cowpea
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> bv. <i>phaseoli</i> bv. <i>trifolii</i>	Peas, vetch Beans Clover
<i>Rhizobium meliloti</i>	Alfalfa, sweet clover
<i>Rhizobium</i> sp. NGR234	many hosts
<i>Azorhizobium caulinodans</i>	<i>Sesbania rostrata</i>

products from mannitol catabolism, tend to have many of their symbiotic genes localized on large plasmids, and induce indeterminate nodules on their host plants (Elkan, 1992). *Bradyrhizobium* spp., on the other hand, are slow growing (7-20 hrs doubling time), produce alkaline products from mannitol catabolism, have their symbiotic genes localized in the chromosome, and induce determinate nodules on their host plants (Elkan, 1992). The genus *Azorhizobium* is unique in that it can elicit stem and root nodules on its host plant and is able to fix nitrogen under symbiotic and free-living conditions (de Bruijn et al., 1988).

AN OVERVIEW OF THE NODULATION PROCESS

The early events of nodule formation involve a signal exchange between the legume plant and the rhizobium. The plant produce signal compounds, called flavonoids, that induce the rhizobium to produce return signals, called Nod signals. Nod signals are oligomers of N-acetylglucoseamine having species-dependent substitutions at the reducing and non-reducing terminal sugars. Nod signals are able to elicit plant responses such as root hair curling, root hair deformation and and root hair branching. Nod signals also induce the root cortical cells re-enter the cell cycle and form a root meristem that eventually develops into the nodule, a specialized plant organ where nitrogen fixation takes place. Some plant species form an indeterminate nodule from division of the ground meristem cells which develop in a manner similar to that of a lateral root with a terminal meristematic region (reviewed by Rolfe and Gresshoff, 1988). On the other hand,

other plant species form determinate nodules from division of subepidermal cortical cells which develop into a rounded nodule without a terminal meristem.

At the site of bacterial attachment, the rhizobia elicit the stimulation and re-orientation of plant cell wall growth, presumably by restructuring the cytoskeleton. As a consequence, the bacteria are trapped within the root hair curl and a tubular structure, the infection thread, is formed. The bacteria invade the developing nodule through the infection thread, which ramifies and penetrates the cells of the developing nodule into which the bacteria are released by a process resembling endocytosis. The released bacteria are surrounded by a membrane of plant origin called the symbiosome membrane (Roth et al., 1988). Upon internalization within the cortical cell, the bacteria differentiate into their mature form capable of nitrogen fixation. The differentiated bacteria are called bacteroids. The bacteroids and the surrounding plant-derived membrane have been collectively termed the symbiosome (Roth et al., 1988). The process of bacteroid maturation is poorly understood and is accompanied by physiological and morphological changes such as alterations in nucleoid structure, heme content and cell wall structure (Sutton et al., 1981; Brewin et al., 1993). The culmination of the maturation process is the “switching-on” of the expression of *nif* and *fix* genes that encode the enzymes necessary for nitrogen fixation. The fixed nitrogen is used by the plant as a nitrogen source and, in turn, photosynthates and amino acids are provided to the bacteroids as carbon, energy and nitrogen sources (Werner, 1992). The process of nodule development has been reviewed by Kijne (1992) and Roth and Stacey (1991).

RELEVANCE OF *Bradyrhizobium*-LEGUME RESEARCH

Extensive studies on rhizobium-legume interactions have been done with the objective of enhancing the contribution of biological nitrogen fixation to agriculture. Such agricultural benefits could include improved yields, development of superior rhizobial inocula, and the transfer of symbiotic nitrogen fixation to non-leguminous plants.

Soybean, which is nodulated by *B. japonicum*, is the major legume crop grown worldwide. In addition to potential agricultural benefits, research on the molecular aspect of the nodulation process can also contribute to the basic biological knowledge of gene regulation, cell-cell recognition, host defense response, and cellular differentiation.

Recent research on the regulation of nodulation and nitrogen fixation genes has revealed that the regulatory circuits involved are highly complex. Such complexity has been attributed to the need of the rhizobia to perceive and respond optimally to varying environmental signals. Current models of rhizobial infection of legumes are largely derived from studies on the genus *Rhizobium*. However, recent research has brought new awareness to the morphological, developmental, and physiological diversity exhibited by rhizobia-legume symbiosis. For example, *Bradyrhizobium* species are distinct from *Rhizobium* species with regard to the infection mechanism, the relatively broader host range, and the formation of determinate nodules. Therefore, it is very likely that the regulation and function of bacterial determinants, as well as the signal pathways, required for nodulation and nitrogen fixation are quite different in these phylogenetically distinct genera. Elucidation of the molecular mechanisms involved in the *Bradyrhizobium*-legume

symbiosis should, therefore, provide new information on the diversity and specialization of bacterial determinants involved in the nodulation process. One goal of such research is to identify common molecular mechanisms found in prokaryote-plant interactions.

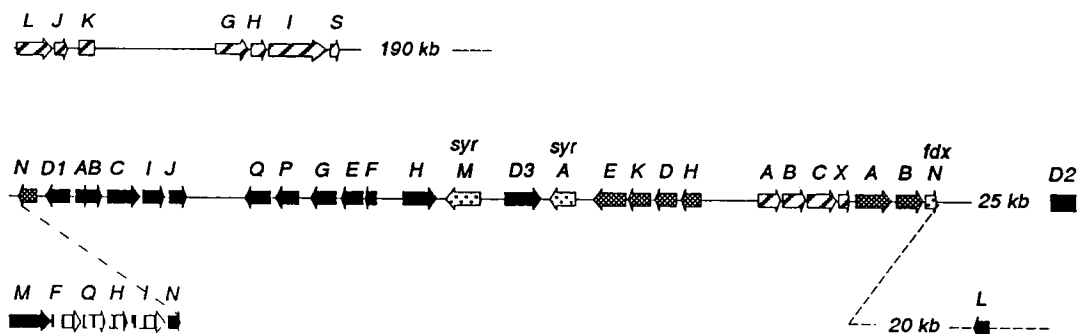
The complexity in the regulatory circuits involved in the regulation of symbiotic gene expression is largely derived from the use of different regulatory proteins to mediate the expression of different subsets of *nod* or *nif/fix* genes in response to specific environmental signals. Recent research has identified several large families of prokaryotic global regulators, such as the LysR family (reviewed by Schell, 1992), the two-component regulatory system (reviewed by Charles et al., 1992), and the recently identified MerR-type regulatory proteins (see below). Although such global regulators are being extensively studied, less is known about how such regulatory systems may interact to integrate the cellular response to the environment. Like in other rhizobia, a LysR-type protein, NodD1, is required in the activation of *B. japonicum nod* gene expression in the presence of plant-produced isoflavones. Unlike other rhizobia, however, recent research in our laboratory has identified additional regulatory proteins which are involved in the regulation of *nod* gene expression. These include NodW, which forms a two-component regulatory system in conjunction with NodV (Sanjuan et al., 1994), and NolaA, which shares sequence homology to the MerR-type regulatory proteins. Therefore, the *B. japonicum*-soybean symbiosis offers an important model system for exploring the cell signalling pathways between host and symbiont, as well as in understanding the integration of regulatory mechanisms involved in the global regulation of cellular metabolism.

CHAPTER II

REVIEW OF RELATED LITERATURE

Extensive research on symbiotic nitrogen fixation has identified and characterized a large number of symbiotic genes that are required for the establishment of effective symbiosis. Such genes can be classified broadly according to the stage in the symbiotic process in which they are required. Genes which are required during the early steps of nodule initiation, such as root hair curling and cortical cell division, are designated as *nod/nol/noe* genes. On the other hand, genes which are required for the later stages of the symbiosis, such as the synthesis and assembly of the bacterial nitrogen fixing apparatus, are designated as *nif/fix* genes. The linkage map of *nod*, *nif*, and *fix* genes of various rhizobia is shown in Figure 1 (Sanjuan et al., 1993). In addition to these two classes of symbiotic genes, more recent research has identified additional genes which function in the intermediate steps of the nodulation process such as infection thread elongation, release of bacteria into plant cells, and bacterial differentiation into mature bacteroids. The first part of this review will present a brief overview of plant function in nodulation, as well as the function and regulation of the different rhizobial symbiotic genes as they relate to the different stages of nodule development. Emphasis will be given to describing the complex regulatory circuits employed by different rhizobia to control the expression of nodulation (i.e. *nod/nol*) genes. The second part of the review will focus on the regulation of *nod* gene expression in *B. japonicum*, with emphasis given to the function of the *nolA* gene.

Rhizobium meliloti RCR2011 plasmid pSym [strain AK631 -----]



R. leguminosarum biovar *viciae* 248 plasmid pRL1JI



Bradyrhizobium japonicum USDA110

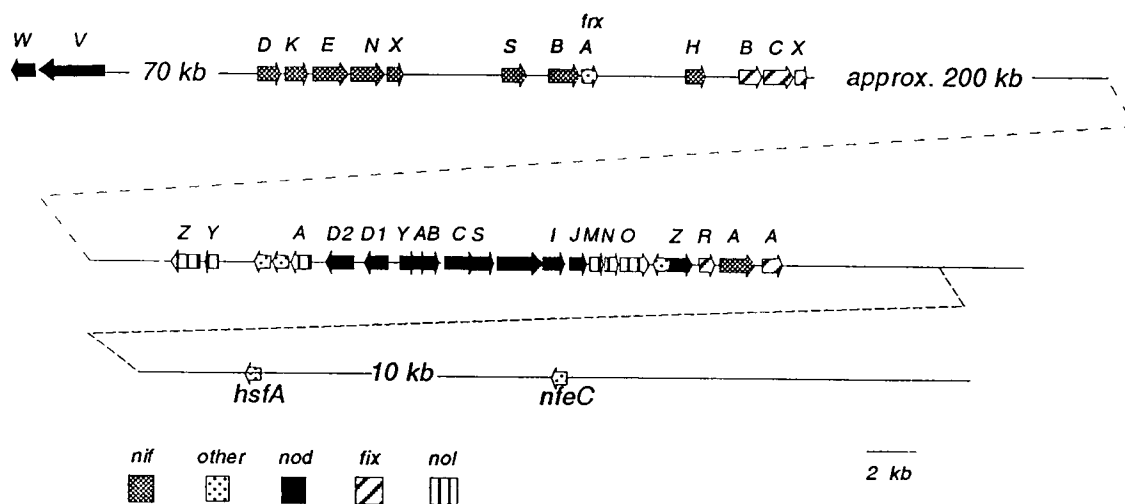


Figure 1: Linkage map of *nif*, *fix* and *nod* genes from various rhizobial species (modified from Sanjuan et al., 1993).

PLANT GENETIC CONTROL OF NODULATION

The observation that specific genotypes of alfalfa can form genuine but non-fixing nodules spontaneously in the absence of *Rhizobium* (termed nodulation in the absence of *Rhizobium*, NAR) illustrates that the entire developmental pathway involved in nodule formation is plant encoded and requires the presence of a particular plant genetic background (reviewed by Caetano-Anolles et al., 1993). It has been suggested that spontaneous nodules may act as a carbon sink, and that normal nodules are modified carbon-storage organs that evolved to include the nitrogen-fixing bacteria (Caetano-Anollés et al., 1993). Plant control of nodule architecture is also demonstrated by the observation that certain *Bradyrhizobium* strains can initiate nodules of different morphologies depending on the host plant. For example, *Bradyrhizobium* strains ANU289 and CP283 induce determinate nodules on siratro, but induce nodules resembling lateral roots in the non-legume *Parasponia* (Scott and Bender, 1990). Another aspect of nodulation that is controlled by the plant is nodule number, also called autoregulation of nodulation or feedback control. Nutman (1952) observed that plants either have few large nodules or many small nodules, indicating that plants possess internal homeostatic mechanisms that control the amount of nodule tissue. The concept of nodulation inhibition was expanded by more recent research (e.g., Bhuvaneswari et al. 1980, 1981; Pierce and Bauer, 1983; Calvert et al. 1984) showing that only the region close to the root tip (i.e. the area containing the emerging root hairs) at the time of inoculation was susceptible to infection. The prior infection of a region of the root prevented further

nodulation in younger regions of the main root, even if accompanied by a second inoculation. Kosslak and Bohlool (1984) have shown, using soybean split root systems, that autoregulation of nodulation is systemic and likely involve the linear transmission of an inhibitor (see below). In addition to feedback control, the degree of nodulation is also inhibited by the presence of nitrate (reviewed by Carroll and Mathews, 1990). Moreover, in addition to nodule architecture and nodule number, the plant also controls key functions necessary for efficient nitrogen fixation, such as carbon and nitrogen translocation, nodule senescence and nodule oxygen supply. The plant function in the control of nodulation and nitrogen fixation, as well as the molecular genetic analysis of nodulation genes in soybean, are reviewed by Gresshoff (1993a, 1993b).

Recent research on plant molecular biology has significantly contributed to the understanding of the plant genetic component necessary for nodulation and nitrogen fixation. Legocki and Verma (1979, 1980) were first to study the tissue-specific expression of plant genes and coined the term nodulins to denote proteins that are nodule-specific. Based on their temporal expression patterns, nodulins are classified as early or late nodulins. Late nodulins are expressed close to the time of the onset of nitrogen fixation by bacteroids. The biochemical function of late nodulins such as leghemoglobin (Lb), glutamine synthetase (GS), and uricase is known (Legocki and Verma, 1979; Verma and Bal, 1976; Verma and Long, 1983). Early nodulins, on the other hand, are expressed prior to the appearance of visible nodules. Unlike late nodulins, the function of early nodulins are not known, and most studies on this class of nodulins are mainly focused on the expression patterns of their gene transcripts. The best characterized early nodulin

genes includes ENOD2, ENOD40 and ENOD12. ENOD2 encodes a hydroxy-proline rich protein which may be a component of the plant cell wall and may function in nodule organogenesis (Franssen et al., 1987; van de Weil et al., 1990; Nap and Bisseling, 1990). ENOD40 expression is rapidly induced by rhizobial or Nod signal inoculation (Kouchi and Hata, 1993) and the transcripts are localized in developing nodules in the pericycle of the root stele and the primary nodule meristem just below the root epidermis. In mature nodules, expression of ENOD40 is localized in the pericycle cells surrounding vascular strands (Kouchi and Hata, 1993). ENOD12, like ENOD2, also encodes a hydroxy-proline rich protein, but the location of its expression differs from that of ENOD2 (Scheres et al., 1990; Journet et al., 1994). There are now reports showing that nodulins are also expressed in other plant organs and tissues. For example, uricase, a nodulin specifically required for ureide synthesis in the uninfected cells of nodules (Kouchi et al., 1989; Nguyen et al., 1985), was also found in soybean embryos (Tajima et al., 1991). ENOD40 has also been found to be expressed in the vasculature of uninfected roots (Kouchi and Hata, 1993). The non-nodule-specificity of nodulin expression raises the possibility of the existence of nodulin multigene families, which express specific members in different organs and cell types. Moreover, these observations also raise the notion that nodulins may have evolved from pre-existing analogous proteins that are utilized during normal plant metabolism and development (Gresshoff, 1993a).

In addition to plant molecular biology, plant genetic techniques have also contributed to the understanding of plant function in nodulation and nitrogen fixation. Several types of symbiotic plant mutants have been identified and these exhibited

phenotypic defects such as non-nodulation, decreased nodulation, supernodulation, or non-nitrogen fixation (reviewed by Caetano-Anolles and Gresshoff, 1991).

Supernodulating mutants exhibit profuse nodulation and a nitrate tolerant nodulation (Nts) phenotype, indicating that they are affected in the autoregulation response. The availability of supernodulating plant mutants, therefore, provided plant geneticists increased experimental conditions to explore in more detail the plant autoregulation of nodulation. Although plant mutants provide experimental means to gain insight into the function and phenotype of the mutation and therefore the affected gene, they do not provide the information on the structure of the gene or its gene product. By using the strategy called positional cloning, the soybean supernodulation (nts) locus was the first nodulation gene to be mapped (see Gresshoff, 1995, for review). However, the nature of the gene corresponding to the soybean nts locus is still to be identified. Genetic strategies which may prove useful in the identification of plant genes involved in nodulation and nitrogen fixation are reviewed recently by Gresshoff et al. (1996).

Based on research findings in plant genetics, as well as in plant cellular and molecular biology, a model for the mechanism involved in autoregulation of nodulation was proposed by Gresshoff (1993). The model, called the auxin burst control (ABC) hypothesis, has two assumptions: (1) auxin negatively regulates nodule formation, and (2) nitrate increases the sensitivity of the plant tissue to auxin. Accordingly, in uninoculated root, cortical cells develop behind the growing root tip and are committed to parenchyma-type differentiation through the exposure to basal levels of auxin. In the absence of bacterial infection, such cells develop into mature root cortex, which is unable to undergo

cell divisions. In the presence of Nod signals produced by the infecting rhizobia, however, cortical cells synthesize high levels of chalcones and flavonoids. This increased levels in chalcone and flavonoids is assumed to inhibit lateral auxin transport from the phloem vessel, thus allowing the induction of cortical cell division below the emerging root hair. This altered auxin balance in the root is subsequently sensed by the plant, which responds by increasing either auxin biosynthesis (presumably in the newly expanding leaves) or auxin translocation to the root. Translocation of auxin into the root cortex then inhibit further cortical cell division, and thus nodulation. Only cell clusters that have advanced sufficiently in size and cytokinin independence, escape the auxin inhibition and develop into nodules. Since nitrate increases the auxin sensitivity of cortical cells, the presence of nitrate makes the cortical cells impervious to the Nod signal-induced decrease in auxin levels, and thus causes reduced nodulation. Supernodulating mutants (i.e. mutations in the *nod* locus) are deficient in auxin burst control and are therefore resistant to nitrate inhibition and are able to develop profuse nodulation.

FUNCTION AND REGULATION OF RHIZOBIAL NODULATION GENES

***nod/nol/noe* Genes**

The nodulation genes (i.e. *nod/nol/noe*) are classified on the basis of having an effect on nodule number, the time course of nodule development, an effect on host range or being regulated by NodD in response to plant-produced flavonoids. Inactivation of

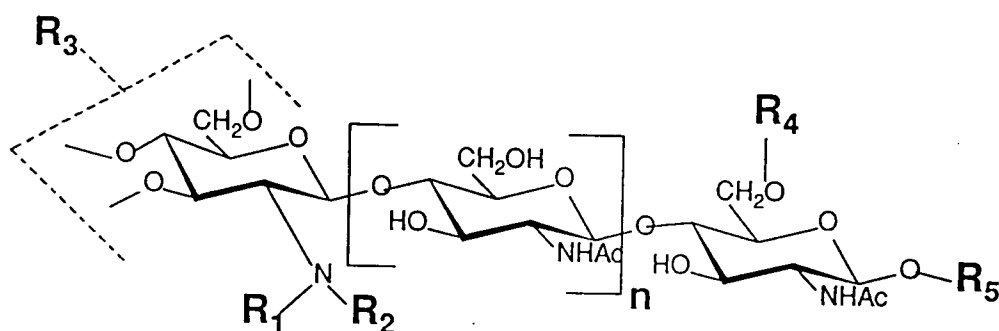
these genes can result in various nodulation phenotypes such as the absence of nodules (Nod⁻), delayed nodulation (Nod^d), or changes in host range. Evidence is now accumulating to suggest that many of the *nod* genes, if not all, are involved directly or indirectly in the production of a “phytohormone” compound, called the Nod signal, which induces root hair curling and cortical cell division. The nodulation genes can be classified into at least three groups (Gottfert, 1993, for review). The common nodulation genes, such as *nodABC*, are found in all rhizobia, and are involved in the biosynthesis of the N-acetylated chitin oligomer which forms the “backbone” of all Nod signals identified so far. The host-specific nodulation genes are not conserved among rhizobia and are responsible for the addition of the various substituents found in Nod signals. Mutations in these genes result in the alteration of the structure and composition of Nod signals produced, leading to modifications in host range. The third group encodes regulatory proteins that control the expression of both common and host-specific nodulation genes. For example, the *nodD* genes are found in all rhizobia and are involved in the flavonoid-dependent expression of the common *nod* genes.

Purification and Characterization of Rhizobial Nod Signals

The purification and characterization of the root deforming factor was first reported for *R. meliloti* by Lerouge et al. (1990). This compound was a tetramer of N-acetylglucosamine, with an N-acylation and acetylation at C-2 and C-6 of the reducing sugar, respectively, and a sulfate group at C-6 of the reducing sugar. The fatty acyl moiety at the non-reducing sugar was identified as C16:2 Δ^{29} . This compound

(subsequently designated as NodRm-1) was active at very low concentrations (i.e. 10^{-11} M) and induced root hair deformation and nodule-like structures on alfalfa but not on the non-host vetch (Lerouge et al., 1990). The *R. meliloti* Nod signal has been chemically synthesized and the synthetic compound has been shown to have the same activity as the compound purified from induced cultures. Such results indicate that NodRm-1 is the bonafide compound which elicits the observed biological effects on alfalfa roots, as opposed to some other contaminating compound present in the purified NodRm-1 factor. The purification and characterization of Nod signals from several rhizobia have been reported during the last few years (Carlson et al., 1994; Dénarié et al., 1996, for recent review). All the Nod signals so far identified consist of a "backbone" of three to five $\beta(1\rightarrow4)$ -linked N-acetylglucosamine residues acylated on the the non-reducing, terminal sugar (Figure 2). Substitutions on the non-reducing sugar include acetylation, carbamylation and N-methylation. Substituents found attached to the reducing, terminal sugar include sulfate, fucose, 2-O-methylfucose, arabinose, acetate, mannose and glycerol.

The fact that Nod signals are composed of an oligosaccharide backbone is interesting in view of the reported role of oligosaccharides in plant development. A class of plant signal molecules, called oligosaccharins (oligosaccharides with regulatory properties), have been characterized from fungal, plant and microbial glycoconjugates. Various oligosaccharins have been shown to activate the plant defense response during infection, to regulate plant hormone response, and affect plant development (Reviewed by Darvill et al., 1989). Likewise, the addition of purified Nod signals to legume roots elicits



<i>Species</i>	R1	R2	R3	R4	R5	n
<i>R. meliloti</i>	H	C16:2 C16:3	Ac(O-6) H	Sulfate	H	1,2,3
<i>R. leg. bv. viciae</i>	H	C18:4 C18:1	Ac(O-6)	H Ac(O-6)	H	2,3
<i>B. japonicum</i>	H	C18:1 C16:0 C16:1	Ac(O-6) H	MeFuc	H	3
<i>B. elkanii</i>	H Me	C18:1	Ac(O-6) H Cb	MeFuc Fuc	H Gro	2,3
NGR 234	Me	C18:1 C16:0	Ac(O-6) H Cb (1,2)	MeFuc AcMeFuc MeFucS	H	3
<i>A. caulinodans</i>	Me	C18:1 C18:0	Ac(O-6) Cb	D-Ara H	H	2,3
<i>R. tropici</i>	Me	C18:1	H	Sulfate	H	3
<i>R. fredii</i>	H	C18:1	H	MeFuc Fuc	H	1,2,3

Figure 2: Structure of Nod signals from various rhizobial species. Abbreviations: Me=methyl; C16:0= palmitic acid; C18:1= vaccenic acid, etc.; Ac= acetyl; Fuc= fucose; MeFuc= methylfucose; Ara= arabinose; Gro= glycerol; Cb= carbamyl; S= sulfate . (This figure is from Carlson, et al., 1994).

several plant developmental responses such as root hair deformation and branching (e.g., Sanjuan et al., 1992; Lerouge et al., 1990), pre-infection thread formation (van Brussel et al., 1992) and cortical cell division (e.g., Sanjuan et al., 1992; Lerouge et al., 1990; van Brussel et al., 1992). Recently, it has been reported that Nod signals can elicit plant cell membrane depolarization (Ehrhardt et al., 1992), changes in the actin cytoskeleton (Allen et al., 1994), rearrangement of the cytoplasmic structures (Allen et al., 1991; Allen et al., 1994) and localized, periodic spikes in cytoplasmic Ca^{+2} levels (Ehrhardt et al., 1996). The ability of Nod signals to trigger these responses at extremely low concentrations (e.g. nM), as well as the specificity of the molecular structure of Nod signals, suggests that highly specific Nod signal receptors are required for recognition. Moreover, such responses also suggest that the host responds to Nod signals via a receptor-mediated signal transduction pathway. However, the nature of the putative receptor and the signal transduction pathway(s) involved still remain to be elucidated.

Functions of Nod Proteins in Nod Signal Biosynthesis and Host-Specific Nodulation

Although numerous early studies had reported the isolation of material from rhizobial cultures that could affect leguminous roots, the first indication of the involvement of *nod* genes in this process came from the work of van Brussel and colleagues (1986). The subsequent elucidation of the Nod signal structure has allowed greater insights as to the possible functions of common and host-specific *nod* gene products. For example, the presence of the N-acetylglucosamine backbone and the fatty acid moiety in all Nod signals so far examined pointed to the involvement of the common

nod genes in the synthesis of these structures. The functions of the *nodABC* gene products have recently been established by using radiolabelled precursors and cell free extracts (Geremia et al., 1994; John et al., 1993; Rohrig et al., 1994). NodC has been shown to be an N-acetylglucosaminyl transferase and thus is involved in the oligomerization of N-acetyl glucosamine monomers (Geremia et al., 1994). NodB deacetylates the terminal non-reducing N-acetylglucosamine residue of chitooligosaccharides (John et al., 1993) and NodA is involved in the N-acylation of the deacetylated oligosaccharide precursor (Rohrig et al., 1994). The products of *nodIJ* share sequence similarity to a family of ATP-binding, membrane-associated transport proteins. It is not known how Nod signals are transported from the cell, although NodI and NodJ are proposed to be involved in the process (Higgins et al., 1986; Schlaman et al., 1990; McKay and Djordjevic, 1993). The various substitutions present in Nod signals, as well as the modifications of the fatty acid moieties, are presumably added by the products of the host-specific genes and are important for host specificity. For example, the NodH, NodP and NodQ proteins of *R. meliloti* are involved in the addition of the sulfate group to the Nod signal produced by this rhizobium (Roche et al., 1991). Strains mutated for *nodH* produce a Nod signal which lacks the sulfate group and are consequently unable to nodulate alfalfa, but are able to nodulate the non-host vetch (Roche et al., 1991). The *nodE* and *nodF* genes of *R. meliloti* and *R. leguminosarum* bv. *viciae* are believed to be involved in the biosynthesis of the highly unsaturated C16 and C18 fatty acids, respectively, found in the Nod signals produced by these rhizobia (Demont et al., 1993). NodE shares sequence similarity to β -ketoacylsynthases (Bibb et al., 1989), whereas NodF has a 4'-phosphopantetheine

prosthetic group analogous to that of the acyl carrier proteins involved in fatty acid biosynthesis (Geiger et al., 1991). *R. leguminosarum* bv. *viciae* strains mutated for *nodEF* produce Nod signals having a mono-unsaturated fatty acid, instead of the highly unsaturated fatty acid group present in the wild-type factor (Demont et al., 1991). Such mutations result in altered host range, again indicating the importance of the Nod signal structure in host specificity. In addition to receptor recognition (see above), it was also proposed that the various substituents found in Nod signals are important in protecting the molecule against degradation by host chitinases (Staehelin et al., 1994a; 1994b). The proposed functions of rhizobial *nod* genes in the biosynthesis and export of Nod signals, as well as in host-specificity, was reviewed recently by Carlson et al. (1994) and Denariè et al. (1996).

Regulation of *nod* Gene Expression

Activation of *nod* gene expression by NodD: Activation of *nod* gene expression requires specific plant-produced flavonoid molecules, the NodD protein and a conserved 47-bp *cis*-acting element found in the promoters of all host-inducible *nod* operons so far examined (reviewed by Gryorgypal et al., 1992; Schlaman et al., 1992b). The *cis*-acting element is called the *nod* box and is the site at which NodD binds to the flavonoid-inducible *nod* promoters (e.g., Rostas et al., 1986; Fisher et al., 1988). To account for the above mentioned requirements, a generalized model for *nod* gene expression was proposed. Accordingly, plant-produced flavonoids interact with NodD, which then undergoes conformational change and binds to the *nod* box. Binding of NodD to the *nod*

box subsequently leads to transcription of the respective genes. The regulation of *nod* gene expression by NodD has been reviewed by Schlaman et al. (1992b) and Gyorgypal et al. (1992). As will be discussed below, recent research has identified additional factors involved in *nod* gene expression, indicating that the regulatory circuits involved are more complex than that envisioned by the above model.

The *nodD* alleles from various rhizobial species are highly conserved and share 70-90% homology with each other. The NodD protein shares sequence similarity to the LysR family of bacterial regulatory proteins (reviewed by Schell, 1992). Sequence comparison of NodD proteins from various rhizobia showed significant sequence similarity at their N-terminal portions, whereas the C-terminal portions are variant. The N-terminal portion of NodD contains a helix-turn-helix DNA binding domain and a "receiver" motif which was proposed to be involved in protein-protein interactions (McIver et al., 1989; Kofoed and Parkinson, 1988). The C-terminal portion of NodD is believed to be involved in flavonoid recognition (e.g., Burn et al., 1987; Horvarth et al., 1987; McIver et al., 1989). The requirement for recognition of specific flavonoids may be the basis for the observed sequence divergence among different NodDs in their C-terminus. However, biochemical evidence showing that NodD interacts directly with flavonoid molecules is still lacking. So far, most of the existing evidence to support flavonoid-NodD interaction comes from mutational and domain-swapping experiments. For example, signal specificity (i.e. flavonoid recognition and host range properties) can be transferred between different rhizobia by exchanging parts of NodD (e.g., McIver et al., 1989; Spaink et al., 1989). Since NodD and flavonoids have been localized in the cell membrane, it has been proposed

that the interaction occurs in this compartment with NodD subsequently translocated into the cytoplasm to activate *nod* gene expression (Recourt et al., 1989; Schlaman et al., 1989). Proteins that can reversibly associate with the cell membrane are called amphitropic proteins (Burn, 1988). The sequence of the NodD protein predicts a hydrophobic α -helix which is presumed to enable NodD to associate reversibly with the cell membrane (Schlaman et al., 1989).

The number of the *nodD* alleles, as well as the effect of *nodD* mutation on nodulation, can vary depending on the rhizobial species examined. For example, *R. leguminosarum* bv. *viciae* and *Azorhizobium caulinodans* have only one *nodD* gene (i.e. *nodD1*), and *nodD* mutants are completely Nod⁻ (Downie et al., 1985; Goethals et al., 1990). In contrast, *R. meliloti* strains 1021 and AK631 have three *nodD* genes (i.e. *nodD1*, *nodD2*, and *nodD3*). Mutation in any one of these *nodD* homologs altered nodulation efficiency in a host-dependent manner, whereas mutations that eliminate the three *nodD* alleles resulted in a Nod⁻ phenotype (e.g., Göttfert et al., 1986; Honma et al., 1990). In addition to variations in the number of *nodD* alleles, different rhizobial species also exhibit differences in the mechanism by which the expression of the *nodD* alleles are regulated. In general, the *nodD* genes are expressed constitutively (Gyorgypal et al., 1992). However, a variety of regulatory mechanisms have been shown to affect this constitutive expression in a species-dependent manner. For example, *nodD* of *R. leguminosarum* bv. *viciae* is negatively autoregulated (Rossen et al., 1985), whereas the *nodD1* of *R. leguminosarum* bv. *phaseoli* is positively autoregulated in response to flavonoids (Davis and Johnston, 1990). The differential expression of different *nodD*

alleles is best demonstrated in *R. meliloti*. In this rhizobium, *nodD1* is constitutively expressed whereas *nodD2* is also constitutively expressed but at a lower level than *nodD1* (Mulligan and Long, 1989). In contrast, *nodD3* expression is regulated in a complex manner involving NodD1, NodD2, NodD3, and SyrM (Mulligan and Long, 1989; Mailett et al., 1990; Kondorosi et al., 1991b). It is proposed that the existence of multiple *nodD* alleles, as well as the differences in the regulation of their expression, contributes to the ability of the rhizobia to nodulate multiple hosts. Accordingly, multiple NodD proteins provide the rhizobia with the versatility to respond and recognize subtle differences in flavonoid patterns produced by various hosts and/or differences in environmental and metabolic conditions.

As previously mentioned, a 47-bp conserved sequence, called the *nod* box, is found upstream of flavonoid-inducible *nod* genes. A consensus sequence for the *nod* box was proposed, consisting of three highly conserved regions of 7, 5, and 25 bp (5' to 3') (Rostas et al., 1986). The NodD proteins of *R. meliloti* and *R. leguminosarum* bv. *viciae* have been shown to bind the *nod* box sequences *in vitro* (Fisher et al., 1988; Hong et al., 1987; Goethals et al., 1992). Like other LysR proteins, NodD binds to the *nod* box independently of the inducer flavonoid (e.g., Fisher et al., 1988), although in some cases, flavonoids can enhance DNA binding ability (e.g., Goethals et al., 1992). Based on *in vitro* footprinting experiments, it was proposed that NodD binds to the *nod* box as dimer or tetramer (Fisher and Long, 1989; Goethals et al., 1992). For rhizobial strains that have more than one *nodD* allele, it is unknown whether heteromers between different NodD proteins can form.

Other Regulatory Proteins: In addition to NodD, other regulatory proteins also participate in the positive or negative regulation of *nod* gene expression. For example, several strains of *R. meliloti* encode a gene, *syrM* (symbiotic regulator) whose predicted product shares sequence similarity (ranging from 30-60%) to the NodD proteins (Mulligan and Long, 1989). SyrM is involved in the flavonoid-independent expression of *nod* genes, as well as in optimum nodulation of the host plant vetch. Moreover, expression of *syrM* and *nodD3* from a multi-copy plasmid resulted in constitutive expression of *nod* genes in *R. meliloti* and a consequent extension of host-range to siratro (Kondorosi et al., 1991a). In addition to increased constitutive expression of *nod* genes, SyrM was also found to enhance extracellular polysaccharide production in *R. meliloti* (Mulligan and Long, 1989). In *B. japonicum*, a homologue of *syrM* is yet to be identified. However, this rhizobium, in addition to NodD1, makes use of another regulatory protein, NodW, to activate *nod* gene expression (Sanjuan et al., 1994). The regulation of *nod* gene expression in *B. japonicum* will be discussed in more detail in the later part of this review.

A repressor protein, NolR, has been identified in certain strains of *Rhizobium meliloti* (e.g., in strain AK631), but not in strain 1021 (Kondorosi et al., 1989). NolR has been shown to bind to a conserved 26 bp sequence in the overlapping *nodD1*, *nodD2* and *nodABC* promoters. The binding site of NolR coincides with the transcriptional initiation sites of these genes, thereby inhibiting transcription (Kondorosi et al., 1991b). Most *R. meliloti* strains and field isolates encode this repressor and the repressor-producing strains are slightly more efficient in alfalfa nodulation. Moreover, mutations in *nolR* resulted in higher expression of *nod* genes in *R. meliloti* strain AK631 leading to a lower nodulation

efficiency. Therefore, it was proposed that negative control of *nod* gene expression is required for optimum nodulation, presumably by fine-tuning the induction of *nod* genes and thus preventing the over-production of Nod signals (Kondorosi et al., 1989).

The expression of inducible *nod* genes is repressed in bacteroids, with the inhibition occurring before the bacteria are released from the infection thread (Schlaman et al., 1991). In *R. leguminosarum*, high expression of *nodABCIJ* from a constitutive promoter has been shown to impair nodulation and abolish nitrogen fixation (Knight et al., 1986). Therefore, the synthesis of bacterial signal molecules, although important during the early stages of nodule initiation, is harmful in bacteroids. The repression of *nod* genes in bacteroids is apparently not due to the absence flavonoids, which were found to be present in nodules. Instead, it was proposed that NodD is present in an inactive form in bacteroids and cannot activate *nod* gene expression. There is also evidence suggesting that a bacteroid-specific repressor is involved in *nod* gene repression (Schlaman et al., 1992a). So far, details on how *nod* genes are repressed in bacteroids are mostly unknown.

Other Factors That Affect Nodule Initiation

Although Nod signals are the primary bacterial determinants for successful infection, nodulation competency can also be limited by other factors. For example, genetic traits that enable the bacteria to survive and colonize the roots of host plants can affect nodulation ability. *R. meliloti* is able to metabolize plant-produced betaines, such as trigonelline and stachydrine (Goldman et al., 1991; Boivin et al., 1991). These compounds apparently induce *nod* gene expression through NodD2 (Phillips et al., 1992).

However, the concentrations required to activate *nod* gene expression are higher relative to flavonoids, thus raising the possibility that these compounds are important as a C and N source, instead of acting as signals. The uptake system for stachydrine was also reported to function in the protection of the rhizobia from osmotic stress (Fougere and Le Rudulier, 1990). Murphy et al. (1987) have identified *R. meliloti* genes that synthesize opine-like compounds in nodules. These compounds are believed to be secreted and stimulate rhizosphere growth of *R. meliloti* strains that have the capacity to metabolize opine-like compounds. A group of rhizosphere-expressed genes (i.e. *rhiABCR*) have been identified in *R. leguminosarum* bv. *viciae* and have been shown to influence nodulation ability (Cubo et al., 1992). The biochemical role of the *rhi* genes has not been established, but it is proposed that they allow the bacteria to metabolize a plant-made metabolite. Recently, it has been reported that RhiR regulates the expression of *rhiABC* in conjunction with an autoinducer molecule, N-acylated homoserine lactone (Gray et al., 1996). In addition to the ability to metabolize plant-produced substrates, rhizobial colonization of plant roots can also be influenced by bacterial chemotaxis towards root exudates (e.g., Barbour et al., 1991; Maxwell and Phillips, 1990), motility (Ames and Bergman, 1981; Mellor et al., 1987) and the ability to attach to host plant roots (Smit et al., 1987; 1989; reviewed by Roth and Stacey, 1991; Loh et al., 1993). It is believed that these bacterial determinants are especially important in the soil (as opposed to laboratory conditions), where the rhizobia encounter various environmental stresses.

The presence of exogenous ammonia has been reported to inhibit *nod* gene expression, as well as nodule initiation (Dusha et al., 1989; Wang and Stacey, 1990). In

R. meliloti, repression of *nod* gene expression by ammonia appears to be mediated by a complex mechanism involving the general nitrogen regulatory system (e.g., NtrC and NtrR), NodD3 and SyrM (Dusha et al., 1993). In *B. japonicum*, the mechanism by which ammonia controls *nod* gene expression is largely unknown, but it has been established that it is independent of the general nitrogen regulatory system (Wang and Stacey, 1990). Dicarboxylic acids (Yuen and Stacey, 1996) and riboflavin (Mellor and Rosendahl, 1994) have also been reported to repress the expression of *nod* genes in *B. japonicum*. Although it is unknown if these types of control have any relevance to nodule initiation, it is proposed that repression of *nod* genes in bacteroids is possibly mediated by the abundance of plant-produced dicarboxylic acids in the nodule.

FUNCTION AND REGULATION OF RHIZOBIAL NITROGEN FIXATION GENES

Function of *nif* and *fix* Genes

The biological reduction of atmospheric nitrogen to ammonia involves an energy-demanding process (i.e. reduction of 1 mole of N_2 to ammonia requires up to 40 moles of ATP) and is catalyzed by nitrogenase (Hill, 1992). Nitrogenase is a metalloenzyme complex composed of two separate components. Component I (also designated as MoFe protein or dinitrogenase) is a tetramer composed of two pairs of nonidentical subunits (α and β). It contains two types of metal centers that are involved in the redox reaction of the N_2 reduction process. The metal centers are organized as four [4Fe:4S] clusters and

two identical FeMo cofactors (FeMoCo). Component II (also called Fe protein or dinitrogenase reductase) is a dimeric protein with one [4Fe:4S] cluster and serves as an electron donor to component I. During catalysis, the Fe protein binds two MgATP molecules, is reduced, associates with and donates a single electron to the MoFe protein. This reduction reaction is coupled to ATP hydrolysis. Nitrogenase can also reduce ethylene (C_2H_2) to acetylene (C_2H_4) (Dilworth et al., 1966; Schollhorn and Burris, 1967). Acetylene reduction activity (ARA) assays are frequently employed to measure nitrogenase activity. In this assay, acetylene and ethylene are separated and quantitated by gas chromatography (Dillworth et al., 1966; Schollhorn and Burris, 1967).

The nitrogen fixation genes are required for the fixation of atmospheric nitrogen and are designated as *nif* or *fix* genes. Mutants defective in any of these genes are able to produce nodules but the resulting nodules do not fix nitrogen (i.e. Nod^+Fix^- phenotype). Rhizobial *nif* genes are homologous to the known *nif* genes of the free-living nitrogen fixing bacterium *Klebsiella pneumoniae* (Arnold et al., 1988) The *fix* genes, on the other hand, are essential for nitrogen fixation but do not have a homologous counterpart in *K. pneumoniae*. The *nif* and *fix* genes are generally organized in distinct clusters whose structure and genomic location are species dependent (Sanjuan et al., 1993). For example, the *nif* and *fix* genes in *R. meliloti* are located in a megaplasmid (i.e. pSym-a) and are organized in several clusters. In contrast, the *nif* and *fix* genes of *B. japonicum* are more closely-linked and are located on the chromosome.

Genetic techniques such as mutagenesis, hybridization with the known *nif* genes, sequence analysis of DNA regions closely linked to known *nif* genes, or mapping of DNA

regions identified by hybridization to bacteroid RNA, have been used to identify *nif* and *fix* genes. The products of *nif* genes are involved in the biosynthesis of dinitrogenase (e.g., *nifDK*; Corbin et al., 1983; Kaluza and Hennecke, 1984; Deneffe et al., 1987), dinitrogenase reductase (e.g., *nifH*; Furhmann and Hennecke, 1984; Norel and Elmerich, 1987) and co-factors necessary for the functions of these proteins (e.g. *nifENB*; Aguilar et al., 1990; Donald et al., 1986). The biochemical functions of the *fix* gene products are generally unknown. Based on sequence homology to other proteins, the *fix* gene products have been proposed to function in bacteroid respiration and electron transport. For example, the FixNOQP proteins are believed to constitute a membrane-bound, cytochrome c-containing heme/copper cytochrome oxidase which may be required to support bacteroid respiration under the conditions of low oxygen present in root nodules (Preisig et al., 1993; Mandon et al., 1993). The FixABCX proteins have been proposed to be involved in electron transport to nitrogenase (Earl et al., 1987; Gubler and Hennecke, 1986).

Regulation of *nif/fix* Gene Expression

The expression of *nif/fix* genes is induced in response to a dramatic environmental change that the bacteria undergo during infection of the plant. For example, the concentration of free oxygen inside the nodule has been reported to be 3-30 nM (Witty and Minchin, 1990; Hunt and Layzell, 1993), as opposed to the 250 μ M (approximate) dissolved oxygen concentration present in standard aerobic cultures. The expression of rhizobial nitrogen fixation genes is controlled by cascades of hierarchically organized

regulatory proteins encoded by *fixL*, *fixJ*, *fixK*, *nifA*, and *rpoN* (reviewed by Fischer, 1994). Their concerted action enables the bacteria to sense optimal environmental conditions required for nitrogen fixation and transduce this information to the level of gene expression. The regulatory activity of these proteins are integrated differently in different rhizobial species, resulting in a species-dependent activation of respective *nif/fix* genes. There are at least two regulatory cascades that control the expression of nitrogen fixation genes in response to low levels of oxygen. The cascade involving NifA controls the expression of *nif/fix* genes in conjunction with RpoN (or σ^{54}). The second cascade involves FixL and FixJ that together form a two-component regulatory system.

It is proposed that NifA, upon sensing the cellular redox state via its cysteine-rich domain, binds to a conserved sequence which is present in most NifA-dependent promoters (Fischer et al., 1988; Hennecke, 1990). This conserved sequence (5'-TGT-N10-ACA-3') is called the upstream activator sequence (UAS) and is located 80 to 50 nucleotides from the transcriptional start site (Alvarez-Morales et al., 1986; Buck et al., 1986; Morrett and Buck, 1988). Binding of NifA to the UAS facilitates the binding of the σ^{54} -RNA polymerase complex to the promoter (Buck et al., 1987). Under aerobic conditions, NifA is expressed at low levels in an apparently inactive form (Kim et al., 1986; Thony et al., 1989). Enhanced expression of active NifA is positively autoregulated and occurs under microaerobic, anaerobic and symbiotic conditions (Ditta et al., 1987; Thony et al., 1989). In addition to the UAS, NifA-dependent promoters contain conserved -12/-24 sequences (5'-TGGCAC-N5-TTGCA/T-3') which are recognized by σ^{54} (Morrett and Buck, 1989; Adams and Chelm, 1984; Buck et al., 1987) as well as

sequences (5'-WATCAAN4TTR-3') inferred to be the binding site of integration host factor (IHF) (Craig and Nash, 1984; Hoover et al., 1990). There is evidence to suggest that the interaction of UAS-bound NifA with the σ^{54} -RNA polymerase complex involves loop formation which is facilitated by IHF (Santero et al., 1989). Examples of genes controlled by NifA include the *R. meliloti nifHDKN* and *fixABCX* genes and the *B. japonicum nifDKENX* and *fixBCX* genes (Aguilar et al., 1987; Earl et al., 1987; David et al., 1988).

FixL is a membrane-bound hemoprotein that has kinase/phosphatase activity (de Phillip et al., 1992; Gilles-Gonzales et al., 1991). The cellular oxygen conditions are sensed by the heme moiety bound to FixL, which in turn modulates its kinase and phosphatase activities. Under low oxygen conditions, FixL is autophosphorylated at a histidine residue (Gilles-Gonzales and Gonzales, 1993; Lois et al., 1993; Monson et al., 1993). By analogy to other two-component regulatory systems, it is proposed that the phosphate in FixL is subsequently transferred to an aspartate residue in FixJ, which then activates gene expression. The binding site of FixJ to the *nif/fix* promoter is unknown, although a common element [C(C/G)NAAT(A/T)T] has been identified in FixJ-activated promoters (e.g., Agron et al., 1992; Batut et al., 1989; Anthamatten and Hennecke, 1991). In *R. meliloti* and *B. japonicum*, the FixLJ cascade activates the expression of another regulatory protein, FixK, which in turn regulates the expression of *nif/fix* genes (e.g., *fixNOQP*, *fixGHIS*). In *R. meliloti* (but not in *B. japonicum*), the FixLJ cascade also activates the expression of NifA (Monson et al., 1993; David et al., 1988).

BACTERIAL DETERMINANTS RELATED TO NODULE INVASION AND BACTEROID MATURATION

Although much is known about the bacterial determinants required during the early (i.e. *nod/nol/noe* genes) and late (i.e. *nif/fix* genes) events of the symbiotic process, relatively little is known about the functions required for the intermediate steps of nodule development. These steps include infection thread elongation, bacterial release into plant host cells, and bacterial maturation into bacteroids. Recent studies show that the genes that specify these functions are likely involved in the signal exchange between the invading bacteria and the plant. For example, the biosynthesis of "surface factors", such as exopolysaccharides (EPS; reviewed by Leigh and Walker, 1994), lipopolysaccharides (LPS; Perotto et al., 1994; Carlson et al., 1987; Stacey et al., 1991; de Maagd et al., 1989) and cyclic β -(1,2) glucan (Dylan et al., 1986; Dylan et al., 1990) has been found to be required for proper infection thread elongation. It has been proposed that these "surface factors" may play a role in the avoidance by the invading bacteria of the plant defense response (Perotto et al., 1994). Recently, a gene (i.e. *bacA*) has been identified in *R. meliloti* which is required for bacterial maturation within the plant cell (Glazebrook et al., 1993). Strains carrying mutations in *bacA* are able invade nodules and are released from infection threads but senesce in the plant cell cytoplasm shortly after release from infection threads. The *bacA* gene has been found to be a homolog of an *E. coli* gene whose product may function in the transport of thiazole ring-containing peptide antibiotics. Therefore, it was proposed that BacA may transport some unknown plant signal peptide

molecule which is required for bacterial differentiation upon release into the plant cell cytoplasm.

Genes which are involved in bacterial metabolism have also been reported to be important for nodule development. Dicarboxylic acids and amino acids are provided by the host plant to the bacteroids as carbon, energy and nitrogen sources (Streeter and Salminen, 1985). Therefore, genes that are involved in the transport of these compounds are necessary for nitrogen fixation. For example, mutations in the *dct* genes which are required for the transport of C4-dicarboxylic acids result in a Nod⁺Fix⁻ phenotype (Finan et al., 1983; Ronson et al., 1981; Humbeck and Werner, 1989). A *R. meliloti* strain which is auxotrophic for leucine has been reported to be defective in the release of bacteria from the infection thread (Truchet et al., 1980). A similar symbiotic phenotype has also been reported for a strain mutated for *hemA*, the gene encoding for δ -aminolevulinic acid synthase which is involved in heme biosynthesis (Dickstein et al., 1991). The mutation in *hemA* causes a very strict auxotrophy for δ -aminolevulinic acid and may block further bacterial growth in planta. Alternatively, the *hemA* mutant may result in the inability of the bacteria to signal the plant to initiate the later stages of bacterial development. Within the context of the second possibility, heme itself or another molecule containing a tetrapyrrole ring could be the signal required for nodule maturation. Recently, a gene required for anthranilate biosynthesis in *R. meliloti*, *trpE*, has been reported to be important in nodule development (Barsomian et al., 1992). The *trpE* gene encodes for anthranilate synthase, the first enzyme in the tryptophan biosynthetic pathway. Mutations in *trpE* resulted in the formation of two types of nodules. The first type contains

bacteroids at its base and is capable of nitrogen fixation although the nodule morphology is different from the normal. The second nodule type lacks bacteroids and cannot fix nitrogen. Interestingly, *R. meliloti* strains carrying mutations in genes necessary for the later steps in the tryptophan biosynthetic pathway (e.g., *trpD*, *trpF*, etc.) formed effective nodules Barsomian et al., 1992). Moreover, *aro* mutants which are blocked in aromatic amino acid biosynthesis at steps prior to anthranilate synthesis, also produced a similar phenotype as the *trpE* mutant (Barsomian et al., 1992). Therefore, it appears that normal nodulation of alfalfa by *R. meliloti* requires bacterial synthesis of anthranilate but not of tryptophan. It has been proposed that anthranilate or some factor derived from anthranilate is required for bacterial maturation or that anthranilate is acting as a siderophore in planta which enables the bacteria to cope with the high demand for iron (Barsomian et al., 1992).

In addition to their inability to fix nitrogen, *nifA* mutant strains in several rhizobia (e.g., *R. meliloti* and *B. japonicum*) are characterized by the induction of nodules that show an altered morphology and ultrastructure (Fischer et al., 1986; Hirsch and Smith, 1987). These mutants induce an increased number of small nodules containing fewer bacteroids that degenerate prematurely. In these nodules, there is a striking accumulation of the phytoalexin glyceollin I in the area of degenerated infected cells (Parniske et al., 1991), which is reminiscent of a hypersensitive response normally observed in incompatible plant-pathogen interactions (Klement, 1982). The concept that rhizobium-legume symbiosis is a controlled disease has been previously proposed (Vance, 1983; Djordjevic, et al., 1987) and is supported by recent reports showing that rhizobia induce

the expression of certain plant defense response genes such as phenylalanine ammonia lyase (Estabrook and Sengupta-Gopalan, 1991) and chalcone synthase (Grosskopf et al., 1993). Therefore, it appears that NifA controls functions that help the bacterial symbiont to overcome potential plant defense reactions and to persist as a nitrogen fixing bacteroid. Moreover, in agreement with the idea that NifA controls the expression of bacterial functions other than those required for nitrogen fixation, it has been reported that NifA is required for the expression of genes (i.e. *nfe1* and *nfe2*) involved in nodulation efficiency and competitiveness (Sanjuan et al, 1989; Sanjuan et al., 1991). Mutations in other nitrogen fixation regulatory genes such as *fixLJ* (Anthamatten and Hennecke, 1991) and *rpoN* (Kullik et al., 1991) also result in the formation of ineffective nodules containing few bacteroids which senesce prematurely. As is the case with NifA, FixLJ and RpoN may also control other yet unknown genes required for bacteroid maturation and persistence. Moreover, the idea that these nitrogen fixation regulatory proteins are sensing signals in addition to low oxygen levels inside the nodule is still an open possibility.

Recently, a host-specific nitrogen fixation (*hsf*) gene has been identified in *B. japonicum*. This gene, *hsfA*, is required for nitrogen fixation in cowpea but not in soybean (Chun et al., 1994). Mutations in *hsfA* elicited nodule formation but microscopic analysis of the nodule formed showed premature senescence of bacteroids. The function of the *hsfA* gene product is still unknown. Earlier reports on host-specific nitrogen fixation have been made in several rhizobia-legume interactions (e.g., Trinick, 1980; Bromfield and Barran, 1990; Stacey et al., 1982). In these cases, however, the genes

involved in the observed phenotype were not identified.

REGULATION AND FUNCTION OF *B. japonicum* *nod* GENES

Bradyrhizobium japonicum USDA 110 nodulates leguminous plants such as soybean (*Glycine max* and *Glycine soja*), cowpea (*Vigna unguiculata*), mungbean (*Vigna radiata*), and siratro (*Macroptilium atropurpureum*). Pigion pea (*Cajanus cajan*) is also nodulated, but ineffectively (Barbour et al., 1992). Recent research in our laboratory has shown that the regulation of *nod* gene expression in *B. japonicum* is quite complex. This rhizobium makes use of both a LysR-type regulator, NodD1, and a two-component regulatory system, NodVW, to activate the expression of *nod* genes in response to plant-produced isoflavone signals. Repression of *nod* gene expression, on the other hand, is mediated by a third regulatory protein, NolA.

Regulatory Proteins involved in *nod* Gene Expression

The NodD1 protein. As in other rhizobia, the induction of *nod* genes (e.g., *nodD1* and *nodYABCSUIJnolMNO*) in *B. japonicum* is dependent on the presence of plant-produced isoflavones (e.g., genistein) and the NodD protein (Banfalvi et al., 1988; Wang and Stacey, 1991; Göttfert et al., 1992). *B. japonicum* possesses two *nodD* genes arranged tandemly as *nodD1nodD2* (Götfert et al., 1992). A deletion mutation of *nodD1nodD2* can be phenotypically complemented by *nodD1* alone, but not by *nodD2* (Götfert et al., 1992). Therefore, it appears that NodD1 is the only functional NodD

protein in *B. japonicum*. The *nodYABCSUIJnolMNO* operon is transcribed 28 bps 3' of a consensus *nod* box sequence (Wang and Stacey, 1991). However, *nodD1* is transcribed 44 bps 3' of a divergent *nod* box sequence which is homologous to the *nodYABC* *nod* box in its 3' end but not in the 5' half (Wang and Stacey, 1991). Other divergent *nod* box-like sequences has also been reported by Göttfert et al. (1989) for *B. japonicum* and by Goethals et al. (1992) for *Azorhizobium caulinodans*. To account for the existence of divergent *nod* box sequences, Wang and Stacey (1991) proposed a consensus sequence for the *nod* box consisting of paired 9-bp repeats. Accordingly, most *nod* box sequences (e.g. *nodYABC* promoter) consist of four repeats, whereas the divergent *nod* boxes (e.g., *nodD1* promoter) possess only two repeats. It was also suggested that the presence of two 9-bp repeats in the *nodD1* promoter may explain the lower flavonoid-inducible expression of this gene, as compared to *nodYABC*. In addition to the model proposed by Wang and Stacey (1991), Goethals et al. (1992) pointed out the conservation of two inverted repeats with the sequence ATC-N₉-GAT, within the known *nod* box sequences. Both of these models predict the binding of multimeric NodD and have the T-N₁₁-A motif proposed to be a general feature of DNA binding sites for LysR-type proteins (Goethals et al., 1992). However, the *nod* box sequence 5' of the *B. japonicum* *nodD1* gene lacks the inverted repeats described by Goethals et al. (1992). In addition to the *nodD1* and *nodYABC* operons, an isoflavone-inducible operon, *nolYZ*, has been recently identified in *B. japonicum*. A *nod* box sequence was also found 5' of the *nolY* coding region. Expression of the *nolYZ* operon was found to be dependent on NodD1, as well as NodW (Dockendorff et al., 1994b).

The NodVW two-component system. As previously mentioned, NodD1 is required for the isoflavone-dependent expression of *nod* genes in *B. japonicum*. However, mutations in *nodD1* only slightly affect nodulation (Nieuwkoop et al., 1987). Therefore, a paradox existed in *B. japonicum* in that the product of the *nodD1* gene is essential for *nod* gene induction and yet mutations in this gene did not affect nodulation. This paradox was resolved by the discovery that a two-component regulatory system, NodV and NodW, can also activate *nod* gene expression (Göttfert et al., 1990; Sanjuan et al., 1994). Göttfert et al. (1990) reported that a *B. japonicum* strain carrying a mutation in *nodW* is unable to nodulate cowpea, siratro, and mungbean, while retaining the ability to nodulate soybean. Subsequently, Sanjuan et al. (1994) showed that deletion of *nodW* and *nodD1* drastically reduced the nodulation ability on all host legumes. Moreover, the expression of the common *nod* genes (i.e. *nodYABC*) from a constitutive promoter restored the ability of the *nodW* mutant strain to nodulate all host plants. Taken together, these results indicated that the NodVW two component system constitutes an alternative pathway, in addition to NodD1, by which plant-produced isoflavones are able to induce the expression of *nod* genes in *B. japonicum*.

A two component regulatory system consists of a sensor protein coupled to a regulatory protein (reviewed by Charles et al., 1992). The sensor protein is usually membrane-bound and can sense environmental signals. On sensing the appropriate signal, the sensor protein autophosphorylates at a histidine residue and transduces the signal by transferring the phosphate to a conserved aspartate residue on its cognate regulator protein. The activated regulatory protein can then bind to the promoter of its target gene

and activate transcription. By sequence analysis, it has been proposed that NodV is a sensor protein that can respond to plant-produced signals and that NodW is its cognate regulator protein (Göttfert et al., 1990). Indeed, recent results in our laboratory have shown that NodV can be phosphorylated *in vitro* and that the phosphorylated NodV can subsequently transfer the phosphate to NodW at a conserved aspartate residue (Loh et al., unpublished data). More importantly, it has been shown that NodW can be phosphorylated *in vivo* upon the addition of genistein and that phosphorylation of NodW is essential for both *nod* gene induction and nodulation (Loh et al. unpublished data).

Repression of *nod* gene expression by Nola. In addition to NodD1 and NodVW, a third regulatory protein, Nola, has been reported to mediate the regulation of *nod* gene expression in *B. japonicum*. As will be discussed in more detail later, the predicted N-terminal helix-turn-helix DNA-binding motif of Nola shares sequence similarity to the MerR family of regulatory proteins (Sadowsky et al., 1991). Unlike NodD1 and NodVW, which activate *nod* gene expression in response to plant-produced isoflavones, Nola has been proposed to act as a negative regulator of *nod* gene expression. Dockendorff et al. (1994a) found that a *B. japonicum* strain deleted for *nodD1*, *nodD2* and *nola* (i.e. strain Bj1267-573) showed wild type levels of expression of a chromosomally-encoded *nodC-lacZ* fusion. This result is unexpected since the induction of *nod lacZ* expression requires both NodD1 and NodW (Sanjuan et al., 1994). Therefore, to explain these results, it was suggested that a gene encoding a repressor (i.e. *nola*) had been deleted in strain Bj1267-573 that allowed for full expression of *nod* genes via the NodVW pathway. To test this hypothesis, a plasmid encoding *nola* was mobilized into

strain Bj1267-573, as well as into strain Bj110-573, a wild type *B. japonicum* harboring a similar chromosomally-encoded *nodC-lacZ* fusion. As predicted, expression of *nolA* from a multi-copy plasmid resulted in a drastic reduction in *nodC-lacZ* expression. However, the reduction in *nodC-lacZ* expression was more pronounced in the wild type background (i.e. Bj110-573) than in the *nodD1nodD2nolA* background (i.e. Bj1267-573). This could indicate that a gene product present in the wild type, but not in the mutant strain, was acting with NolA to affect repression. As previously mentioned, *B. japonicum* encodes two tandem copies of *nodD*. Although NodD2 shares 62% amino acid identity to NodD1, its function in nodulation, if any, has yet to be demonstrated (Göttfert et al., 1992). Moreover, unlike *nodD1* whose expression has been shown to be induced by isoflavones, nothing is known on how *nodD2* expression is regulated. Recently, it has been suggested that homologues of NodD2 in *Bradyrhizobium* sp. (*Arachis*) NC92 (Gillette and Elkan, 1996), as well as in *Rhizobium* sp. NGR 234 (Broughton, personal communication), may act negatively to regulate *nod* gene expression. Therefore, it would be interesting to determine if NodD2 also acts as a repressor in *B. japonicum*. Moreover, it is important to determine if NolA mediates *nod* gene repression by directly binding to the promoters of *nod* genes or indirectly by regulating the expression of other proteins, such as NodD2.

The importance of a negative regulatory pathway for *nod* gene expression was first elucidated in *R. meliloti*. In this rhizobium, a repressor, NolR, binds to *nod* promoters at a site overlapping the RNA polymerase binding site blocking transcription (Kondorosi et al., 1991). Repression of *nod* gene expression is important for optimum nodulation (Kondorosi et al., 1989). As in *R. meliloti*, the *nolA* gene of *B. japonicum* is important

for optimum nodulation (Dockendorff et al., 1994a). Therefore, like NodR, NodA may be involved in the fine control of *nod* gene expression and thus the amount of Nod signals produced by *B. japonicum*.

Proposed Model for *nod* Gene Regulation in *B. japonicum*.

Based on recent research in our laboratory, the following model for the regulation of *nod* gene expression in *B. japonicum* was proposed: NodD1 can interact with isoflavones and bind to the *nod* box sequences found upstream of the *nodD1*, *nodYABCSUIJnolMNO* and *nolYZ* operons activating the transcription of these genes. In addition to NodD1, the membrane-bound NodV can also recognize isoflavone plant signals and transduces this signal via phosphorylation of NodW. The phosphorylated NodW, in turn, activates the expression of nodulation genes. The NodD1 and NodVW systems can complement each other for the nodulation of soybean, while the NodVW system is required for the nodulation of other legume hosts such as mungbean, cowpea, and siratro. To explain this host-specificity, the model predicts that soybean can produce isoflavones that can interact with either NodD1 or NodV, whereas cowpea, mungbean and siratro produce isoflavones that are specifically recognized by NodVW. Not much is known concerning how NodA fits into this model. It is possible that NodA, like NodR, binds to the promoters of *nodD1* and *nodYABC*, thus preventing the transcription of these genes. However, it is also conceivable that NodA does not act directly on these promoters, but rather mediates *nod* gene repression indirectly through other proteins.

Production of Nod signals in *B. japonicum*

Induction of the *B. japonicum* *nod* genes by soybean seed extract or genistein results in the synthesis and secretion of a Nod signal having a pentameric oligo-chitin backbone, substituted at the reducing end with 2-O-methylfucose and acylated with a C18:1 fatty acid at the non-reducing end [i.e. NodBjV (C18:1, MeFuc)] (Sanjuan et al., 1992). Lesser amounts of an acetylated version of this Nod signal are also produced. The effects of mutations in a number of *nod/nol* genes on the production of Nod signals have been studied. Mutations in *nodB* or *nodC* resulted in the absence of any detectable Nod signal synthesis (Sanjuan et al., 1992). These results reflect the proposed function of these common *nod* genes in the synthesis of the lipo-chitin oligosaccharide backbone. Mutation of the *nodZ* gene resulted in the production of a Nod signal lacking the 2-O-methylfucose substituent and an inability to nodulate siratro. However, the *nodZ* mutant retained the ability to nodulate soybean (Stacey et al., 1994). Indeed, transfer of the *nodZ* gene of *B. japonicum* to *Rhizobium leguminosarum* bv *viciae*, which produces Nod signals which are not modified by 2-O-methylfucose resulted in the production of fucosylated Nod signals (Lopez-Lara et al., 1996). The *Rhizobium leguminosarum* biovar *viciae* strain expressing NodZ gained the ability to nodulate non-host legumes such as siratro, soybean and cowpea. Luka et al. (1993) identified three genes, *noIMNO*, which are part of the *nodYABCSUIJ* transcript. Analysis of the Nod metabolites produced by a *nolO* mutant strain indicated that NodO may play a role in Nod signal fucosylation in conjunction to NodZ (Luka et al., 1993). However, the sequence of *nolO* shares significant similarity to *nodU*. The *nodU* gene of *A. caulinodans* and *Rhizobium* sp.

NGR234 has been suggested to encode a carbamoyl transferase (Broughton, personal communication). Therefore, the role of Nolo in Nod signal biosynthesis has yet to be determined.

Involvement of *nolA* in Genotype-Specific Nodulation of Soybean

Competition between inoculant strains and indigenous rhizobia is generally recognized as a major obstacle to the goal of improving legume productivity via inoculation with improved rhizobia. This is referred to as the rhizobium competition problem and several laboratories are using molecular genetic approaches to address this problem. For example, efforts have been made to identify host and bacterial determinants that will improve the competitive ability of inoculum strains (reviewed by Bottomley, 1991; Triplett and Sadowsky, 1991). Cregan and Keyser (1986) identified several soybean genotypes which restrict nodulation by *B. japonicum* strain SD6-1c, a representative of the USDA123 serogroup. Two soybean genotypes (Plant Introduction lines, PI377578 and PI371607) were found to restrict nodulation by strain SD6-1c, as well as by 20 other *B. japonicum* USDA123 strains (Keyser and Cregan, 1987). Sadowsky et. al. (1991) investigated the genetic basis of this nodulation restriction. DNA from strain USDA 110 (not restricted on PI377578) was transferred into strain SD6-1c by conjugation. Transconjugants were then tested for nodulation on permissive (i.e. cultivar Williams) and non-permissive (i.e. PI377578) soybean lines. A single gene present in strain USDA110, *nolA*, when transferred to strain SD6-1c, enabled this strain to nodulate the non-permissive soybean. The mechanism by which *nolA* mediates genotype-specific

nodulation is still unclear. One hypothesis is that *NolA* regulates the expression of genes involved in the production of specific Nod signals which are recognized by certain soybean genotypes. However, the effect of *nolA* on Nod signal production has not been examined. Moreover, it remains to be tested whether *nolA* is indeed the sole bacterial determinant present in *B. japonicum* USDA110 that allows it to overcome the nodulation restriction offered by the restricting soybean lines.

NolA Belongs to the MerR Family of Bacterial Regulatory Proteins

The *nolA* coding sequence predicts a protein having an N-terminal helix-turn-helix DNA binding motif which shares sequence similarity to the conserved DNA-binding domains of the MerR family of regulatory proteins (Figure 3). This family includes the MerR proteins from various bacterial species (Summers, 1992), *Streptomyces lividans* TipA_L (Holmes et al., 1993), *E. coli* SoxR (Amabile-Cuevas and Demple, 1991; Wu and Weiss, 1991), and *Bacillus subtilis* BmrR (Ahmed et al., 1994). Members of this protein family have been shown to bind to the promoters of the genes that they regulate and to activate transcription upon binding of an inducer molecule. These inducer molecules appear to interact with the C-terminal domain of the protein. For example, the C-terminal domains of MerR and SoxR show some sequence similarity around cysteine residues involved in binding the metal ligands mercury and iron, respectively (Helman et al., 1990; Hidalgo and Demple, 1994). The C-terminal region of TipA does not contain cysteine residues, but this domain has been shown to bind the ligand thiostrepton (Holmes et al., 1993). Likewise, the C-terminal domain of BmrR is thought to bind various inducers,

		Helix-Turn-Helix Motif	
		← 000 →	
BmrR	8	IGEVSKLANVSIKALRYYDKIDLFKPAYVDPDTSYRYYTDSQLIHLDLIKSLKYIGTPLEEM	
MerR	10	IGELADKCGVNKETIRYYERLGLIPEP_ERTEKGYRMYSQQTVDRLHFIKRMQELGFTLNEI	
TipA_L	5	VGQVAGFAGVTVRTLHHYDDIGLLVPS_ERSHAGHRRYSADLDRLQQILFYRELGFPLDEV	
NolA	13	IGELAEATGVTVRTLHHYEHTGLLAAT_ERTEGGHRMYDRESGQRVHQIRALRELGFSLVEI	
SoxR	14	PGEVAKRSGVAVSALHFYESKGLITSI_RNSGNQRRYKRDVRLRYVAIIKIAQRIGIPLATI	
Consensus		-G-----V-----Y----L-----R-Y-----I-----G-----	

Figure 3: Comparison of the N-terminal amino acid sequence of the MerR-type regulatory proteins. Residues identical in all five sequences are indicated in the consensus sequence. A block of ten amino acid residues found within the NolA and TipA_L helix-turn-helix regions is boxed. The number indicate the amino acid number corresponding to the first residue in each sequence.

including rhodamine (Ahmed et al., 1994).

MerR, SoxR, TipA_L and BmR are functionally similar in that they are all involved in controlling the expression of genes that provide resistance to toxic compounds. MerR strongly induces the expression of the mercury resistance genes encoded by the *mer* operon in response to the presence of Hg(II) and represses the expression of these genes in the absence of Hg(II). MerR also acts as an autorepressor of its own expression, both in the presence or absence of Hg(II). The action of the *mer*-encoded proteins results in the reduction of Hg(II) to metallic Hg(0) which is chemically inert and volatile (reviewed by Misra, 1992). SoxR controls the first step of a redox-sensitive superoxide stress pathway (Amabile-Cuevas and Demple, 1991; Greenberg et al., 1990; Nunoshiba et al., 1992; Wu and Weiss, 1991). Reduction of the SoxR iron-sulfur center by an intracellular redox signal, such as superoxide or nitric oxide, activates SoxR, which in turn induces *soxS* transcription. SoxS then activates the transcription of at least nine additional genes whose products defend the cell against oxidative stress and may also confer cellular resistance to antibiotics and activated macrophages (Greenberg et al., 1993; Nunoshiba et al., 1993; Tsaneva and Weiss, 1990). The expression of the *tipA* gene of *S. lividans* has been shown to be autogenously regulated in response to the presence of the peptide antibiotic thiostrepton (Holmes et al., 1993). The *tipA* gene encodes two proteins. TipA_L contains the N-terminal helix-turn-helix DNA-binding motif and TipA_S is N-terminally truncated and lacks this region. The synthesis of TipA_L and TipA_S is controlled translationally by the choice of two alternative ATG start codons (Holmes et al., 1993). The normal physiological function of TipA is still unclear, although it has been proposed

to be involved in the biosynthesis of hormone-like peptide(s) which may play a role in actinomycete differentiation. The most recently identified member of the MerR family is BmrR (Ahmed et al., 1994). BmrR activates the transcription of the *bmr* gene that encodes a multidrug transporter. More than 20 different inducers of BmrR have been identified (e.g., rhodamine, kanamycin, benzylamine, etc.). These chemicals are all toxic to the cell and have in common the fact that they can be transported by the multidrug transporter (Ahmed et al., 1994).

The promoter binding sites for MerR, SoxR, TipA_L, and BmrR have been determined (Heltzel et al., 1987; Hidalgo and Demple, 1994; Holmes et al., 1993; O'Halloran et al., 1989; O'Halloran and Walsh, 1987). These proteins bind to a site between the -10 and -35 consensus sequences within the promoters of the genes that they regulate. The promoters have the unique feature that the -10 and -35 regions are separated by 19-bp, not the normal 16-17 bp. In each case, this 19 bp region contains an inverted repeat. Binding of the activator protein to the region of dyad symmetry results in the distortion of the spacer DNA. This distortion alters the relative separation and orientation of the -10 and -35 promoter regions, allowing them to interact more effectively with the RNA polymerase. Indeed, *in vitro* biochemical analysis has shown that Hg(II)-MerR binding to its target DNA induces a 33-degree unwinding of the DNA helix, allowing alignment of the -10 and -35 promoter regions (Ansari et al., 1992). Moreover, deletions within the *mer* promoter to give spacing of 17-18 bp between the -10 and -35 regions resulted in a high level of constitutive gene expression that did not require MerR (Parkhill and Brown, 1990). Likewise, analysis of mutant MerR proteins that can activate

transcription in the absence of Hg(II) showed that the degree of DNA distortion they cause upon binding is correlated to their transcriptional activity (Parkhill et al., 1993).

RATIONALE FOR PRESENT WORK

The *B. japonicum nola* gene predicts a DNA-binding protein that shares sequence similarity to the MerR family of regulatory proteins. This gene was initially identified by its ability to mediate genotype-specific nodulation of soybean (Sadowsky et al., 1991). Subsequently, Dockendorff et al. (1994a) reported that Nola is involved in the repression of *nod* gene expression and is important for optimum nodulation. However, the lack of a *B. japonicum nola* mutant strain has precluded more detailed functional studies on the role of *nola* in symbiotic nitrogen fixation. In the present work, two *B. japonicum* strains mutated for *nola* were constructed. Using these strains, the importance of *nola* to the ability of *B. japonicum* to nodulate its plant hosts and fix dinitrogen was investigated. In addition to these functional studies, the mechanism by which *nola* expression is regulated at the transcriptional and translational levels was also determined. The sequence similarity of Nola to the MerR-type regulatory proteins suggests that it functions as a transcriptional regulatory protein. The results presented in this present work strongly support this hypothesis and a DNA sequence to which Nola may bind was identified.

CHAPTER III

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

All bacterial strains and plasmids used in this study are listed in Table 2. Details on strains and plasmids generated in this study are given in the text.

CULTURE MEDIA AND GROWTH CONDITIONS

Bradyrhizobium japonicum strains were grown at 30°C in modified RDY medium (So *et al.* 1987) for routine growth and plant tests. HM salt medium (Cole and Elkan, 1973) supplemented with 0.1% arabinose was used for growth of *B. japonicum* when conducting matings, extracting nucleic acids, and obtaining cell lysates for Western Blot analysis. Minimal medium (Bergersen, 1961) was used for β -galactosidase activity assays and production of Nod metabolites. For routine growth, *E. coli* strains were cultured in LB or M9 medium (Sambrook *et al.* 1989) at 37°C. M9 medium (Sambrook *et al.* 1989) supplemented with 0.5% (w/v) casamino acids was used for β -galactosidase activity assays of *E. coli* cells. The following antibiotics were used for plasmid selection: with *E. coli*, ampicillin (Ap, 200 μ g/ml), kanamycin (Km, 30 μ g/ml), tetracycline (Tc, 25 μ g/ml), streptomycin (Sm, 30 μ g/ml) and spectinomycin (Sp, 100 μ g/ml); with *B. japonicum*,

Table 2: Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Source or Reference
Bacterial strains		
<i>B. japonicum</i>		
USDA110	wild type	USDA, Beltsville, MD
USDA123	wild type	USDA, Beltsville, MD
BjD21	<i>nolA</i> , Sp ^r , Sm ^r	This study
BjB3	<i>nolA</i> , Sp ^r , Sm ^r	This study
Bj110-573	USDA110, chromosomal <i>nodC-lacZ</i> fusion, Tc ^r	Dockendorff et al., 1994a
Bj1221	Bj110-573 harboring pTE3Ω	This study
BjtrpD2-1	Bj110-573 harboring pBGtrpD2-1	This study
Bj613	<i>nodW</i> , Sp ^r , Sm ^r	Göttfert et al., 1990
Bj586	<i>nodD1</i> , Km ^r	Göttfert et al., 1992
<i>E. coli</i>		
JM109	<i>endA1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> (r _K ⁻ , m _K ⁺), <i>relA1</i> , <i>supE44</i> , (<i>lacproAB</i>) [F', <i>traD36</i> , <i>proAB</i> , <i>lacI^qZ</i> M15]	Messing 1983
S17-1	RP4 2-Tc::Mu-Km::Tn7 <i>pro</i> , <i>hsdR</i> , <i>recA</i>	Simon et al. 1983
HMS174(DE3)pLyS	F ⁻ , <i>recA</i> , r _{K12} ⁻ , m ⁺ , DE3 lysogen, <i>plysS</i> plasmid, Cm ^r , Rif ^r	Studier et al. 1990

Table 2 (continued)

Strain or plasmid	Relevant characteristics	Source or Reference
Plasmids		
pRK2013	RK2, Tra ⁺ , Km ^r	Figurski and Helinski 1979
pRK290	RP4, Mob ⁺ Tc ^r	Ditta et al., 1980
pTE3	Tc ^r , <i>trp</i> promoter	Egelhoff and Long 1985
pTE3Ω	Tc ^r , Sp ^r /Sm ^r cassette, <i>trp</i> promoter	This study
pUC19	Ap ^r	Yanisch-Perron et al. 1985
pBluescriptII SK ⁺	Ap ^r	Stratagene
pRSETB	Ap ^r , T7 promoter	Invitrogen, San Diego, CA
pNM480	Ap ^r , promoterless <i>lacZ</i>	Minton 1984
pNM482	Ap ^r , promoterless <i>lacZ</i>	Minton 1984
pHP45Ω	Sp ^r /Sm ^r , Ω cassette	Prentki and Krisch 1984
pSUP202	RP4 <i>mob</i> , Tc ^r , Ap ^r , Cm ^r	Simon et al. 1983
TD310	3.0 kb <i>EcoRI</i> - <i>Bam</i> H1 fragment clone of <i>nodD1nodD2</i> in pUC18, Ap ^r	T. Dockendorff
pZB22	<i>nodD1-lacZ</i> , Tc ^r , Ap ^r	Banfalvi et al., 1988

Table 2 (continued)

Strain or plasmid	Relevant characteristics	Source or Reference
pZB32	<i>nodY-lacZ</i> , Tc ^r , Ap ^r	Banfalvi et al., 1988
pRJ1248	<i>nodD2-lacZ</i> , Tc ^r	Góttfert et al. 1992
pBGAlac1	<i>nolA-lacZ</i> , Ap ^r	This study
pBGAlac1-H	<i>nolA-lacZ</i> , Ap ^r	This study
pBGAlac1-EX	<i>nolA-lacZ</i> , Ap ^r	This study
pBGAlac14	<i>nolA-lacZ</i> , Ap ^r	This study
pBGAlac4	co-integrate of pBGAlac1 and pRK290, Tc ^r , Ap ^r	This study
pBGAlac36	cointegrate of pBGAlac14 and pRK290, Tc ^r , Ap ^r	This study
pBG301	4.5 Kb <i>EcoRI-PstI</i> fragment with Ω insertion in <i>nolA</i> cloned into pSUP202, Sm ^r , Sp ^r , Tc ^r	This study
pBG303	4.5 Kb <i>EcoRI-PstI</i> fragment with deletion and Ω insertion in <i>nolA</i> cloned into pSUP202, Sm ^r , Sp ^r , Tc ^r	This study
pBGtrpA-88	1.2 Kb <i>SalI-BglII</i> clone of <i>nolA</i> in pTE3, Tc ^r	This study
pBGtrpD2-1	1.4 Kb <i>BamHI-SalI</i> clone of <i>nodD2</i> in pTE3 Ω , Tc ^r , Sm ^r , Sp ^r	This study
pBG24	1.2 kb <i>SalI-BglII</i> fragment clone of <i>nolA</i> cloned into pUC19, Ap ^r	This study
pBGT7-A2	<i>nolA</i> clone in pRSETB, Ap ^r	This study

Table 2 (continued)

Strain or plasmid	Relevant characteristics	Source or Reference
pBG101	4.5 kb <i>EcoRI-PstI</i> fragment clone of <i>nodD2nolA</i> in pUC19, Ap ^r	This study

kanamycin, streptomycin, spectinomycin and tetracycline (100 µg/ml), and chloramphenicol (Cm, 30 µg/ml).

ENZYMES, ISOTOPES AND DNA PRIMERS

Unless otherwise specified, all enzymes used in this study were purchased from Promega (Madison, WI) or Amersham Life Science Inc. (Arlington Heights, IL), and were used following the protocols specified by the manufacturer. Radioisotopes were obtained from ICN Biomedical (Irvine, CA). Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

GENETIC TECHNIQUES

Plasmid transformations into *E. coli* cells were done as described by Sambrook et al. (1989). Tri-parental matings between *E. coli* donors and *B. japonicum* recipients were done as follows: *B. japonicum* strains, as recipient, were grown in HM medium at 30° C until an OD₆₀₀ of 0.5-1.0 was reached. *E. coli* donors and helper (containing pRK2013) strains were grown overnight in LB medium. One ml of each culture was pelleted using a microfuge at 14,000 rpm for 1 min. The cells were resuspended in 1 ml of HM medium and re-centrifuged. Cells were resuspended in 1 ml HM medium, mixed, and spotted onto sterile 0.2 µM filter discs which were placed on the surface of HM agar plates. Plates were then incubated for 48 hrs at 30°C. Cells were suspended in 1 ml RDY medium and

were plated onto RDY medium containing the appropriate antibiotics. As controls, recipients, donors and helpers were also plated individually. In cases where the donor *E. coli* strain is S17-1, biparental matings were done as described above, except that the helper strain was not included in the mating.

DNA ISOLATION AND MANIPULATION

Plasmid Isolation from *E. coli*

Plasmids from *E. coli* were isolated using the alkaline lysis method as described in Sambrook et al. (1989) or by using the Magic™ Miniprep DNA Purification System (Promega Corporation, Madison, WI).

Chromosome Isolation from *B. japonicum*

Chromosomal DNA from *B. japonicum* was isolated as follows: Cultures were grown in HM medium until late logarithmic phase. Three ml of the culture were pelleted and the pellet was resuspended in 1.5 ml of 0.5% Sarkosyl in TNE buffer (Sambrook et al. 1989). The cells were again pelleted and resuspended in 0.45 ml of TE buffer (Sambrook et al. 1989). To the resuspended cells, 0.15ml of 5% SDS solution in TE buffer and 0.15 ml of a 2.5 mg/ml solution of Proteinase K was added, mixed and incubated at 37°C for 1 hr. The solution was extracted with phenol/chloroform 3-5 times, each time mixing the extraction mixture by gently vortexing for 20-40 secs. The liquid phase containing the DNA was then precipitated by adding two volumes of ethanol.

Isolation of DNA Fragments

DNA restriction endonuclease fragments used in sub-cloning were isolated by electrophoresis in agarose gels as described by Sambrook et al. (1989), followed by excision from the gel and purification of the DNA fragment using the Geneclean II kit (Bio 101 Inc., La Jolla, CA).

Southern Blotting and Hybridization

Chromosomal DNA (5 µg) was cut with the appropriate restriction endonuclease and electrophoresed in an agarose gel. Transfer of the electrophoresed DNA to Nylon filters (Amersham Life Science Inc., Arlington Heights, IL) was done as described by Sambrook et al. (1989). All prehybridization and hybridization steps were carried out following the protocol specified by Amersham. The filters were washed at high stringency as follows: twice (15 mins each time) at 65°C with 2X SSC, once for 30 mins at 65°C with 2XSSC, 0.1% SDS, and finally for 10 mins at 65°C with 0.1X SSC. The filters were air-dried and autoradiographed with Kodak X-OMAT AR film at -80°C. Radioactive probes were labelled with [α -³²P]dATP using the Prime-a-Gene® System of Promega (Madison, WI).

DNA Sequencing

DNA sequences were determined by the dideoxy chain termination method (Sanger et al. 1977) using oligonucleotide primers labelled with either [γ -³²P]ATP or [α -

³²P]dATP. Double strand sequencing was done using either the deltaTaq™ Cycle Sequencing Kit or the Sequenase™ T7 polymerase from United States Biochemical (Cleveland, OH) following the manufacturer's recommended protocol. DNA templates for sequencing were prepared using the Magic™ Miniprep DNA Purification System (Promega Corporation, Madison, WI). Preparation of polyacrylamide sequencing gels, electrophoresis, gel fixation and autoradiography were done as described by Chun (1994).

PLASMID CONSTRUCTIONS

Construction of Plasmids Encoding *nolA-lacZ* Fusions

The N-terminal *nolA-lacZ* fusion encoded in pBGAlac1 was constructed by cloning the 1.5 Kb *Bam*HI fragment from pTD320 containing *nolA* into the *Bam*HI site of pNM480 (Minton, 1994). Two additional plasmids were constructed to delete *nolA* sequences 5' of the *lacZ* fusion site of pBGAlac1. pBGAlac1-H was constructed by cutting pBGAlac1 with *Hind*III, and ligating the fragment containing the *nolA-lacZ* fusion into the *Hind*III site of pNM480. To construct pBGAlac1-EX, pBGAlac1 was cut with *Xba*I and *Eco*RI, releasing promoter sequences and the 5' terminus of the *nolA* coding region. The fragment containing the truncated *nolA-lacZ* fusion was filled-in with Klenow DNA polymerase, then self-ligated. In addition to the C-terminal *nolA-lacZ* fusions, a plasmid encoding an N-terminal *nolA-lacZ* fusion was constructed as follows: A 596 bp *Nhe*I fragment containing the promoter and 5' terminus of the *nolA* coding region was blunt-ended by filling-in the 5' overhanging ends with Klenow DNA

polymerase. This fragment was ligated into the *Sma*I site of pNM482 (Minton, 1984) to give pBGAlac14. The *nolA-lacZ* in-frame fusion site in pBGAlac1 or pBGAlac14 was confirmed by DNA sequence analysis using the dideoxynucleotide chain termination method of Sanger et al (1977). To conjugate pBGAlac1 or pBGAlac14 into *B. japonicum*, these plasmids were co-integrated with the conjugative plasmid pRK290 (Ditta et al 1980) by restricting pBGAlac1 or pBGAlac14 with *Eco*RI and ligating into the *Eco*RI site of pRK290. The resulting co-integrate (PBGAlac4 or pBGAlac36) was transformed into *E. coli* S17-1 (Simon et al., 1983) and then mobilized into different *B. japonicum* strains by biparental mating as described by Banfalvi *et al.* (1988).

Construction of Plasmids Expressing *nolA* or *nodD2* from the *trp* Promoter

Plasmid pBGtrpA-88 was constructed in order to express *nolA* from the *trp* promoter of pTE3 (Egelhoff and Long, 1985). This plasmid was constructed by isolating a 1.2 Kb *Sal*I-*Bgl*II fragment containing *nolA*, filling-in the 5' overhanging ends with Klenow DNA polymerase and then adding *Nsi*I linkers [5'-d(TGCATGCATGCA)-3', United States Biochemical, Cleveland, OH). After digestion with *Nsi*I, this fragment was ligated into the *Pst*I site of pTE3. Plasmid pBGtrpD2-1 expresses *nodD2* from the *trp* promoter of pTE3 and was constructed as follows: A 1.4 kb *Bam*HI-*Sal*I fragment encompassing *nodD2* was cloned into the *Bam*HI-*Sal*I site of pUC19. The resulting plasmid was cut with *Bam*HI and *Pst*I and the fragment containing *nodD2* was cloned into the *Bam*HI-*Pst*I site of pTE3Ω. pTE3Ω was constructed by inserting the Sp/Sm cassette of pHP45Ω into the *Bgl*II site of pTE3. Gene expression from the *trp* promoter was

determined by RNA slot-blot hybridization.

Construction of a Plasmid Expressing a His-Tagged Nola Fusion Protein from a T7 Promoter

To produce high amounts of Nola for antibody production, the polyhistidine-tag system was utilized to overexpress Nola as a fusion protein from the T7 promoter of the expression vector pRSETB (Invitrogen, San Diego, CA). The plasmid used in the overexpression of Nola was constructed as follows: Based on the *nola* sequence, oligonucleotide primers containing *Bgl*II (5'-GGAGATCTGAACAGAGCTACACCAA-3', for coding strand sythesis) or *Eco*R1 ((5-TAGAATTCGTCAGTAAGGCTGATCC-3', for non-coding strand synthesis) restriction sites were used to PCR-amplify the *nola* coding region. The amplified fragment was isolated, blunt-ended with Klenow DNA polymerase, phosphorylated using T4 polynucleotide kinase, and blunt-end ligated using T4 ligase to form concatemers. After digestion with *Bgl*II and *Eco*RI, the fragment was cloned into the *Bam*H1-*Eco*RI sites of pRSETB. The resulting plasmid (pBGT7A-2) was transformed into *E. coli* HMS174(DE3)(pLysS) which harbors a chromosomal T7 RNA polymerase under the control of the *lac* promoter (Studier et al., 1990). The in-frame translational fusion in pBGT7A-2 was confirmed by DNA sequence analysis using the dideoxynucleotide chain termination method of Sanger et al. (1977).

CONSTRUCTION OF *B. japonicum nola* MUTANT STRAINS BY INTERPOSON MUTAGENESIS

The *nola* mutant strain BjD21 was constructed as follows: A 4.5 Kb *EcoRI-PstI* fragment encompassing *nola* was cloned into pUC19 (Yanisch-Perron et al., 1985). This plasmid (pBG101) was cut at the *XbaI* site internal to the *nola* coding region and the 5' overhanging ends were blunted with Klenow DNA polymerase. A 2.0 Kb *SmaI* fragment containing the Sp/Sm resistance cassette of pHP45 Ω was ligated to the *XbaI* site. The resulting plasmid was cut with *EcoRI-PstI* to release the pUC19 vector and the fragment carrying the mutated *nola* was ligated to the *EcoRI-PstI* site of pSUP202. The resulting plasmid (pBG303) was transformed into *E. coli* S17-1 and conjugated into *B. japonicum* USDA 110. Transconjugants expressing Sp/Sm resistance and Tc sensitivity, indicating double cross-over events, were selected. The mutation in BjD21 was confirmed by Southern blot hybridization.

The second *nola* mutant strain, BjB3, was constructed as follows: pBG101 was cut with *NheI*, releasing a 600 bp fragment containing sequences 5' to the *nola* start codon and the 5' terminus of the *nola* coding region. The larger *NheI* fragment was filled-in with Klenow DNA polymerase and ligated to the 2.0 Kb *SmaI* fragment containing the Sp/Sm resistance cassette of pHP45 Ω . The resulting plasmid was cut with *PstI-EcoRI* to release the pUC19 vector and the fragment containing the Ω insertion was ligated to the *PstI-EcoRI* site of pSUP202. The resulting plasmid (pBG301) was transformed into *E. coli* S17-1 and conjugated into *B. japonicum* USDA 110. Marker exchange events were

selected as above and confirmed by Southern hybridization.

RNA ISOLATION AND MANIPULATION

RNA Isolation

Extraction of RNA from *B. japonicum* was done as follows: Cells were grown in HM or RDY medium containing the appropriate antibiotics until an OD₆₀₀ of 0.8-1.0 was reached. Cells were diluted to an OD₆₀₀ of 0.25 in 100 ml HM or RDY medium and soybean seed extract (prepared as described by Smit et al., 1992) was added at 20 ul/ml. Cells were incubated at 30°C with shaking for another 6 hrs and then pelleted. Cells were resuspended in 4 ml of solution containing 20 mM sodium acetate (pH 5.5) and 1 mM EDTA. Once the cells were completely resuspended, SDS was added to a final concentration of 0.5%. An equal volume of hot phenol (65°C) equilibrated with 20 mM sodium acetate (pH 5.5) was added, mixed, and incubated in a 65°C water bath for 5 mins. Organic and aqueous phases were separated by centrifugation and the aqueous phase was then mixed with phenol/chloroform/isoamyl alcohol (25:24:1) solution. The RNA in the aqueous phase was precipitated by the addition of two volumes of ethanol and 0.1 volume of 3M sodium acetate (pH5.2), incubated overnight at -20°C, and centrifuged. The resulting RNA pellet was resuspended in 0.1X TE and then treated with RNase-free DNaseI to remove contaminating DNA. The amount of RNA yield was measured by reading the OD₂₆₀ (Sambrook et al., 1989, for quantitation of RNA) and the integrity of the RNA was examined by agarose gel electrophoresis.

RNA Dot Blot Hybridization

RNA (5-20 µg) was blotted onto Nylon filters (Amersham Co., Arlington Heights, IL) as described by Sambrook et al. (1989). All the steps in the pre-hybridization, hybridization and washing of Nylon filters were done at 65°C as described by Sambrook et al. (1989). Radioactive DNA probes were prepared using the Prime-a-Gene® System of Promega (Madison, WI).

PRIMER EXTENSION

The transcriptional start sites of *nolA* and *nodD2* were determined by primer extension as described by Chun and Stacey (1994). The following oligonucleotide primers were used: for *nolA*, 5'-d(GTGTGCTCATAATGGTGCAGCGT)-3' (23-mer) and 5'-d(GTTACTCCGGTCGCCTCTGCAA)-3' (22-mer) which are complementary to bases +76 to +96 and +47 to +68, respectively; and for *nodD2*, 5'-d(GCTAATTGGTCTTGCCGGTTCCG)-3' (23-mer) and 5'-d(GCAGATCAGCCCAGTGTTTCGTCA)-3' (23-mer), which are complementary to bases -221 to -199 and -280 to -258, respectively. The bases are numbered such that +1 is the first base in the *nolA* or *nodD2* coding region. End-labelling of the primers was carried out for 30 mins at 37°C in a 20 µl reaction solution containing 10 pmole of primer DNA, 320 µCi of [γ -³²P]ATP, 10 U of T4 polynucleotide kinase, and 1x buffer as suggested by the supplier (USB, Cleveland, OH). The kinase reaction was stopped by incubating at 65°C for 5 mins. The unincorporated [γ -³²P]ATP was removed by precipitating the DNA

twice by the addition of 200 μ l ethanol and 2 μ l of 3M sodium acetate (pH 5.2) at -80°C for 20 mins. The labelled primer was resuspended in 25 μ l of sterile deionized water. Approximately 1×10^6 cpm of the labelled primer was added to 50 μ g of RNA and precipitated by the addition of 0.3M sodium acetate and ethanol. The pellet was washed with 70% ethanol and resuspended in 30 μ l of hybridization buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.1 mM EDTA). The hybridization mixture was denatured at 95°C for 3 mins, incubated at 63°C for 1.5 hrs, and cooled slowly to $35-40^{\circ}\text{C}$. The annealed DNA primer/RNA was precipitated by the addition of 0.3 M sodium acetate and ethanol, and the pellet was used in a reverse transcription reaction. The following was added to each annealed primer/RNA pellet: 7 μ l of 2 mM dNTP mix (2 mM each of dATP, dCTP, dGTP and dTTP), 44 U RNasin, 11 μ l sterile deionized water, 1X reverse transcriptase buffer (Promega Corporation, Madison, WI), and 40 U of AMV reverse transcriptase (Promega Corporation, Madison, WI). The tubes were incubated at 45°C for 1.5 hrs. Subsequently, 2 μ l of 2.5 M EDTA and 10 U RNaseA were added to each reaction and the tubes were incubated at 37°C for an additional 30 mins. After incubation, 200 μ l of sterile deionized water was added to each tube and the solution was extracted with 250 μ l of phenol/chloroform. The aqueous phase was separated by centrifugation and DNA was precipitated by the addition of 0.3M sodium acetate and two volumes of ethanol. The reverse transcription products were electrophoresed on acrylamide gels as described by Chun (1994). Size standards were obtained using the same primers in a dideoxysequencing reaction using a plasmid encoding *nolA* (pBG24) or *nodD2* (pTD310) as template.

β-GALACTOSIDASE ACTIVITY ASSAYS

The β-galactosidase activity of *B. japonicum* strains harboring *lacZ* gene fusions was assayed as described by Banfalvi *et al.* (1988). The induction of *nod-lacZ* expression by the isoflavone genistein (2 μM) or soybean seed extract (20 μL/ml) was done as described by Yuen *et al.* (1995). The β-galactosidase activity of *E. coli* cells was assayed as follows: cells were grown overnight in M9 medium (Sambrook *et al.* 1989) supplemented with 0.5% (w/v) casamino acids, 20 μg/ml tryptophan and the appropriate antibiotics. Overnight cultures were diluted 20-fold into fresh M9 medium in the presence or absence of tryptophan. β-galactosidase activity were measured 2 hours after subculture following the method described by Banfalvi *et al.* (1988).

PRODUCTION OF ANTIBODIES AGAINST NolA

Protein Purification

E. coli cells harboring pBGT7A-2 were grown to an O.D.₆₀₀=0.5 and were induced with 1 mM IPTG. Induced cells were allowed to grow for an additional three hours, harvested by centrifugation and lysed by sonication. The cell lysate was centrifuged at 31,000 xg for 20 minutes. The proteins in the resulting supernatant (soluble fraction) and pellet (insoluble fraction) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel (Laemmli, 1970). Protein bands were visualized by staining with Coomassie blue. Since the NolA fusion protein was detected

only in the insoluble fraction, the protein was purified as follows: the insoluble fraction containing inclusion bodies was washed once with phosphate-buffered saline (PBS; 137mM NaCl, 3mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄) containing 2M urea, three times with PBS containing 1% TritonX-100 and once with PBS. These series of washings removed most of the contaminating proteins (as analyzed by SDS) and resulted in a preparation containing predominantly (approximately 90%) the Nola-polyhistidine-tagged fusion protein. The washed inclusion bodies were solubilized in gel sample buffer (65mM Tris- HCl, pH 6.8, 10% glycerol, 1% sodium dodecyl sulfate, 150mM β -mercaptoethanol, and 0.0005% bromphenol blue) and separated by SDS-PAGE on a preparative 12% gel. The gel was lightly stained with Coomassie blue and the band corresponding to the Nola fusion protein was excised. The protein was electroeluted from the gel slice following the method described by Harlow and Lane (1989) and concentrated by ultrafiltration using Centricon-10 (Amicon, Inc., Beverly, MA). Protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL).

Immunization and Preparation of Antibodies

Immunization of female New Zealand white rabbits was done by subcutaneous and intramuscular injections of approximately 500 ug of gel-purified protein emulsified in complete Freund's adjuvant. The rabbits were given booster injections at 5-week intervals with approximately 200 ug of the gel-purified protein emulsified in incomplete Freund's adjuvant. Blood samples were collected via ear bleeds 7-10 days after each booster injections. Serum was processed using standard methods (Hurn and Chantler, 1980).

Immunoglobulin G (IgG) specific for Nola was affinity-purified basically as described by Gu et al. (1994). Briefly, washed inclusion bodies containing the His-tagged Nola fusion protein were solubilized in 6M guanidine-HCl and loaded onto a Sepharose 6B column (Sigma, St. Louis, MO) previously activated with Ni^{+2} following the protocol recommended by Novagen (Madison, WI). The column was washed with 15 volumes of Wash Buffer A (20mM imidazole, 0.5M NaCl, 20mM Tris-HCl, pH 7.9), then with 15 volumes of Equilibration Buffer (150 mM NaCl, 50 mM Tris-HCl, pH7.4). Crude antiserum was applied to the column and incubated at room temperature for 30 minutes. The column was then washed with 5 column volumes of Equilibration Buffer, followed by 5 column volumes of Wash Buffer B (2M NaCl, 50 mM Tris-HCl, pH 7.4). To elute the antibody, the column was first incubated in 1 column volume of 4M MgCl_2 for 15 minutes, following which an additional 1 column volume of 4M MgCl_2 was added, and the eluate collected. The eluate, containing the affinity-purified antibody, was dialyzed against water for one hour, and then against PBS exhaustively at 4°C. Preadsorption of the affinity-purified antibody was done as follows: acetone powder of *B. japonicum* strain BJB3 was prepared as described by Harlow and Lane (1989) and was added to the affinity-purified anti-Nola antibody preparation at 1% (w/v) final concentration. The mixture was incubated at 4°C for 30 mins, and was used in Western Blot experiments as described below.

WESTERN BLOTS

B. japonicum cells were harvested by centrifugation, washed with PBS buffer, and resuspended in the same buffer. Cells were lysed by sonication and the cell lysates were centrifuged at 31,000 xg for twenty minutes. Proteins contained in the supernatant were separated by SDS-PAGE through a 12% gel and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA) using a Hoeffer Scientific (San Francisco, CA) electrophoretic transfer apparatus. Membranes were blocked by incubating in TBS (20mM Tris-HCl, pH7.5; 0.5M NaCl) containing 5% BSA (TBS-BSA) for 2 hours. Membranes were then incubated overnight with 1:300 dilution of the purified anti-NolA antibody in TBS-BSA followed by washing three times with TTBS [TBS containing 0.05% Tween 20 (Sigma Inc., St. Louis, Mo)]. The membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Bio-Rad Laboratories, Hercules, CA) in TBS-BSA for 1 hour and then washed three times with TTBS. Immunoreactive bands were developed using nitroblue tetrazolium (NBT; Pierce, Rockford, IL) and 5 bromo-4 chloro-3 indoyl phosphate (BCIP; Pierce, Rockford, IL) as substrates for bound alkaline phosphatase.

ANALYSIS OF NOD SIGNALS BY THIN LAYER CHROMATOGRAPHY

B. japonicum cells were grown until an O.D.₆₀₀ of 0.5-0.6 was reached and cells were pelleted by centrifugation. Cells were washed once with minimal medium, and

diluted in this medium to an O.D.₆₀₀ of 0.1. Cells were then induced by the addition of soybean seed extract at 20 µL/ml. At the time of induction, 50 µCi of [1-¹⁴C]acetate was added and the culture was incubated overnight. Induced cells were pelleted by centrifugation. The supernatant was extracted with 1-butanol and applied to octadecyl silica thin layer chromatography plates (Sigma, St. Louis, Mo) as described by Sanjuan et al. (1992). Plates were dried and exposed to X-ray film (Kodak X-Omat AR) for 2-4 days at room temperature.

PLANT INFECTION TESTS

Seeds of *Glycine max* (soybean) (genotypes cv. Essex, PI371607 and PI417566) and *Vigna unguiculata* (cowpea) cv. Caloona were surface-sterilized as described by Nieuwkoop et al. (1987). After germination, seedlings were transferred into sterile Leonard jars containing vermiculite and half-strength plant nutrient solution (Wacek and Brill, 1976). Plants were grown in a greenhouse under 16-hr daylight. Nitrogen fixation activity was detected by the acetylene reduction activity assay (Wacek and Brill, 1976) with a Shimadzu GC-8A gas chromatograph equipped with a six-foot (ca.2-m) Poropak R column. The detector was maintained at 100°C and the column at 75°C.

LIGHT AND ELECTRON MICROSCOPY

Nodules formed on soybean and cowpea were excised at 14 and 21 days post-

inoculation and 1mm discs were taken from the center of each nodule. Tissue samples were prepared as described by Roth and Stacey (1989). For light microscopy, 1 μ m thick sections were stained with toluidine blue and photographed with a Nikon microphot optical microscope. For electron microscopy, 150 nm thick sections were stained and then examined and photographed with a Hitachi H-600 electron microscope operating at 75 kV.

CHAPTER IV

RESULTS

SYMBIOTIC ROLE OF THE *B. japonicum nola* GENE

The previous report by Sadowsky et al. (1991) indicated that *NolA* was involved in mediating genotype-specific nodulation of soybeans. In addition, Dockendorff et al. (1994a) reported that conjugation of a plasmid encoding *nola* into a *B. japonicum* strain deleted for *nodD1*, *nodD2* and *nola* significantly improved nodulation ability. These reports point to the importance of *nola* in the ability of *B. japonicum* to effectively nodulate its plant hosts. However, the lack of a *B. japonicum nola* mutant strain had precluded more detailed functional studies on the role of *nola* in nodulation. In the present study, two *B. japonicum* strains with mutations in *nola* were constructed by interposon mutagenesis. Using these strains, the importance of *nola* in the ability of *B. japonicum* to nodulate and establish a nitrogen-fixing symbiosis with its plant hosts was examined.

Construction of *B. japonicum nola* Mutants Strains

Two *nola* mutant strains of *B. japonicum* were constructed by interposon mutagenesis. The mutation in strain BJD21 (constructed using pBG303, Figure 4) was made by insertion of the spectinomycin (Sp)/streptomycin (Sm) cassette of pHP45Ω into the *nola* coding region. However, the site of this mutation did not preclude the possibility

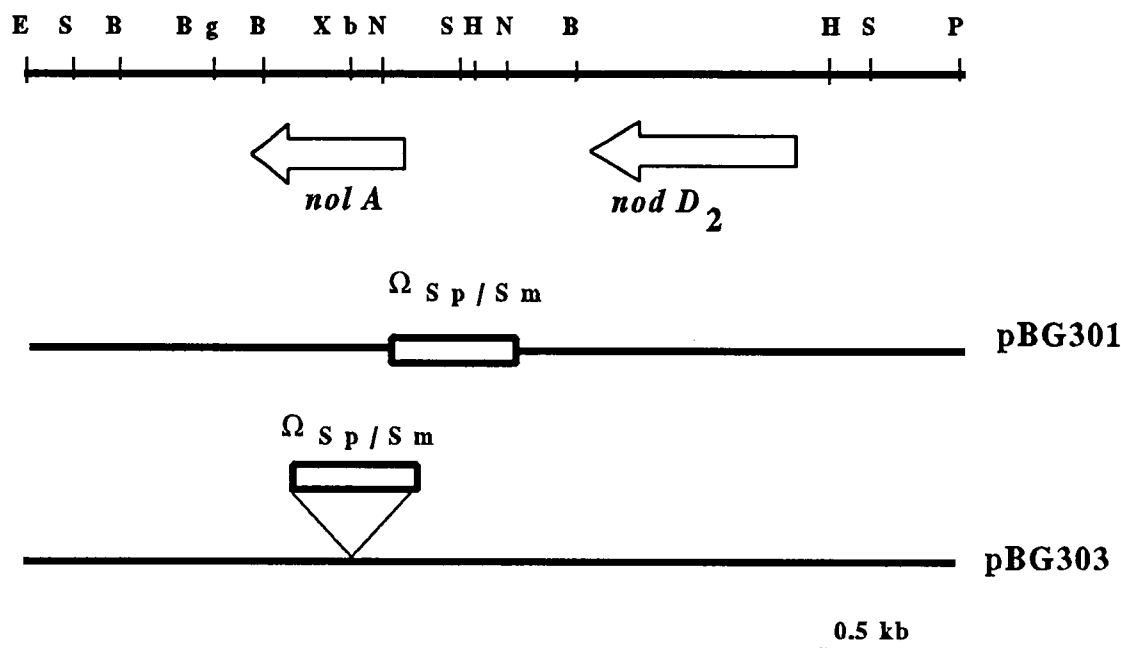


Figure 4: Construction of *B. japonicum* *nolA* mutant strains by interposon mutagenesis. Restriction map of the *nod* gene region of the *Bradyrhizobium japonicum* chromosome under study (top). The position of the *nolA*, *nodD1*, and *nodD2* genes are shown immediately below the map. (Only the pertinent restriction sites are shown: E=*EcoRI*, S=*SalI*, B=*BamHI*, Bg=*BglII*, Xb=*XbaI*, N=*NheI*, P=*PstI*) Below the map are the plasmid constructs used in the mutagenesis of *nolA*. PBG301; pSUP202 plasmid (not shown) containing a 4.5 kb *EcoRI*-*PstI* fragment (thin line) into which the Ω Sp/Sm resistance cassette (open box, labelled) was cloned so as to delete the *nolA* promoter and 5' region of the coding sequence. pBG303; pSUP202 plasmid (not shown) containing a 4.5 kb *EcoRI*-*PstI* fragment (thin line) with the Ω Sp/Sm resistance cassette (open box, labelled) inserted into the *XbaI* site so as to disrupt the *nolA* coding region. The DNA regions flanking the Ω cassette provided the DNA needed for marker exchange by double homologous recombination.

that a N-terminally truncated NolA protein could be made in this strain. Therefore, a second mutant strain, BjB3 (constructed using pBG301, Figure 4), was made in which the 5' end of the *nolA* coding sequence, as well as sequences upstream of the putative ATG start codon, were deleted. The *nolA* mutations in strain BjD21 and BjB3 were confirmed by Southern blot hybridization analysis of the chromosomal DNA isolated from each strain (data not shown).

Nodulation and Nitrogen Fixation Phenotype of *nolA* Mutant Strains on Soybean and Cowpea

The nodulation and nitrogen fixation phenotypes of the *nolA* mutant strains BjD21 and BjB3 were tested on *Glycine max* (soybean) cv. Essex and *Vigna unguiculata* (cowpea) cv. Caloona. Nodulation efficiency was examined by determining the nodule number and fresh weight produced per plant, whereas nitrogen fixation was measured by acetylene reduction activity. As shown in Figure 5, the *nolA* mutant strain BjD21 showed grossly defective nodulation and nitrogen fixation on cowpea plants. Similar results were obtained for mutant strain BjB3 (data not shown). Nodulation efficiency of the mutant strain was about half that of the wild type, even at 30 days post-inoculation (PI). The nitrogen fixation activity shown by the mutant strain was only 10% of the wild type at 14 and 21 days PI, and increased to approximately 30% of the wild type level at 30 days PI. Cowpea plants inoculated with the mutant strain resembled uninoculated control plants in that their leaves turned chlorotic (Figure 6). This gross defect in nodulation and nitrogen fixation by the mutant strain was not observed in soybean. As shown in Figure 5, soybean

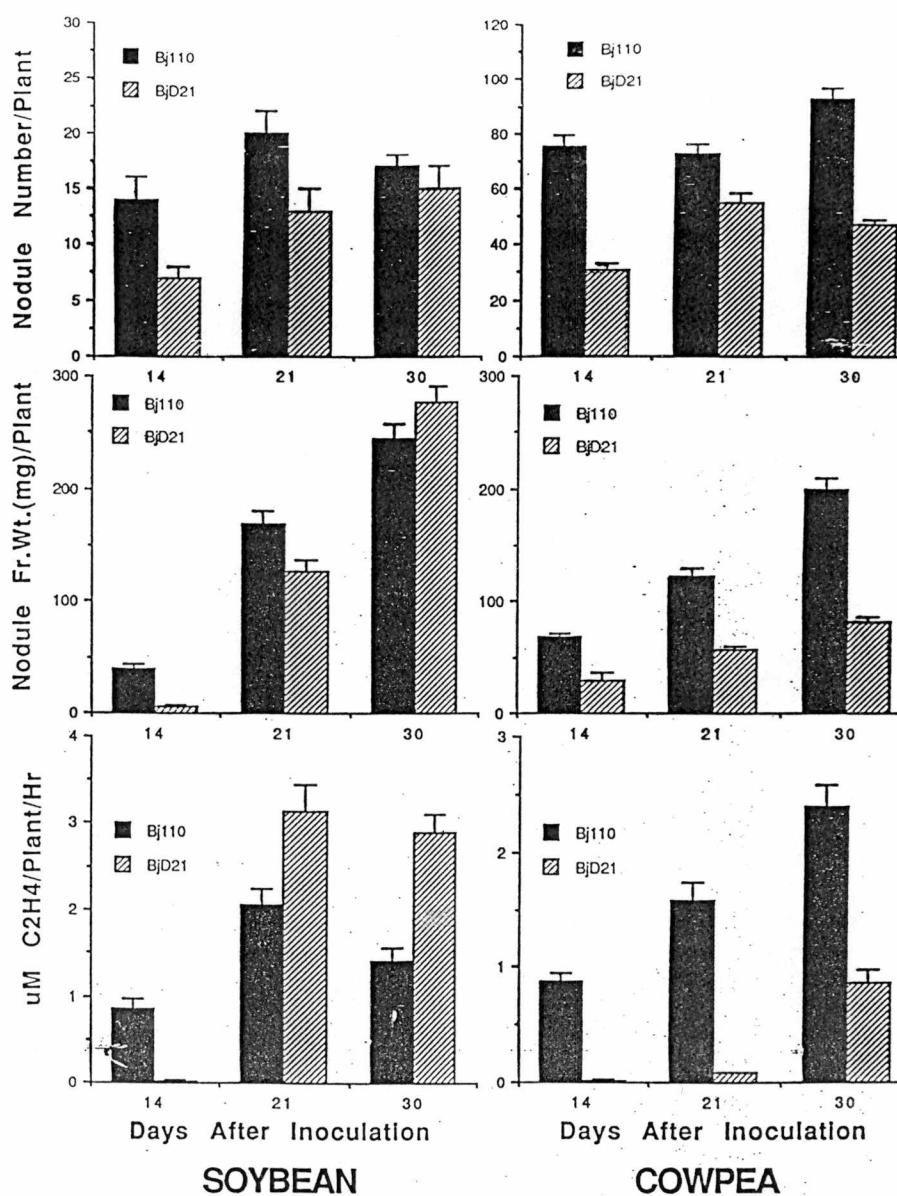


Figure 5: Nodulation and nitrogen fixation of *B. japonicum* USDA110 and a *nolA* mutant strain, Bjd21, on soybean and cowpea. Top panel, nodule number per plant at 14, 21 and 30 days post inoculation (PI). Middle panel, nodule fresh weight (mg) per plant at 14, 21, and 30 days PI. Lower panel, acetylene reduction activity ($\mu\text{M C}_2\text{H}_4$ formed per plant per hour) at 14, 21 and 30 days PI. Results presented are the means of 18 plants assayed over two trials (standard error is shown above each bar).



A

B

C

Figure 6: Chlorosis of cowpea plants inoculated with a *B. japonicum nola* mutant strain, BjD21. These plants are 21-day-old cowpea inoculated with BjD21 (A) and USDA110 (B). The uninoculated plants (C) are shown for comparison.

plants inoculated with the mutant strain BjD21, as compared to plants inoculated with the wild-type strain, showed only a slight delay in nodulation and even a higher degree of nitrogen fixation at 21 and 30 days PI.

Microscopic Examination of the Nodules Induced by the *nolA* Mutant Strain BjD21 on Soybean and Cowpea (These studies were conducted in collaboration with Dr. John Dunlap, University of Tennessee.)

Nodules formed by mutant strain BjD21 were examined by light and electron microscopy and compared to those formed by the wild type. Light microscopy revealed that soybean nodules induced by the the wild type at 14 days PI contained larger infected cells and a higher ratio of infected to uninfected cells than nodules formed by the mutant strain (Figure 7A and C). When examined by electron microscopy, plant cells infected by the wild type typically contained symbiosomes with two or three bacteroids. These bacteroids had significant internal deposits of polyhydroxybutyrate (Figure 7B). In contrast, plant cells infected by the mutant had symbiosomes that typically contained a single bacteroid. The mutant bacteroids showed little internal polyhydroxybutyrate (Figure 7D).

Soybean nodules induced by strain BjD21 at 21 days PI showed a similar ratio of infected to uninfected cells to that found in nodules formed by the wild type (Figure 8A and C). At 21 days PI, the bacteroids formed by both the wild type and mutant contained significant amounts of polyhydroxybutyrate (Figure 8B and D) and were morphologically indistinguishable from each other.

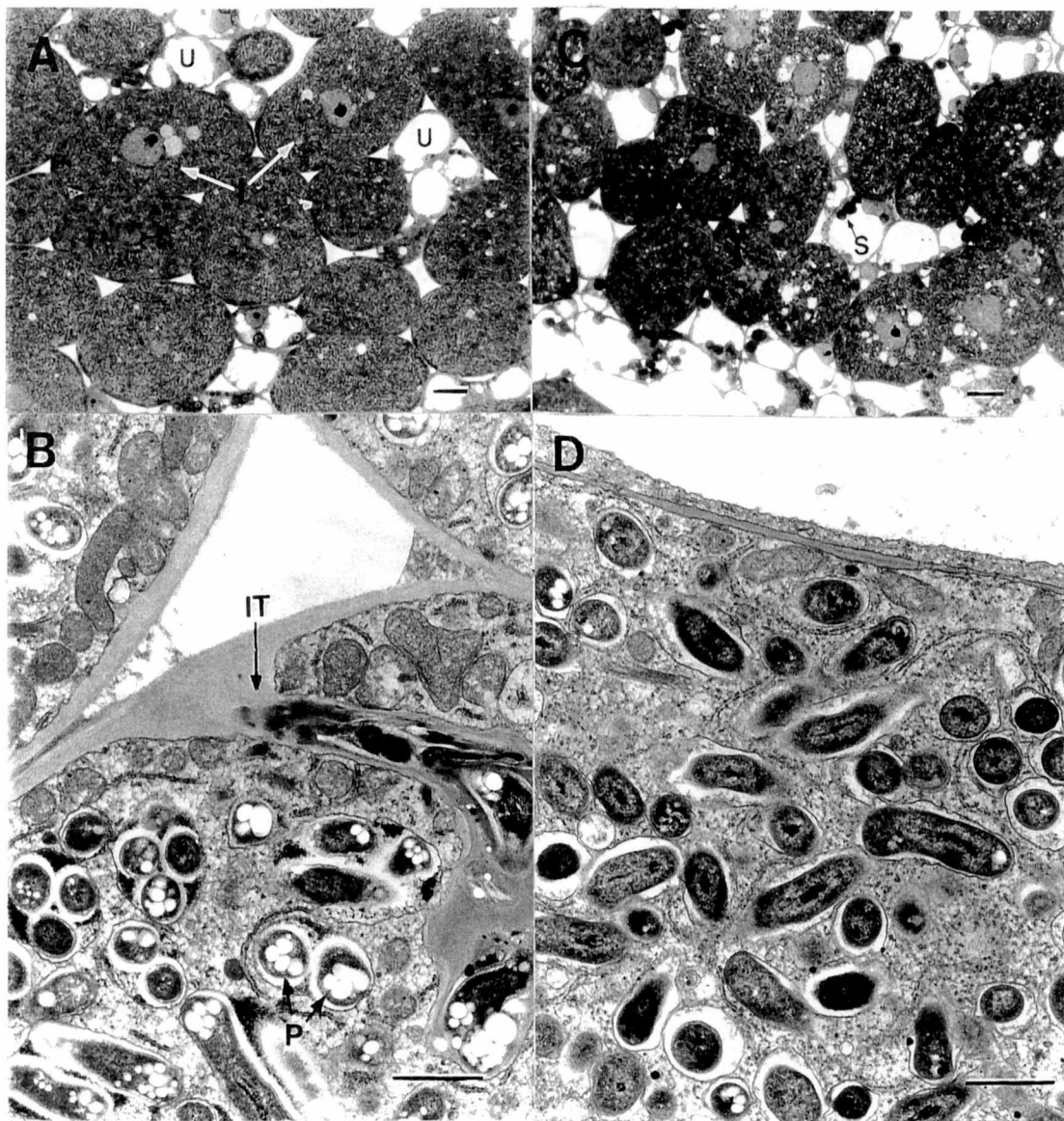


Figure 7: Microscopic examination of soybean nodules induced by *B. japonicum* USDA110 and a *nolA* mutant, BjD21, at 14 days PI. Light and electron micrographs of nodules induced by strain USDA110 are shown in panels A and B, respectively. Light and electron micrographs of nodules induced by strain BjD21 are shown in panels C and D, respectively. Magnification: Light micrographs A and C, bar=10 μ M; Electron micrographs B and D, bar=1 μ M. Abbreviations: I=infected cell, U=uninfected cell, IT=infection thread, P=polyhydroxybutyrate, S=starch granules.

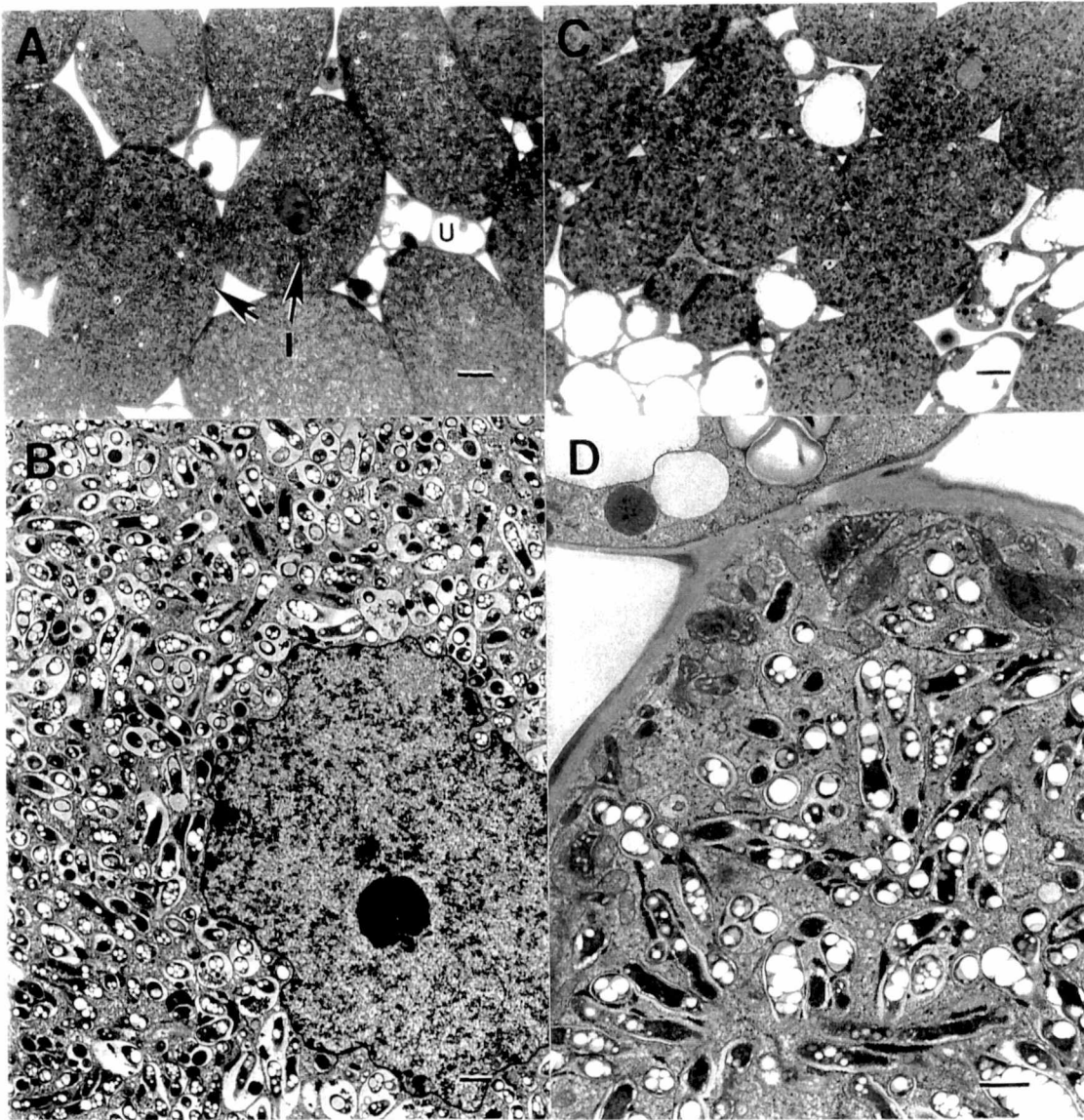


Figure 8: Microscopic examination of soybean nodules induced by *B. japonicum* USDA110 and a *nolA* mutant, BJD21, at 21 days PI. Light and electron micrographs of nodules induced by strain USDA110 are shown in panels A and B, respectively. Light and electron micrographs of nodules induced by strain BJD21 are shown in panels C and D, respectively. Magnification: Light micrographs A and C, bar=10 μ M; Electron micrographs B and D, bar=1 μ M. Abbreviations: I=infected cell, U=uninfected cell.

Cowpea nodules formed by the wild-type and mutant strain BjD21 were also analyzed by light and electron microscopy at 14 and 21 days PI. Light microscopy revealed that nodules formed by the mutant strain had a lower ratio of infected to uninfected cells at both 14 and 21 days PI as compared to nodules induced by the wild type (Figure 9A and C; Figure 10A and C). Electron microscopy revealed that nodule cells infected by the wild-type strain at 14 days PI exhibited symbiosomes typically containing multiple bacteroids (Figure 9B). In contrast, cells infected by strain BjD21 at 14 days PI contained only a few apparent symbiosomes that typically contained only a single bacteroid (data not shown). Most of the mutant bacteroids were found embedded in a large vesicular-like structure containing fibrous material (Figure 9D). The origin of these structures is unknown but they clearly do not resemble normal symbiosomes.

At 21 days PI, cowpea nodule cells infected by the wild-type strain were filled with symbiosomes typically containing several bacteroids (Figure 10B). In contrast, cells infected by the mutant strain contained fewer bacteroids. As was the case at 14 days PI, no typical symbiosomes were present in the mutant infected nodule cells (Figure 10D and E). Fine fibrillar material was loosely distributed throughout the mutant infected cells (Figure 10D). Even though the mutant bacteroids were not found within symbiosomes, they did not appear to be in a state of degradation (Figure 10E). The host cellular content, on the other hand, appeared to be degraded (Figure 10D).

Nodulation Phenotype of the *nolA* Mutant Strains on Soybean Genotypes PI371607 and PI417566 (These studies were done in collaboration with Dr. Michael Sadowsky,

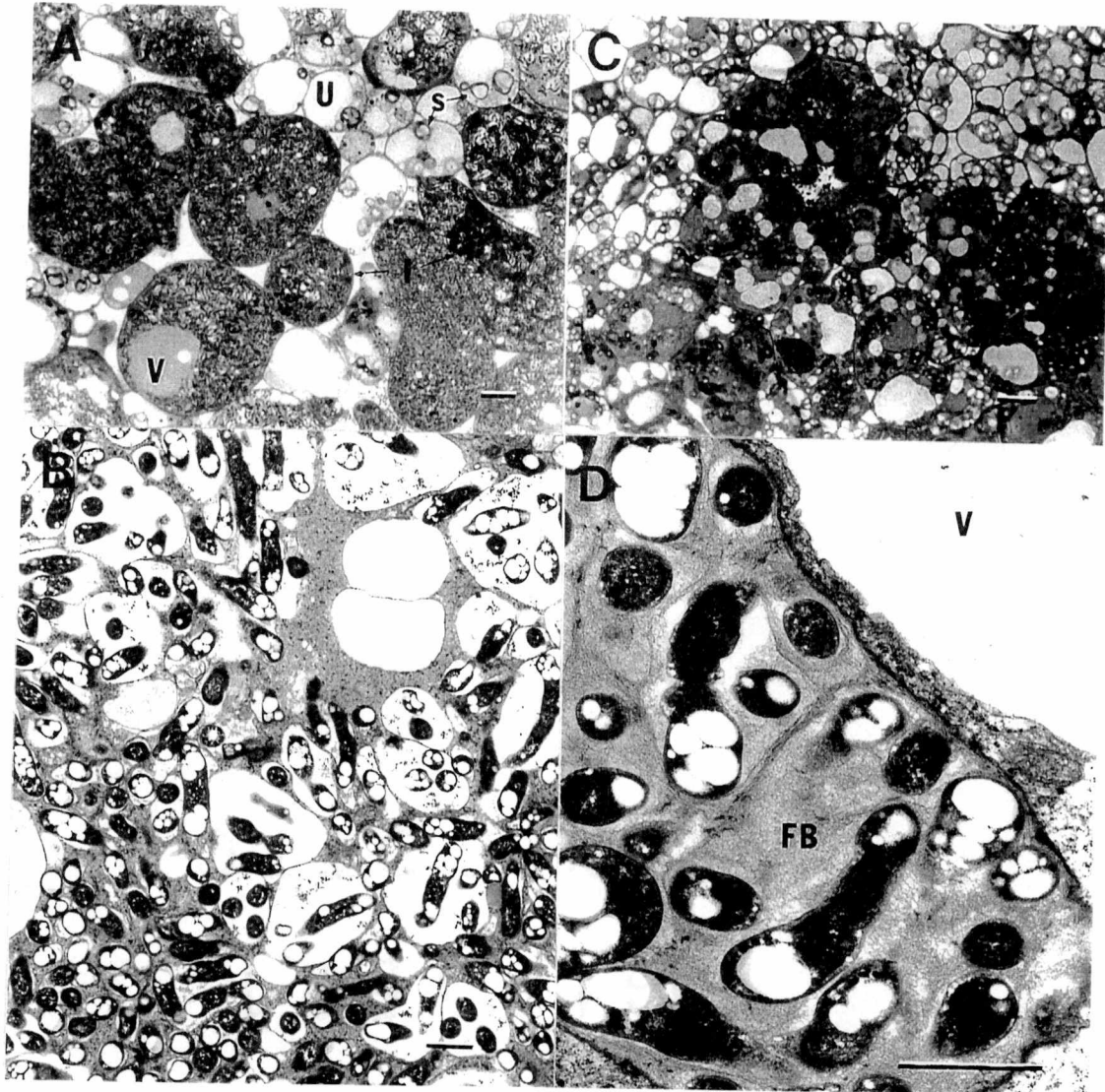


Figure 9: Microscopic examination of cowpea nodules induced by *B. japonicum* USDA110 and a *nolA* mutant, BJD21, at 14 days PI. Light and electron micrographs of nodules induced by strain USDA110 are shown in panels A and B, respectively. Light and electron micrographs of nodules induced by strain BJD21 are shown in panels C and D, respectively. Magnification: Light micrographs A and C, bar=10 μ M; Electron micrographs B and D, bar=1 μ M. Abbreviations: I=infected cell, U=uninfected cell, V=vesicle, FB=fibrous material, S=starch granules.

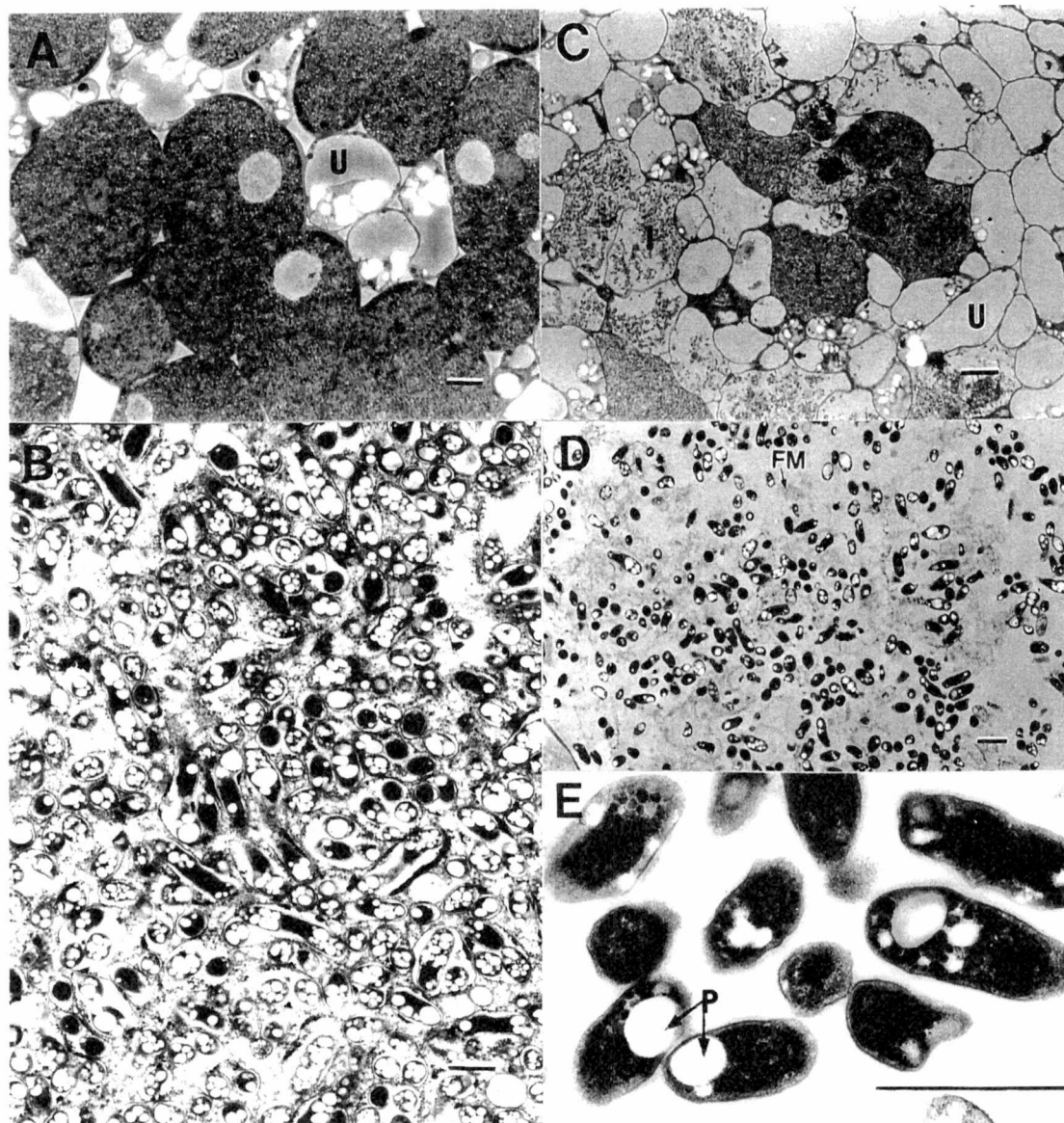


Figure 10: Microscopic examination of cowpea nodules induced by *B. japonicum* USDA110 and a *nolA* mutant, BjD21, at 21 days PI. Light and electron micrographs of nodules induced by strain USDA110 are shown in panels A and B, respectively. A light micrograph of a nodule induced by strain BjD21 is shown in panel C. Electron micrographs of nodules induced by strain BjD21 are shown in panels D and E. Magnification: Light micrographs A and C, bar=10 μ M; Electron micrographs B, D and E, bar=1 μ M. Abbreviations: I=infected cell, U=uninfected cell, FM=fibrillar material, P=polyhydroxybutyrate.

University of Minnesota.)

Two soybean genotypes (Plant Introduction lines, PI377578 and PI371607) were found to restrict the nodulation of *B. japonicum* strain SD6-1c, as well as of 20 other *B. japonicum* USDA123 strains (Keyser and Cregan, 1987). These soybean genotypes, however, do not restrict the nodulation of *B. japonicum* strain USDA110. Conjugation of a plasmid encoding the *nolA* gene of strain USDA110 into strain SD6-1c was found to enable this latter strain to overcome the host restriction. These data suggest that *nolA* gene function may be essential for the ability of strain USDA110 to nodulate soybean line PI377578 or PI371607. To test this hypothesis, the nodulation phenotype of the *nolA* mutant strain BJD21 on soybean line PI371607 was determined. As shown in Table 3, strain BJD21 was able to nodulate PI371607, indicating that NolA is not the bacterial determinant essential for the ability of strain USDA110 to nodulate this soybean genotype. Similar to the wild-type strain USDA110, strain BJD21 was restricted for nodulation on soybean genotype PI417566. Therefore, although transfer of the *nolA* gene to strain SD6-1c could overcome genotype-specific nodulation restriction, the *nolA* gene does not appear to be critical to mediating genotype-specific nodulation by strain USDA110.

ROLE OF *nolA* IN THE REGULATION OF *nod* GENE EXPRESSION

Previous reports indicated that maximum induction of a *nodD1-lacZ* or *nodY-lacZ* fusion in *B. japonicum* requires both NodD1 and NodW (Sanjuan et al. 1994). However,

Table 3: Nodulation of different *B. japonicum* strains on *G. max* genotypes PI371607 and PI417566.

Strain	Nodule number/plant ^a	
	PI371607 ^b	PI417566 ^c
USDA110 (wild type)	25 A	1 B
BjD21 (<i>nolA</i>)	27 A	1 B
USDA123 (wild type)	3 B	31 A

^a Values are means of triplicate samples. Numbers in a column followed by the same letter are not significantly different at $p=0.05$.

^bSoybean genotype that restricts nodulation of *B. japonicum* strain USDA123

^cSoybean genotype that restricts nodulation of *B. japonicum* strain USDA110

strains deleted for *nodD1*, *nodD2* and *nolA* (i.e. strains Bj1267 and Bj329; Sanjuan et al. 1994) showed wild-type levels of *nod* gene induction. These results led to the suggestion that a gene encoding a repressor (i.e., *nolA*) had been deleted in these strains which allowed for *nod* gene induction mediated by NodW alone (Dockendorff et al. 1994a, Sanjuan et al. 1994). Subsequently, it was found that the expression of NolaA from a multicopy plasmid inhibited the isoflavone-mediated induction of a *nodC-lacZ* fusion, suggesting that *nolA* encodes a repressor (Dockendorff et al. 1994a). Since *nolA* sequence predicts a protein having a helix-turn-helix DNA-binding motif, it was hypothesized that NolaA mediates the repression of *nodD1* and *nodYABCSUIJnolMNO* expression by binding to the promoters of these genes and thereby inhibiting transcription. In this study, the role of NolaA in the regulation of *nod* gene expression (i.e. *nodD1*, *nodD2*, *nodY*, and *nolA*) was further examined. The effect of NolaA on the profile, as well as the quantity, of the Nod metabolites produced by *B. japonicum* was also determined.

Effect of NolaA on the Expression of *nodD1* and *nodYABCSUIJnolMNO* Operons

To further elucidate the role of NolaA in *nod* gene regulation, plasmids encoding a *nodD1-lacZ* or *nodY-lacZ* fusion were mobilized into *B. japonicum* wild-type (USDA 110) and *nolA* mutant (BjD21 and BjB3) strains. The β -galactosidase activity of the fusions was assayed in the presence or absence of flavonoid compounds, i.e. soybean seed extract and genistein, which are known to induce the expression of *nodD1* or *nodY*. Surprisingly, the basal and induced expression of *nodD1* and *nodY* in both mutant strains was comparable to that of the wild type, as shown in Table 4. These results contradict

Table 4: Expression of *nodY* and *nodD1* in *B. japonicum* wild-type and *nolA* mutant strains.

	β - galactosidase activity ^{a,b} (U)		
	Uninduced	+SSE ^c	+genistein
<i>nodY-lacZ</i>			
USDA110 (wild type)	31 $\pm$ 2	1268 $\pm$ 21	650 $\pm$ 43
BjD21 (<i>nolA</i>)	36 $\pm$ 9	1623 $\pm$ 23	786 $\pm$ 19
BjB3 (<i>nolA</i>)	29 $\pm$ 9	1488 $\pm$ 109	724 $\pm$ 98
<i>nodD1-lacZ</i>			
USDA110 (wild type)	7 $\pm$ 1	36 $\pm$ 5	19 $\pm$ 4
BjD21 (<i>nolA</i>)	6 $\pm$ 1	31 $\pm$ 4	21 $\pm$ 5
BjB3 (<i>nolA</i>)	5 $\pm$ 1	34 $\pm$ 9	22 $\pm$ 3

^a Units using chlorophenolred- β -D-galactopyranoside (CPRG) as a substrate

^b Values are the means of two independent determinations; each determination was done in three replicates. The standard deviation is indicated.

^c Soybean seed extract

was conducted in the previous hypothesis that NodA directly mediates the repression of *nod* gene expression by binding to the promoters of the *nodDI* and *nodYABCSUIJnolMNO* operons (Dockendorff et al. 1994a). It is possible that NodA mediates the repression of *nod* gene expression indirectly through another protein.

Effect of NodA on the Production of Nod Metabolites (This study collaboration with Jonathan Cohn, The University of Tennessee)

To determine if a mutation in *nolA* results in a change in the profile and/or quantity of Nod signals being produced, the Nod metabolites produced by the wild-type (strain USDA 110) and *nolA* mutant (strain BjD21) strains were analyzed by thin layer chromatography (TLC). As shown in Figure 11, the profile of Nod metabolites produced by the wild-type and *nolA* mutant strains were comparable. The major band (indicated by an arrow in Figure 11) detected for each strain corresponds to the wild-type Nod signal, called NodBj-V (C18:1, Me-Fuc). This Nod metabolite was purified and characterized previously by Sanjuan et al. (1994). Other bands were also detected in the TLC plate and these represent the minor forms of Nod signal produced by *B. japonicum* USDA110 (Minami et al., 1996). There was no detectable difference in the quantity of Nod metabolites produced by either the wild-type or *nolA* mutant strain. These results are consistent with the fact that mutations in *nolA* did not affect the expression of the *nodDI* and *nodYABCSUIJnolMNO* operons.

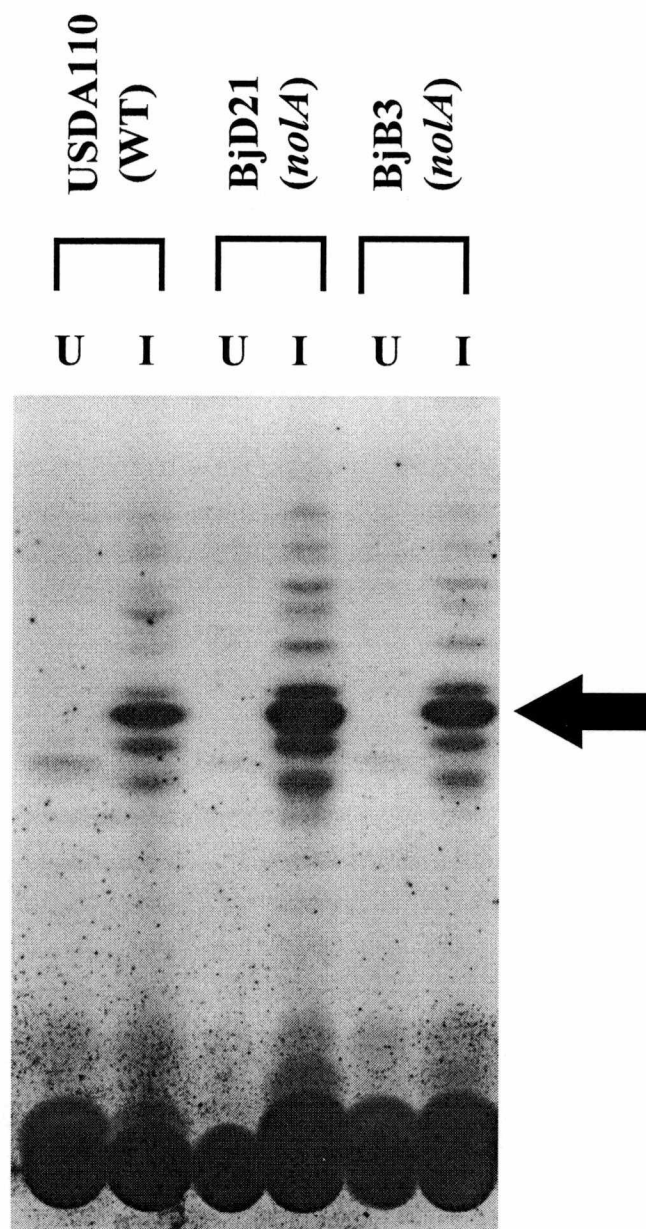


Figure 11: Analysis of Nod metabolites produced by *B. japonicum* USDA110 and the *nolA* mutant strains, BjD21 and BjB3, by thin layer chromatography (TLC). For each strain, Nod metabolites were analyzed from uninduced cells (U), and cells induced with soybean seed extract (I). The arrow indicates the major Nod metabolite produced by strain USDA110, NodBj-V(C18:1, MeFuc).

Effect of *NolA* on the Expression of *nodD2*

B. japonicum has two copies of *nodD*, *nodD1* and *nodD2*, which are arranged tandemly (Göttfert et al., 1992). The expression of *nodD1* has been reported to be positively autoregulated by the NodD1 protein in the presence of plant-produced isoflavones. Although NodD2 shares 62% amino acid identity with NodD1, NodD2 cannot complement the function of NodD1 as a positive regulator of *nod* gene expression in response to plant-produced isoflavones. No function has yet been reported for the NodD2 protein. Moreover, no work has been done to examine how the expression of *nodD2* is regulated. In this study, the effect of *nolA* on the expression of *nodD2* was examined. A plasmid encoding a *nodD2-lacZ* fusion was mobilized into *B. japonicum* wild-type (USDA110) and *nolA* mutant (BjD21 and BjB3) strains, and the β -galactosidase activity was determined in the presence or absence of plant-produced isoflavones. As shown in Table 5, the expression of *nodD2* was significantly lower in the mutant strains as compared to the wild type. Moreover, unlike *nodD1*, the expression of *nodD2* was not induced by the addition of isoflavones. These results indicate that *nodD2* expression is independent of isoflavones and that *NolA* is likely involved in the positive regulation of *nodD2* expression.

Effect of *nodD2* on *nod* Gene Expression

As previously mentioned, *NolA* may mediate *nod* gene repression indirectly through another protein. A likely candidate is NodD2, a homologue of which has been shown to be involved in the repression of *nod* gene expression in *Bradyrhizobium* sp.

Table 5: Expression of *nodD2* in *B. japonicum* wild-type and *nolA* mutant strains.

	β -galactosidase activity ^{a,b} (U)		
	Uninduced	+SSE ^c	+genistein
USDA110 (wild type)	178 $\pm$ 20	184 $\pm$ 16	160 $\pm$ 11
BjD21 (<i>nolA</i>)	23 $\pm$ 5	36 $\pm$ 3	19 $\pm$ 4
BjB3 (<i>nolA</i>)	31 $\pm$ 4	29 $\pm$ 5	25 $\pm$ 5

^a Units using CPRG as a substrate

^b Values are the means of two independent determinations; each determination was done in three replicates. The standard deviation is indicated.

^c Soybean seed extract

(*Arachis*) NC92 (Gillette and Elkan, 1996). To test whether the *B. japonicum* NodD2 has a repressive effect on *nod* gene expression, NodD2 was expressed from the *trp* promoter encoded on pTE3 Ω and its effect on the expression of a chromosomal *nodC-lacZ* fusion was tested. As shown in Table 6, the expression of NodD2 from the constitutive *trp* promoter results in a drastic reduction of *nodC-lacZ* expression. Therefore, it is possible that the observed reduction in the expression of a *nodC-lacZ* fusion in a *B. japonicum* strain harboring multi-copy *nolA* (Dockendorff et al. 1994a) was due to a consequent elevation of NodD2 expression in this strain.

Positive Autoregulation of *nolA* Expression

The *nolA* DNA sequence predicts a translation product possessing a N-terminal, helix-turn-helix DNA binding motif which shares sequence similarity to the MerR-type regulatory proteins (Sadowsky *et al.* 1991). Studies on MerR and TipA, two proteins belonging to this family of regulatory proteins, have shown that they autoregulate their own expression (Lund *et al.*, 1986; Holmes *et al.* 1993). To examine the possibility that the expression of *nolA* is autoregulated, a plasmid encoding a *nolA-lacZ* fusion was constructed (i.e., pBGAlac1, Figure 12). In addition to this *nolA-lacZ* fusion, the *nolA* gene was cloned into plasmid pTE3 in order to express the Nola protein from the *trp* promoter (i.e., pGBtrpA-88, Figure 12). This promoter can be regulated in *E. coli* by the presence or absence of tryptophan, but is constitutively expressed in *B. japonicum*. *E. coli* cells were co-transformed with pBGAlac1 (containing the *nolA-lacZ* fusion) and pGBtrpA-88. As controls, co-transformations were also done using the vectors pTE3 or

Table 6: Expression of a chromosomal *nodC-lacZ* fusion in the presence or absence of NodD2.

Strain	β -galactosidase activity ^{a,b} (U)		
	Uninduced	+SSE ^c (-tryptophan)	+SSE (+tryptophan) ^d
Bj1221 (<i>nodC-lacZ</i> ; pTE3 Ω)	13 $\pm$ 6	400 $\pm$ 6	400 $\pm$ 12
BjtrpD2-1 (<i>nodC-lacZ</i> ; pBGtrpD2-1)	21 $\pm$ 3	44 $\pm$ 5	47 $\pm$ 7

^a Units using CPRG as a substrate

^b Values are the means of two independent determinations; each determination was done in three replicates. The standard deviation is indicated.

^c Soybean seed extract

^d added at 20 μ g/ml of culture

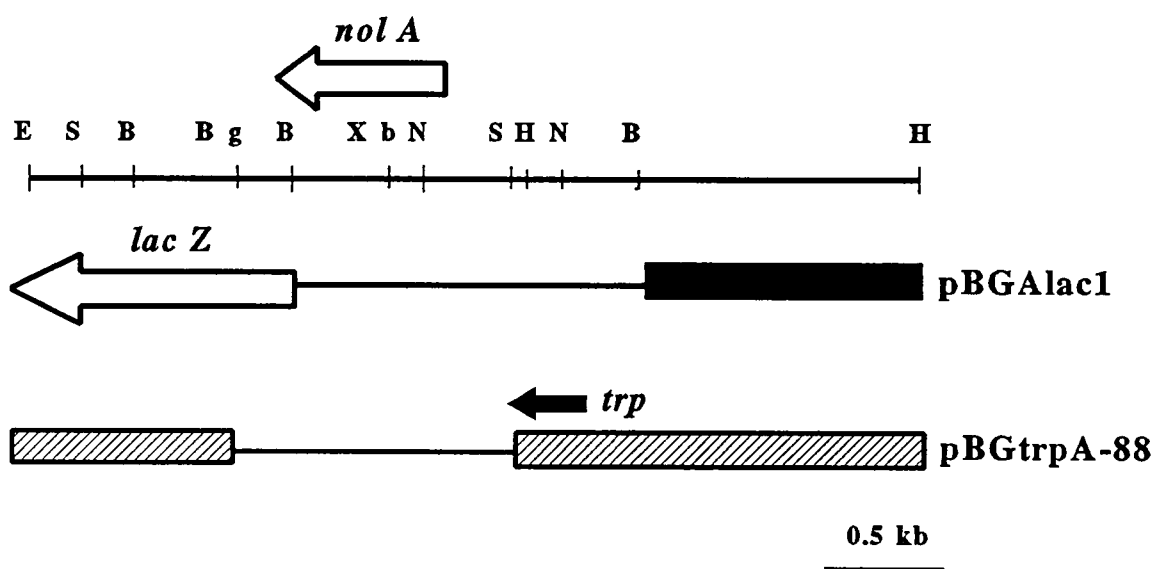


Figure 12: Construction of plasmids pBGAlac1 and pBGtrpA-88. Restriction map of *B. japonicum* chromosome encompassing the *nolA* gene. The position of *nolA* is shown above the map. (Only the pertinent restriction sites are shown. E=*EcoRI*, S=*SalI*, B=*BamHI*, Bg=*BglII*, Xb=*XbaI*, N=*NheI*, H=*HindIII*.) pBGAlac1: the thick filled line represents the pNM480 vector into which the *nolA* promoter and coding sequence (thin line) was cloned to produce an in-frame fusion with *lacZ* (open arrow, labelled). pBGtrpA-88: the thick striped line represents the pTE3 vector into which the *nolA* gene (thin line) was cloned so as to be expressed from the constitutive *trp* promoter (filled arrow, labelled).

pNM480 (see Methods). β -galactosidase activity assays were done on logarithmically growing cells in the presence or absence of tryptophan in the growth medium. As shown in Table 7, high expression of the *nolA-lacZ* fusion required the presence of NolA (i.e. in the presence of pBGtrpA-88 and the absence of tryptophan in the growth medium), suggesting that NolA acts as a positive regulator of its own expression in an *E. coli* background.

To confirm *nolA* autoregulation in *B. japonicum*, plasmid pBGAlac1 was cointegrated with the conjugative plasmid pRK290 and mobilized into different *B. japonicum* backgrounds. Consistent with the notion that *nolA* is positively autoregulated, *nolA-lacZ* expression was reduced six-fold, as compared to the wild type, in the *nolA* mutant strains BjD21 and BjB3 (Table 8). The expression of the *nolA-lacZ* fusion in the *nodW* mutant strain Bj613 was comparable to that of the wild type, suggesting that NodW is not involved in the regulation of *nolA*. However, the expression of *nolA* in the *nodD1* mutant strain Bj5686 was reduced approximately two-fold compared to the wild type. Therefore, in addition to NolA, maximum expression of *nolA* is mediated by NodD1.

THE *nolA* GENE ENCODES TWO PROTEINS

The *nolA* coding sequence predicts a protein having an N-terminal helix-turn-helix DNA-binding motif which shares sequence similarity to the DNA-binding domains of the MerR-type regulatory proteins. Moreover, the sequence also predicts two possible ATG start codons for translation (Figure 13). Translation from the first ATG (ATG1) would

Table 7: Expression of a *nolA-lacZ* fusion in *E. coli* in the presence or absence of Nola.

Plasmid/fusion	β - galactosidase activity ^{a,b} (U)	
	+ tryptophan ^c	- tryptophan
pBGAlac1 (<i>nolA-lacZ</i>)		
+pTE3	26 $\pm$ 11	28 $\pm$ 9
+pBGtrpA-88	30 $\pm$ 3	351 $\pm$ 43
pNM480 (vector)		
+pTE3	0	0
+pBGtrpA-88	0	0

^a Units using CPRG as a substrate

^b Values are the means of two independent determinations; each determination was done in three replicates. The standard deviation is indicated.

^c tryptophan was added at 20 μ g/ml growth culture

Table 8: Expression of a *nolA-lacZ* fusion in *B. japonicum* wild-type and mutant strains.

Strain (Phenotype)	β -galactosidase activity ^{a,b} (U)
USDA110 (wild type)	595 $\pm$ 13
BjD21 (<i>nolA</i>)	108 $\pm$ 34
BjB3 (<i>nolA</i>)	96 $\pm$ 12
Bj586 (<i>nodDI</i>)	274 $\pm$ 8
Bj613 (<i>nodW</i>)	550 $\pm$ 62

^a Units using CPRG as a substrate

^b Values are the means of two independent determinations; each determination was done in three replicates. The standard deviation is indicated.

Figure 13: Diagram of the *nolA* coding region showing the two putative ATG start codons, ATG1 (filled box) and ATG2 (open box). The putative ribosome binding site preceding each start codon is indicated. The exact locations of the *lacZ* fusions encoded in pBGAlac1 and pBGAlac14 are shown (capital letters show *nolA* sequence; lower case letters show *lacZ* sequence). The putative N-terminal helix-turn-helix DNA-binding motif is underlined.

RBS
 TTGAAATTGAACAACATG M N R A T P R R R R W R I G
 GAACTTGCAGAGCGGACCGGAGTAACGGTGCGCACGCTGCACCATATGAGCACACT
 E L A E A T G V T V R T L H Y E H T

G G A C T G C T A G g g g g a t c c g t c g a c c t g lacZ pBGAlacI4 RBS
 G G A C T G C T A G C A G C G A C A G A G C G C A C T G A G G G C G G T C A C C G G A T G T A T G A C C G C
 G L L A A T E R T E G G H R M Y D R

A T C C T G A A G G T A C T T G C A C G A T T A A G C G C G C C C
 I L K V L A R L S A P

lacZ GGTTGGgatacgtcg pBGAlacI
 C G C A G T C T G G C T G T T G G G A T C C C T G T C T A A T G C A A T A T C T C G A T C T T G C C C T T
 R S L A G W D P C L M Q Y L D L A L

G G C G G A T T G G A G G A T C A G C C T T A C T G A
 G G L E D Q P Y * * *

give a full-length protein (NolA_L) that has the putative N-terminal helix-turn-helix DNA-binding domain. Translation from the second ATG (ATG2) would give a truncated protein (NolA_S) which lacks the DNA-binding domain. In this study, the possibility that the *nolA* gene encodes two proteins was examined.

The N-terminal and C-terminal *nolA-lacZ* Fusions are Differentially Expressed

In order to study the possibility that *nolA* can be translated from two putative ATG start codons, the expression of *nolA-lacZ* fusions which can be translated from either of these ATG start codons was determined. As previously mentioned, the expression of a plasmid-encoded *nolA-lacZ* fusion (i.e. pBGAlac1) requires the *nolA* gene product, indicating that *nolA* expression is positively autoregulated. The site of LacZ fusion to NolA encoded on pBGAlac1 is in the C-terminal end of NolA (Figure 13 and 14). Therefore, translation of this fusion could occur at either of the putative ATG start codons. In addition to pBGAlac1, additional plasmids encoding a *nolA-lacZ* fusion were constructed (Figure 14). pBGAlac1-H or pBGAlac1-EX was derived from pBGAlac1, but *nolA* sequences upstream of the *lacZ* fusion site were deleted. Therefore, both of these plasmids encode C-terminal *nolA-lacZ* fusions identical to that encoded in pBGAlac1. In contrast, plasmid pBGAlac14 encodes an N-proximal *nolA-lacZ* fusion lacking the putative ATG2 start site (Figure 13 and 14). Translation of NolA from plasmid pBGAlac14 should presumably occur from ATG1.

Plasmids pBGAlac1, pBGAlac1-H, pBGAlac1-EX, and pBGAlac14 were individually transformed into an *E. coli* strain capable of expressing NolA from the *trp*

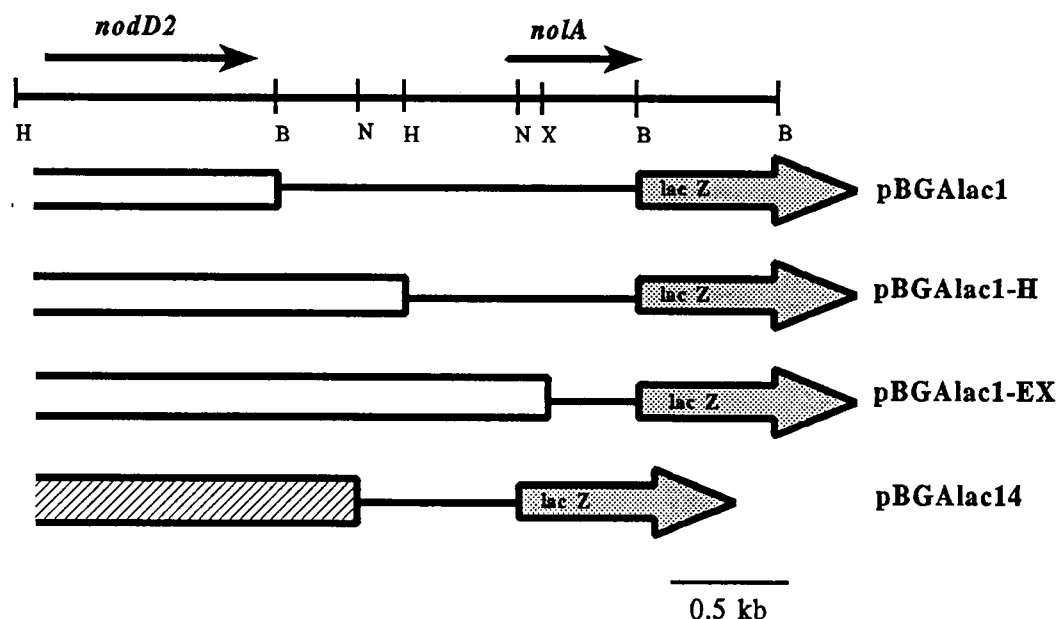


Figure 14: Construction of plasmid-encoded N-terminal and C-terminal *nola-lacZ* fusions. Restriction map of *B. japonicum* USDA110 chromosome showing the location of *nola* and *nodD2*. (Only the pertinent restriction sites are shown: H=*Hind*III, B=*Bam*HI, N=*Nhe*I, X=*Xba*I). Below the restriction map are shown the various *nola-lacZ* fusions used in the study. pBGAlac1 and pBGAlac1-H, the open rectangles represent the pNM480 vector into which the *nola* promoter and coding sequence (thin black line) was cloned to produce an in-frame C-terminal fusion with *lacZ* (filled arrows, labelled); pBGAlac1-EX, DNA sequence internal to the *nola* coding region was cloned into pNM480 (open rectangle) to give a similar C-terminal *nola-lacZ* fusion as in pBGAlac1 and pBGAlac1-H, except that the *nola* promoter and the 5' end of the *nola* coding region was deleted; pBGAlac14, striped rectangle represents the pNM482 vector into which the *nola* promoter and 5' coding region was cloned to give an in-frame N-terminal *nola-lacZ* fusion. The *nola-lacZ* fusion sites in pBGAlac1 and pBGAlac14 were confirmed by DNA sequencing, and are diagramed in more detail in Figure 13.

promoter encoded in the vector pTE3 (i.e. pBGtrpA-88). In *E. coli*, the expression of NolA from the *trp* promoter is repressible by the presence of tryptophan in the growth medium. As a control, the *nolA-lacZ* fusion plasmids were also transformed into an *E. coli* strain harboring only the vector pTE3. As shown in Table 9, the expression of the *nolA-lacZ* fusion encoded on pBGAlac1 or pBGAlac1-H was induced by at least 5-fold when the *nolA* gene was expressed; i.e., in the presence of pBGtrpA-88 and the absence of tryptophan in the growth medium. These data are consistent with the results presented in the previous section showing that the expression of *nolA* is positively autoregulated. However, elevated expression of the *nolA-lacZ* fusion encoded on pBGAlac14 was not observed when NolA was expressed in *E. coli*. This low expression of the pBGAlac14 fusion was not due to the absence of *cis*-acting promoter sequences since pBGAlac1-H, which contains a shorter promoter region than pBGAlac14, gave high β -galactosidase activity. Therefore, these results suggest that either the ATG1 translational start site is not used or that expression from this start site is regulated differently from that measured by the C-terminal *nolA-lacZ* fusions (e.g., pBGAlac1). Specifically, these results suggest that the expression of NolA_L from ATG1 is independent of the *nolA* gene product. The lack of detectable expression of the fusion encoded on pBGAlac-EX was expected since the *nolA* region present in this construct lacks both the transcriptional and translational start signals.

To test whether the N-terminal *nolA-lacZ* fusion encoded on pBGAlac14 was active, this plasmid and plasmid pBGAlac1 were individually cointegrated with pRK290 to form plasmids pBGAlac36 and pBGAlac4, respectively. These plasmids were conjugated

Table 9: Effect of Nola_L on the expression of N-terminal or C-terminal *nola-lacZ* fusions in *E. coli*.

Plasmid	Tryptophan	β -galactosidase activity ^{a,b} (U)	
		pTE3	pBGtrpA-88
pBGAlac1	+	38	26
	—	37	318
pBGAlac1-H	+	75	68
	—	84	452
pBGAlac1-EX	+	0	0
	—	0	0
pBGAlac14	+	4	2
	—	6	7

^a units using CPRG as substrate

^b values are the means of 2 independent determinations, each determination was done in 3 replicates; the standard deviations are <15%

into *B. japonicum* wild-type strain USDA110 and the *nolA* mutant, strain BjD21. As shown in Table 10, *nolA* expression from plasmid pBGAlac1 was reduced six-fold, as compared to the wild type, in the *nolA* mutant background. These results are consistent with previously presented data indicating that expression of the C-terminal fusion is positively autoregulated (i.e. Tables 7, 8 and 9). However, lower expression of the N-terminal *nolA-lacZ* fusion encoded on pBGAlac14 was not observed in the *B. japonicum nolA* mutant background. Therefore, consistent with the data in Table 9, expression of this fusion does not require the *nolA* gene product. The β -galactosidase activity encoded by plasmid pBGAlac14 was significantly increased by the addition of soybean seed extract, but not by the addition of genistein (Table 10). Genistein is a known inducer of *nod* gene expression in *B. japonicum* (e.g, Banfalvi et al. 1988). Other isoflavones, such as daidzein, known to induce *nod* gene expression, as well as other flavonoids, such as luteolin, were also tested but were unable to induce *nolA* expression from plasmid pBGAlac14 (data not shown). Therefore, it would appear that soybean seed extract contains an unidentified compound capable of inducing *nolA* expression. Taken together these data show that Nola_L expression, presumably from ATG1, is inducible by soybean seed extract, but does not require Nola. In contrast, expression of the C-terminal *nolA-lacZ* fusion, presumably initiated from ATG2, is not inducible but requires Nola. Since only Nola_L would contain the N-terminal, helix-turn-helix DNA-binding motif, it can be postulated that it is Nola_L that is required to express the C-terminal *nolA-lacZ* fusion.

Table 10: Expression of the N-terminal or C-terminal *nolA-lacZ* fusion in *B. japonicum* wild-type and *nolA* mutant strains

Strain		β -galactosidase activity ^{a,b} (U)	
		pBGAlac4	pBGAlac36
USDA 110 (wild type)	uninduced	582	102
	+SSE ^c	521	237
	+genistein	582	117
BjD21 (<i>nolA</i>)	uninduced	74	116
	+SSE	80	258
	+genistein	76	95

^a units using CPRG as substrate

^b values are the means of 2 independent determinations, each determination was done in 3 replicates; the standard deviations are <15%

^c soybean seed extract

***nolA* Encodes Two Proteins Differing in Molecular Weight.** (This study was done in collaboration with Dr. John Loh, Univ. Tennessee.)

To obtain high amounts of NolA for antibody production, the NolA polypeptide was expressed in *E. coli* from a T7 promoter. The NolA polypeptide was expressed as a His-tagged fusion protein with an expected molecular weight of 30.5 kD. SDS-PAGE analysis of *E. coli* cells expressing the fusion protein showed a unique band of approximately 33-34 kD (Figure 15). This discrepancy in the apparent and expected molecular weight could be due to the predicted high content of basic amino acids for the fusion protein. This band was detected only in the insoluble fraction, indicating that it was produced in the form of inclusion bodies. Subsequent washing of the inclusion bodies with urea and detergent removed most of the contaminating proteins and resulted in a preparation containing predominantly (approximately 90%) NolA-polyhistidine tagged protein. The NolA polypeptide was further purified by excising the protein from a preparative SDS-PAGE gel, followed by electroelution. The resulting protein (Figure 15, laneA) was used to immunize female New Zealand white rabbits.

Based on the *nolA* DNA sequence, the predicted molecular weight of NolA_L (starting from ATG1) and NolA_S (starting from ATG2) would be 27 and 22 kd, respectively. Western blot analysis of cell extracts of *B. japonicum* wild type and *nolA* mutant strains was performed using the antibody directed against the NolA polypeptide expressed in *E. coli*. As shown in Figure 16, two immunoreactive bands were observed in the cell lysates from the wild-type strain. The apparent molecular weights of the bands are 29,000 and 25,000, only slightly larger than the predicted molecular weights of NolA_L

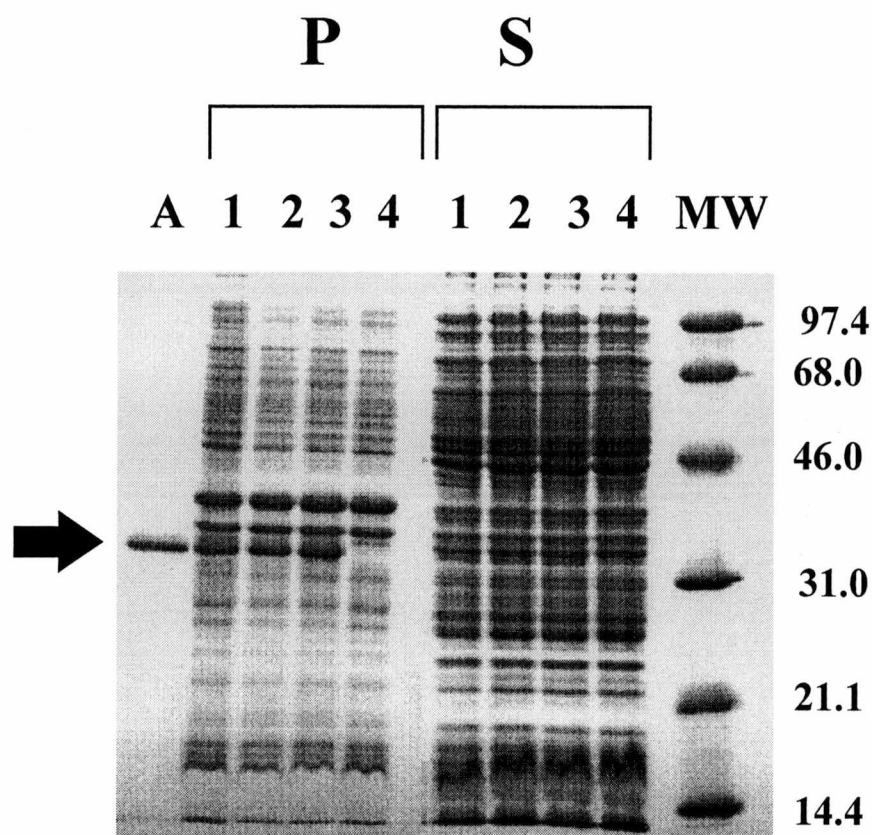


Figure 15: Expression and purification of a His-tagged Nola fusion protein in *E. coli*. SDS-PAGE analysis of soluble (S) and insoluble (P) proteins from *E. coli* cells induced with 1mM IPTG for two hrs. Lanes 1, 2, and 3; cell lysates from cells transformed with pBGT7-A2 that expresses Nola as a His-tagged fusion protein from the T7 promoter encoded on the vector pRSETB. Lane 4; cell lysates from cells transformed with the vector pRSETB. Lane A, purified His-tagged Nola; Lane MW; molecular weight markers (kDa). The arrow indicates the Nola fusion protein.

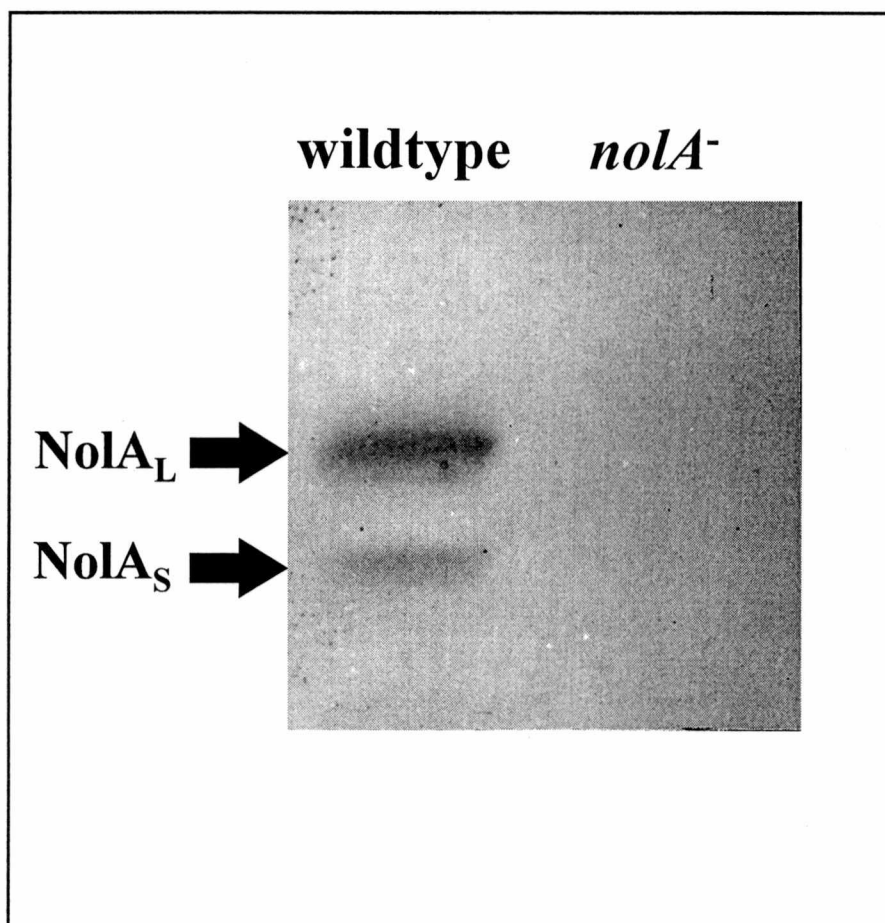


Figure 16: Western Blot analysis of cell extracts from *B. japonicum* wild-type (i.e. strain USDA110, lane 1) and *nolA* mutant (i.e. strain BjB3, lane 2) strains using polyclonal antibody against NolA. The two immunoreactive bands are indicated as NolA_L and NolA_S.

and Nola_S, respectively. This difference between the apparent and expected molecular weights could be attributed to the predicted high content of basic amino acids in Nola. The Nola immunoreactive bands were not observed in cell lysates from the *nolA* mutant strain (Figure 16). The polyclonal anti-Nola antibody used in the Western Blot analysis described above was affinity purified and was preadsorbed with acetone powder made from the *nolA* mutant strain BJB3 (see Materials and Methods). Western Blot analysis of *B. japonicum* wildtype and *nolA* mutant cell extracts was also done using an affinity-purified anti-Nola antibody which was not preadsorbed with strain BJB3 acetone powder. In this case, the two immunoreactive bands described above were also observed in the cell extract from the *nolA* mutant strain (data not shown). In this case, the immunoreactive bands could represent proteins which were cross-reacting with the anti-Nola antibody. However, there is also the possibility that a second copy of *nolA* is encoded by *B. japonicum*.

DETERMINATION OF THE TRANSCRIPTIONAL START SITES OF *nolA* AND *nodD2*

Transcriptional Start Sites of *nolA*

The differential expression of the N-terminal and C-terminal *nolA-lacZ* fusions and the two forms of Nola detected by Western blot analysis, clearly indicate that *nolA* encodes two proteins via translation from two alternative ATG start codons. Therefore, the mechanism by which Nola_L and Nola_S are expressed was investigated. Specifically,

this study examined whether the expression of the two proteins could be controlled transcriptionally via transcription from two promoters, or post-transcriptionally via the use of alternative ATG start codons to translate a single mRNA transcript. As stated previously, *tipA* has also been shown to encode two proteins (Holmes et al., 1993). Since transcription of *tipA* initiates at a single site, it was postulated that expression of TipA_L and TipA_S is regulated post-transcriptionally (Holmes et al., 1993). In contrast, as shown in Figure 17, transcription of *nolA* initiates at two, distinct sites. These results were confirmed using two, independent primers. The first transcriptional start site (from promoter P1) is found 82 bases upstream of ATG1. The second transcriptional start (from promoter P2) is found 7 bases upstream of ATG1 and is immediately downstream of a putative NolA_L binding site (Figure 17). The location of this putative NolA_L binding site is based on its similarity to the promoters of other MerR-type regulatory proteins; i.e., conserved -10 and -35 hexamers separated by 19 bp with an imperfect inverted repeat contained within this 19-bp.

Transcriptional Start Site of *nodD2*

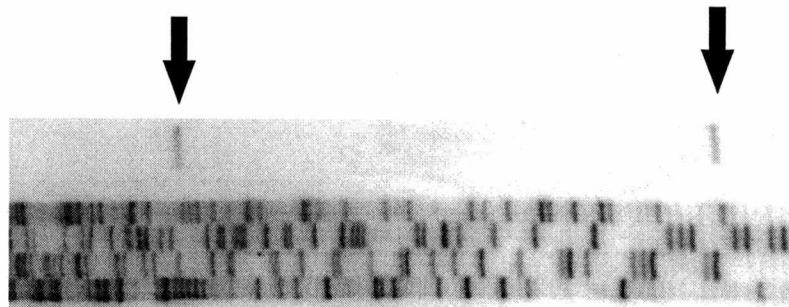
As indicated by previous results, *nodD2* expression requires the *nolA* gene product. Examination of the DNA sequence upstream of the predicted *nodD2* ATG start codon revealed a putative NolA_L binding site that has significant homology to the P2 promoter of *nolA*. Indeed, the results of our primer extension experiments localized the *nodD2* transcriptional start site 7-bp downstream of this putative NolA_L binding site (Figure 18). These results provide further support to the idea that NolA_L acts as

Figure 17: Determination of the transcriptional start sites of *nolA*. **A.** Results of primer extension experiments using 50 µg of RNA from uninduced *B. japonicum* cells (lane 1) and from cells which were induced with soybean seed extract (lane 2). A DNA sequencing ladder is shown for comparison. The two *nolA* transcripts are indicated by arrowheads. **B.** The *nolA* promoter region showing the location of the two transcriptional start sites, designated as P1 and P2. The location of ATG1 and ATG2 relative to the transcriptional start sites are shown. A putative Nola_L binding site located immediate upstream of P2 is also shown as characterized by the presence of -10 and -35 hexamers (boxed) separated by 19 bps, with a dyad symmetry (arrows) found within the 19-bp region.

A

SSE

GATC - +



B

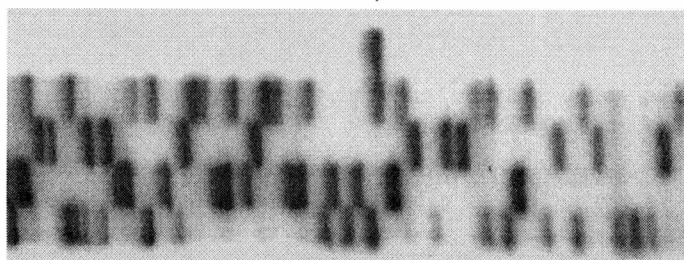
GCGAACTTGT TGATCACCCCT TTCCTCTGAG GGTTCATCAG CCGCCATTCT
 AAGGATAGTC ACCACTCGCG CGCATGAATT AAAGGCGATT CAACTGGAGC
 TTAGAAATCC GCTTGAATCA TACGTGACGT CAGGTTGTAG GCTTTGAAAT
 TGAACAACAT GAACAGAGCT ACACCAAGAC GCGCTCGATG GCGCATTTGGC
 GAACTTGCAG AGGCGACCGG AGTAAACGGTG CGCACGCTGC ACCATTATGA
 GCAACCTTGA CTGCTAGCAG CGACAGAGCG CACTGAGGGC
 GGTCACCGGA TGTATGACCG CGAAAGCGGG
 ATG2

P1 ↑
 -35
 -10
 P2 ↑
 ATG1

Figure 18: Determination of the transcriptional start site of *nodD2*. **A.** The *nodD2* transcript (indicated by an arrow) as determined by primer extension experiments using 50 µg of RNA extracted from uninduced *B. japonicum* cells. A DNA sequencing ladder is shown as comparison. **B.** The *nodD2* promoter region showing the location of the transcriptional start site and the proposed *nodD2* ATG start codon. The putative Nola_L binding site found immediately upstream of the *nodD2* transcriptional initiation site is also shown. The putative -10 and -35 hexamers are boxed, and the inverted repeats are indicated by arrows.

A

G A T C



B



positive transcriptional regulator of *nodD2* expression, as well as that of *nolA*.

CHAPTER V

DISCUSSION

REGULATION OF *nod* GENE EXPRESSION BY *NolA*

Previously, Dockendorff et al. (1994a) suggested that *NolA* is a repressor of *nod* gene expression in *B. japonicum*. This suggestion was based on the fact that a strain deleted for *nodD1*, *nodD2*, and *nolA* showed elevated *nod* gene expression and that expression of *nolA* from a multi-copy plasmid resulted in a significant reduction in *nodC-lacZ* activity. Repression of *nod* gene expression has also been reported in other rhizobia. For example, a repressor protein, *NolR*, has been identified in certain strains of *Rhizobium meliloti*. *NolR* has been shown to bind to a 26-bp site in the overlapping *nodD1* and *nodABC* promoters, thereby inhibiting the transcription of these genes. *NolA* and *NolR* show no sequence similarity. However, since the *nolA* gene predicts a protein having a helix-turn-helix DNA binding domain, it was hypothesized that *NolA* mediates the repression of *nod* gene expression in *B. japonicum* by binding to the promoters of these genes. To test this hypothesis, the expression of a plasmid-encoded *nodD1-lacZ* or *nodY-lacZ* fusion was measured in *B. japonicum* wild-type (i.e. strain USDA110) or *nolA* mutant (i.e. strains BJD21 and BJB3) backgrounds. Contrary to the proposed hypothesis, mutations in *nolA* did not result in higher expression of *nodD1* or *nodY*. Therefore, unlike

NolR, NolA does not appear to act directly on the promoters of these genes to mediate repression.

The expression of *nodD2*, unlike *nodD1*, is independent of plant-produced isoflavones and requires the *nolA* gene product. The latter observation is interesting in view of recent reports suggesting that proteins homologous to NodD2 are involved in the repression of *nod* gene expression in *Bradyrhizobium* sp. (*Arachis*) NC92 (Gillette and Elkan, 1996) and *Rhizobium* sp. NGR234 (Broughton et al., personal communication). Therefore, to determine if the *B. japonicum* NodD2 protein can repress *nod* gene expression, NodD2 was expressed from a constitutive promoter and its effect on the expression of a chromosomal *nodC-lacZ* fusion was determined. Indeed, elevated expression of NodD2 resulted in a drastic reduction of *nodC* expression. Since NolA is required for NodD2 expression, it is conceivable that NolA mediates the repression of *nod* genes indirectly, possibly through NodD2. The idea that NolA acts in concert with NodD2 is also suggested by the results reported by Dockendorff et. al. (1994a) showing that the repressive effect of NolA is more pronounced in the presence of NodD2. The mechanism by which NodD2 mediates *nod* gene expression is presently unclear. One possible mechanism is that NodD2 interferes with the positive regulatory function of NodD1. Studies on several rhizobial species have indicated that NodD1 binds to the promoters of *nod* genes as a dimer (Egelhoff and Long, 1989; Goethals et. al., 1992; Wang and Stacey, 1991). Since NodD2 shares 62% amino acid homology to NodD1, it is possible that these two NodD proteins can form a heterodimer. Formation of such a heterodimer could inactivate NodD1 or form an inactive complex when bound to the *nod* gene promoter.

ROLE OF *nolA* IN NODULATION AND NITROGEN FIXATION

The nodulation and nitrogen fixation phenotypes of the *nolA* mutant strains BjD21 and BjB3 were tested on *Glycine max* (soybean) cv. Essex and *Vigna unguiculata* (cowpea) cv. Caloona. When compared to the wild type, the *B. japonicum nolA* mutant strains showed only a slight delay in soybean nodulation. This slight delay in nodule development was also apparent when nodules induced by the wild-type and mutant strains were examined by light and electron microscopy. For example, at 14 days PI, plant cells infected by the wild type showed more bacteroids per symbiosome and the bacteroids had more internal deposits of polyhydroxybutyrate. At 21 days PI, however, plant cells infected by the wild-type and mutant strains were morphologically indistinguishable from each other.

In contrast to soybean plants infected by the *nolA* mutants strains, cowpea plants nodulated by the mutant strains showed a drastic reduction in nodulation efficiency and nitrogen fixation activity. Microscopic examination of cowpea nodules infected by strain BjD21 showed the lack of clearly identifiable symbiosomes. At 14 days PI, bacteroids were in large vesicular-like structures filled with fine granular material. The nature of this material is unknown, as is the origin of these structures. It is possible that the vesicular-like structures represent improperly formed symbiosome membranes or symbiosome membranes that have started to degenerate. By 21 days, it appeared as if these structures had disintegrated leaving the bacteroids free in the cytoplasm. In some respects this resembles the effects of early nodule senescence (c.f., Fischer *et al.*, 1986; Kullik *et al.*,

1991; Anthamatten and Hennecke, 1991). However, contrary to such reports, the morphology of the mutant bacteroids did not suggest a state of degradation, except for their lack of symbiosome membranes. This is also suggested by the fact that these nodules showed low, but measurable levels of nitrogen fixation (i.e., 10% of the wild-type level). Thus, it may be that the *nolA* mutants induce the early degeneration of the symbiosome membrane, but without inducing a rapid onset of senescence. Nodule morphology was not followed beyond 21 days to see if bacteroid degradation did eventually occur.

Negative control of *nod* gene expression has been proposed to be important for optimum nodulation, presumably by fine-tuning the induction of *nod* genes and thus preventing the over-production of Nod signals (Kondorosi et al., 1989). This proposal stemmed from studies in *Rhizobium meliloti* where a repressor protein, NolR, has been identified. Most *R. meliloti* strains and field isolates encode this repressor and the repressor-producing strains are slightly more efficient in alfalfa nodulation. It was also found that in *R. meliloti* strain AK631, mutations in *nolR* resulted in higher expression of *nod* genes and a consequent lower nodulation efficiency (Kondorosi et al., 1989; Kondorosi et al., 1991b). By analogy to NolR, Dockendorff et al. (1994a) proposed that NolA is required for optimum nodulation in *B. japonicum* by fine-tuning the amount of Nod signal produced by this rhizobium. However, it is unlikely that the reduced nodulation efficiency of the *B. japonicum nolA* mutant strains on soybean and cowpea is due to an effect on Nod signal production. This statement is supported by the results presented in this study showing that the expression of a *nodY-lacZ* or *nodDI-lacZ* fusion was not affected in the *nolA* mutants. Moreover, analysis of the lipo-chitin Nod signals

produced by the *nolA* mutant strains by thin layer chromatography indicated that the mutants produce identical Nod signals as the wild type and in equivalent amounts. It seems more likely that NolA plays an important role in nodulation by regulating other, as yet, unidentified genes. Previous reports have found that nodulation competency can also be limited by factors other than the nodulation signals produced. These factors may be due to general rhizosphere genes that enable the bacteria to survive and colonize the roots of host plants, such as the ability to metabolize plant-produced substrates and attach to plant root hairs (see Chapter II for detailed discussion of factors affecting nodule initiation). Therefore, it is possible that NolA functions in nodulation by mediating the expression of gene(s) that specify any of these bacterial determinants.

Previously, we reported that mutations in the *hsfA* gene led to a loss of nitrogen fixation ability on cowpea, but not on soybean (Chun et al. 1994). However, ultrastructural analysis of soybean nodules infected by the *hsfA* mutant did reveal minor differences from wild-type nodules. Thus, the phenotype of both the *nolA* and *hsfA* mutants is more severe on cowpea, than on soybean. This indicates clearly that there are differences in the response of soybean and cowpea plants to *B. japonicum* infection. A difference in the response of soybean and cowpea plants to *B. japonicum* inoculation was also reported by Bauer et al. (1985). The ability of different *B. japonicum* strains to nodulate cowpea and soybean plants was tested and it was found that cowpea required 100-fold-higher inoculum levels to initiate early nodulation responses. Likewise, although the regulatory proteins NodD1 and NodW can functionally complement one another with regard to soybean nodulation, only NodW is essential for cowpea nodulation (Sanjuan et

al., 1994; Göttfert et al., 1990).

Although much is known about the bacterial determinants required during the early (i.e. *nod/nol/noe* genes) and late (i.e. *nif/fix*) events of the symbiotic process, relatively little is known about the functions required for the intermediate steps of nodule development (e.g., infection thread elongation, release into plant cells, and bacterial differentiation into mature bacteroids). Studies on various *Rhizobium* and *Bradyrhizobium* species have identified several genes that are required for bacteroid maturation. Rhizobia that carry mutations in these genes are able to initiate nodules but are defective in the formation of mature bacteroids. These genes have been proposed to be important in proper bacteria-plant signalling [e.g., genes for the biosynthesis and/or transport of exopolysaccharide (reviewed by Leigh and Walker, 1994), cyclic β -(1,2) glucan (Dylan et al., 1986), plant-produced signal peptides (Glazebrook et al., 1993)], bacterial metabolism inside the plant [e.g., genes for the transport of C4-dicarboxylic acids (Finan et al., 1983), anthranilate and leucine biosynthesis (Dickstein et al., 1991; Truchet et al., 1980), and bacteroid respiration (e.g., Thony-Meyer et al., 1989)], and regulation of nitrogen fixation [e.g., *nifA* (Fischer et al., 1986), *rpoN1/N2* (Kullik et al., 1991), or *fixLJ* (Anthamatten and Hennecke, 1991)]. Judging from the nodule ultrastructure of plants inoculated by the *nolA* mutants, it is very likely that NolA mediates the expression of genes that are important for bacteroid development and maintenance. Moreover, such genes are host-specific in that they would be required for proper bacteroid maturation in cowpea but not in soybean. Previous reports suggested that premature nodule senescence may be due to the induction of plant host defense response

(Werner et al., 1985; Parniske et al., 1991). For example, ineffective soybean nodules accumulate the phytoalexin glyceollin I (Werner et al., 1985; Parniske et al., 1991), and show early loss of symbiosome membrane (Werner et al., 1985). The latter observation is interesting in view of the results presented in this study showing that cowpea nodules induced by the *nolA* mutant strains contained bacteroids having an atypical symbiosome membrane. This atypical symbiosome morphology could represent an early stage of bacteroid degeneration. Therefore, NolA may be involved in mediating the expression of bacterial determinant(s) that suppress plant host defense response.

FUNCTION OF *nolA* IN GENOTYPE-SPECIFIC NODULATION

Cregan and Keyser (1986) identified several soybean genotypes (e.g., Plant Introduction lines, PI377578 and PI 371607) which restrict nodulation of *B. japonicum* strain SD6-1c, but not strain USDA110. Subsequently, Sadowsky et al. (1991) found that a gene in strain USDA110 (i.e. *nolA*), when conjugated into strain SD6-1c, allowed this latter strain to overcome host restriction. Subsequent DNA hybridization experiments showed that there was no correlation between the presence of the *nolA* gene and the ability to nodulate the restricting soybean genotypes (i.e. certain strains were found to lack *nolA* but were still able to nodulate the restricting soybean genotypes) (Sadowsky et al., 1991). These data suggest that genotype-specific nodulation may be controlled by multiple bacterial genes. Regardless of this report, in this study, experiments were performed to test the role of the *nolA* gene in mediating genotype-specific nodulation by

strain USDA110. Results presented in this study showed that the *nolA* mutant (i.e. strain BjD21), similar to the wild-type USDA110, was able to nodulate soybean genotype PI 371607. Moreover, similar to the wild-type USDA110, the *nolA* mutant strain was restricted on soybean genotype PI471566. Therefore, *nolA* does not seem to play a critical role in the ability of strain USDA110 to nodulate PI371607, nor in the nodulation restriction of strain USDA110 by soybean genotype PI471566.

REGULATION OF *nolA* EXPRESSION

The *nolA* gene of *B. japonicum* encodes two proteins designated as Nola_L and Nola_S. This statement is supported by the differential regulation of N-terminal and C-terminal translational *nolA-lacZ* fusions and by the observation of two protein bands on Western blots using an anti-NolA antibody. There are only a few procaryotic genes in which ribosomes initiate translation from one continuous reading frame at two sites (Holmes et al., 1993; Park et al., 1993; Seol et al., 1994). A notable example is the *tipA* gene of *Streptomyces lividans* whose expression, like that of *nolA*, is autoregulated. The *tipA* gene can be translated from two alternative ATG start codons to give a full-length protein (TipA_L) having an N-terminal, helix-turn-helix DNA-binding motif and an N-terminally truncated protein (TipA_S) that lacks the N-terminal DNA-binding domain. TipA_L functions as a MerR-type regulator that activates transcription upon binding the inducer molecule thiostrepton at its C-terminal domain. It has been proposed that TipA_S functions in the autogenous control of *tipA* expression. This proposal is based on the fact

that TipA_S possesses the same C-terminal, thiostrepton-binding domain as TipA_L and, therefore, could compete for the binding of thiostrepton. The thiostrepton sequestered by binding to TipA_S would be unavailable for interaction with TipA_L, thereby modulating the activity of this transcriptional regulator. All the known MerR-type regulatory proteins require an inducer molecule which binds to the C-terminal domain (Figure 19) and is necessary for transcriptional activity (Helman et al., 1990; Hidalgo and Demple, 1994; Holmes et al., 1993; Ahmed et al., 1994). All these inducers are compounds toxic to bacterial cells and the genes that the MerR-type proteins regulate are involved in conferring resistance to these inducing compounds. Therefore, it is likely that such an inducer molecule is required for NolA_L activity. Once this molecule is identified it should be possible to test whether NolA_S functions in a similar manner to that of TipA_S to affect the activity of NolA_L.

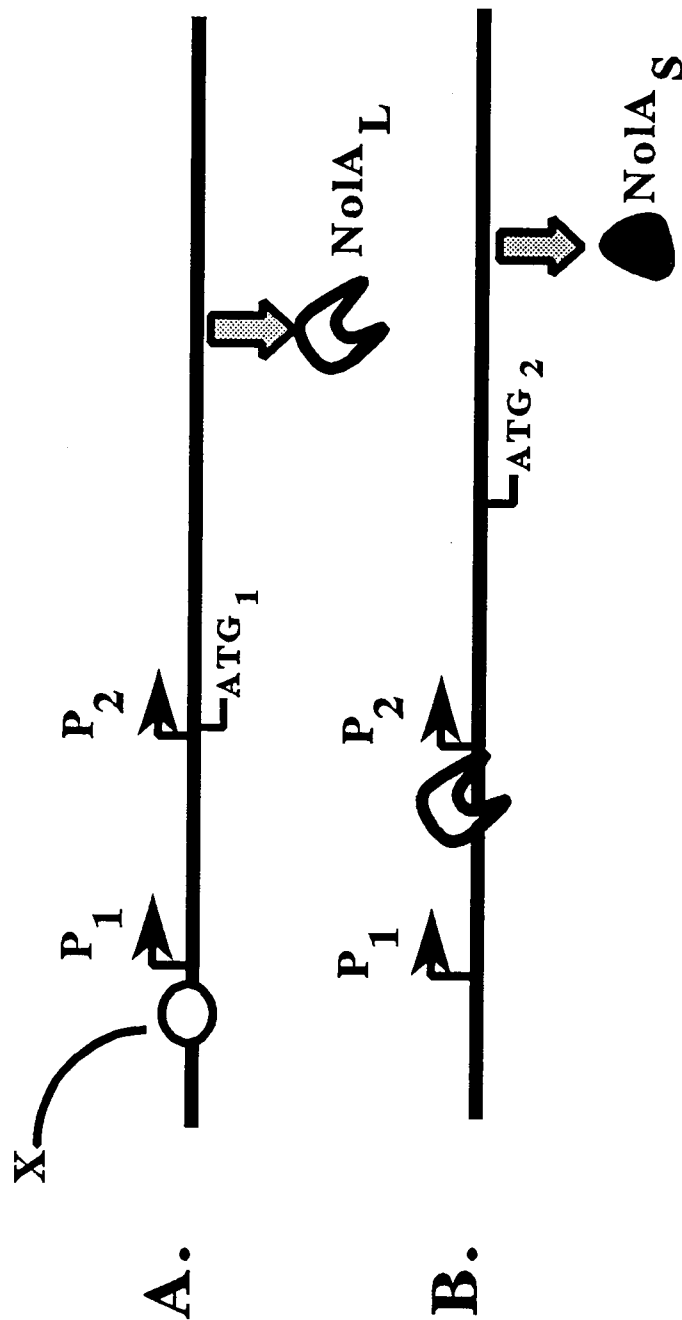
In contrast to *tipA* expression, *nolA* transcription initiates at two, distinct start sites. The P1 promoter appears to mediate the inducible expression of NolA_L, while the P2 promoter appears to require NolA (presumably NolA_L) for activity. Whereas TipA_L and TipA_S expression has been proposed to be regulated post-transcriptionally, the presence of the P1 and P2 promoters and their location suggest a mechanism by which NolA_L and NolA_S expression could be transcriptionally controlled. Transcription from P1 starts 82 bases upstream of ATG1, whereas transcription from P2 starts 7 bases from the ATG1 codon and is immediately downstream of the putative NolA_L DNA-binding site. The respective locations of P1 and P2 suggest a model (Diagrammed in Figure 20) for the transcriptional control of NolA_L and NolA_S expression that should be useful for predicting

REGULATOR	CO-REGULATOR	ACTIVATES
NolA _L	?	<i>nolA, nodD2</i>
TipA _L	thiostrepton	<i>tipA</i>
MerR	mercury	<i>merT</i>
SoxR	superoxide	<i>soxS</i>

Figure 19: Co-activators and genes controlled by the MerR-type regulatory proteins.

Figure 20: Proposed model for the transcriptional control of Nola_L and Nola_S expression.

An inducer present in soybean seed extract (X) induces *noIA* transcription from P1. This allows for efficient translation from ATG1, leading to the production of NoIA_L.



NoIA_L, produced as in A, binds to the *noIA* promoter and initiates transcription from P2. The spacing of P2 relative to ATG1 leads to inefficient translation from ATG1, favoring translation from ATG2. This gives rise to the production of NoIA_S. Note that production of NoIA_L is inducible, while production of NoIA_S requires NoIA_L.

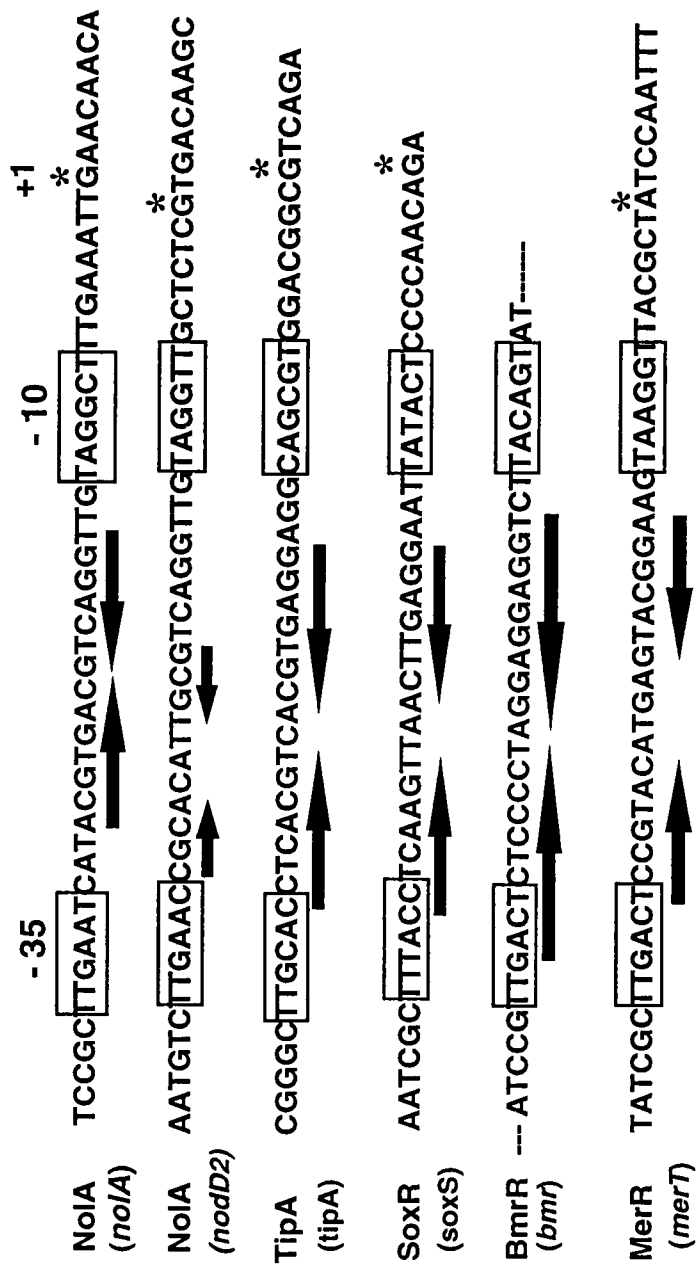
future experiments. In this model, initiation of transcription from P1 results in the production of Nola_L by translation from ATG1. Transcription from P1 is not regulated by Nola_L, as indicated by the results of the *nola-lacZ* studies (i.e., pBGAlac14), as well as by the absence of a putative Nola_L DNA-binding site upstream of P1. Rather, transcription from P1 is induced by an unidentified cellular factor(s) (indicated by “X” in Figure 20) present in *B. japonicum* which can be activated by soybean seed extract. Once Nola_L is produced, this protein can bind to the P2 promoter and initiate transcription from P2. However, the mRNA made from P2 would have only 7 bases before ATG1, likely resulting in inefficient translation from this start site. This fact alone may be sufficient to account for the use of ATG2 when transcription is initiated from P2. Translation from ATG2 results in the production of Nola_S. This model predicts that Nola_S production would require Nola_L, which is consistent with the results of the *nola-lacZ* fusion studies (e.g., pBGAlac1). It is possible that Nola_S can also be translated from the mRNA initiated at P1. However, the 5-fold higher relative expression of β -galactosidase from the C-terminal *nola-lacZ* fusion encoded on pBGAlac1, as compared to that from pBGAlac14, suggests that such expression would be negligible. This also likely explains why no inducible expression is seen from pBGAlac1.

In addition to the *nola* gene, *nodD2* expression also requires Nola. Likewise, the expression of *nola* and *nodD2* in *Bradyrhizobium* sp. (*Arachis*) NC92 has been reported to be regulated by a common mechanism requiring Nola (Gillette and Elkan, 1996). Examination of the *B. japonicum nodD2* promoter revealed a putative Nola_L DNA-binding site and primer extension experiments localized the transcriptional start site of

nodD2 immediately downstream of this putative promoter. Comparison of this putative Nola_L-binding site to that found in the *nolA* promoter (i.e. upstream of P2) showed that the DNA sequence of the two sites are highly identical (Figure 21). The *nodD2* transcriptional start site is somewhat unusual since it is 328 bp upstream of the predicted ATG start codon for NodD2. However, such a situation is not without precedent. For example, the *syrM* and *nodD3* genes of *R. meliloti* are transcribed from a start site 499 bp and 659 bps 5' of their ATG start codons, respectively (Barnette et al., 1996).

The promoter regions recognized by the MerR-type regulatory proteins contain conserved -10 and -35 hexamers which are separated by 19 bps, rather than the typical 17 bps. An inverted repeat is found within these 19- bps intervening sequences. The putative Nola_L binding site in the promoters of *nolA* and *nodD2* shares significant sequence similarity to the DNA target sites of other MerR-type regulatory proteins (Figure 22). The putative DNA binding site of Nola_L is most similar to that recognized by TipA_L. This is not surprising since the helix-turn-helix DNA binding domains of Nola_L and TipA_L share 10 identical amino acids in their second, so called recognition helix. Efforts to directly show the interaction of Nola_L with *nolA* promoter fragments using gel-shift assays (Fried and Crothers, 1981) were unsuccessful (data not shown). These failures may have been due to methodical problems, e.g., difficulty in isolating active Nola_L. Alternatively, Nola_L binding to DNA may require the postulated, unidentified co-inducer. Additional experiments will be required to show that Nola_L does bind to DNA.

Figure 22: Comparison of the putative Nola_L DNA binding site to those recognized by the MerR-type regulatory proteins. The -10 and -35 hexamers which are separated by 19 bps are boxed. The transcriptional start sites are indicated by asterisks (*). The inverted repeats are indicated by arrows.



CONCLUDING REMARKS

Our current model for *B. japonicum* *nod* gene regulation predicts that transcription is controlled through the concerted action of specific members of three families of global regulators (diagrammed in Figure 23). Induction of *nod* gene expression occurs in response to plant-produced isoflavones mediated by a LysR-family protein, NodD1, and a member of the two-component regulator family, NodW. It is a unique and rather curious feature of *B. japonicum* that these two, apparently redundant systems function to activate *nod* gene expression. One explanation is that NodW is required for the nodulation of alternative hosts, such as cowpea, for which NodD1-mediated *nod* gene expression is insufficient. A member of the MerR-family of transcriptional regulators, NolA, does not appear to directly repress *nod* gene expression. Instead, NolA may activate the expression of additional genes, perhaps NodD2, that, in turn, repress *nod* gene expression. However, it is becoming obvious that *nod* gene regulation in *B. japonicum* is very complex, and involves the interplay of different regulatory factors. Therefore, attributing the repressive effects of NolA expression to an effect on *nodD2* alone may be a simplistic way of interpreting the data presented in this study. At present, the biological need for such complexity in *nod* gene regulation is unclear but no doubt arises from the interesting biology surrounding the interaction of this bacterium with its variety of host plants.

NolA can exist in two forms of which only one (i.e., NolA_L) possesses the N-terminal, helix-turn-helix DNA-binding motif and, therefore, can act as a transcriptional regulatory protein. Phenotypic studies have shown that NolA is required for the critical

Figure 23: Proposed Model for *nod* gene regulation in *B. japonicum*.

initial steps in nodule initiation and infection. Moreover, examination of cowpea nodules formed by a *nolA* mutant suggests that NolA is also involved, at least on this host, with later steps in nodulation involved in bacteroid formation and maintenance. These phenotypes have been attributed to a proposed role for NolA_L as a DNA-binding transcriptional regulatory protein. Indeed, NolA_L is required for the expression of NolA_S, as well as NodD2. Moreover, the data presented in this study, though not conclusive, provide strong evidence suggesting that NolA_L regulates the expression of *nolA* and *nodD2* by binding to a conserved site found in the promoters of these genes. The variety of phenotypes on different hosts suggests that, in addition to *nodD2* and *nolA*, NolA_L likely is involved in regulating other, as yet unidentified, genes. These genes could specify bacterial determinants which are involved in the early events of nodule initiation, as well as in bacteroid maturation. The identification of the putative DNA binding site of NolA_L, which was done in this study, could be helpful in the future identification of such genes. The function of the truncated form of NolA (i.e. NolA_S) is still unknown. By analogy to other proteins (e.g., TipA_S), NolA_S may play a role in modulating the regulatory activity of NolA_L. However, this and much more about NolA remains to be investigated.

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VITA

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