To the Graduate Council:

I am submitting herewith a dissertation written by Thomas C. Dockendorff entitled "Novel Regulatory Mechanisms for the Nodulation Genes 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.

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NOVEL REGULATORY MECHANISMS FOR THE NODULATION GENES OF *BRADYRHIZOBIUM JAPONICUM*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Thomas C. Dockendorff

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ABSTRACT

Genetic studies were carried out to ascertain the functions and mechanisms of regulation for a new flavonoid induced locus in *Bradyrhizobium japonicum* USDA110. These studies revealed that this locus is comprised of two open reading frames that have been designated *nolY* and *nolZ*. These genes have been found to be regulated by NodD1 and NodW, a manner similar to the regulation of the *nodD1* and *nodYABC* genes of USDA110. Mutational analysis of *nolY* and *nolZ* showed that these genes were not essential for nodulation of host plants.

Studies were undertaken to analyze *nod* gene expression in USDA110. The use of different *nod* gene regulatory mutants for these studies produced several novel results. The *nodD2* gene product might be important for regulation of *nodYABC*, *nodD1*, and *nolYZ*. Expression of *nodYABC* and *nodD1*, but not *nolYZ*, in a strain deleted of *nodD1, nodD2, nolA*, and two open reading frames downstream of *nolA*, was found to be similar to that of wild-type strains upon isoflavone induction. This result suggested that some type of repression unique to *nodYABC* and *nodD1* had been relieved. The product of the genotype-specific nodulation gene *nolA* is proposed to be a repressor of *nodD1* and *nodYABC*. The phenotype of a *nodD1 nodD2 nolA* deletion (*i.e.* poor nodulation, wild-type levels of common *nod* gene expression) suggests that other unknown genes are necessary for efficient nodulation of host plants.

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LIST OF ABBREVIATIONS

M	molar
mM	millimolar
μM	micromolar
l	liter
ml	milliliter
μl	microliter
m	meter
nm	nanometer
g	times gravity
hr	hour
bp	basepair
kb	kilobase
OD_{600}	optical density at 600 nm
R_f	relative migration
SDS	sodium dodecyl sulfate
EDTA	ethylenediaminetetraacetic acid
SSC	saline sodium citrate
SSPE	saline sodium phosphate EDTA
Ap^r	ampicillin resistant
Km^r	kanamycin resistant
Sm^r	streptomycin resistant
Spc^r	spectinomycin resistant
Tc^r	tetracycline resistant
Tc^s	tetracycline sensitive

CHAPTER I

INTRODUCTION

Members of the genera *Rhizobium, Bradyrhizobium,* and *Azorhizobium* are Gram negative, soil-dwelling bacteria that have the unique ability to infect roots (*Azorhizobium* can also infect stems) of certain classes of leguminous plants to form nitrogen fixing symbioses. Nitrogen fixation takes place in a specialized organ, the nodule, that develops on host plant roots or stems in response to the infection. In this symbiosis, atmospheric nitrogen is reduced to ammonia by the bacteria. The ammonia moves to host plant cells, and is converted to a suitable organic form that is then transported to other plant tissues. In return, the bacteria receive a carbon source from the host plant in order to derive energy required for nitrogen fixation. This process is thought to be unique to these bacterial genera, the actinomycete *Frankia*, and with few exceptions, the legume family of plants (See Long, 1989; Nap and Bisseling, 1990, for reviews).

Benefits from the *Rhizobium*-legume interaction have been noted since the earliest days of agriculture in the form of enriched soil fertility after legume cultivation. Hellriegel and Wilfarth (1888) and Beijerinck (1888) were the first to demonstrate the role that rhizobia and root nodules play in the establishment of an effective symbiosis. Since then, the *Rhizobium*-legume interaction has been extensively studied in

attempts to derive agricultural benefits such as improved yields, development of rhizobial inocula, and the transfer of symbiotic nitrogen fixation to non-leguminous crops. These studies have brought insights into symbiotic relationships and other plant-microbe interactions, and may facilitate studies on plant developmental processes and mechanisms of signal transduction.

Rhizobial classification

Early studies on *Rhizobium*-legume interactions demonstrated that certain rhizobial strains were limited to a certain subset of legumes that they could successfully nodulate. Host range and other physiologic parameters have therefore been used as the basis for assigning genus and species to rhizobial strains, although such methods have long been challenged (see Elkan, 1992 for discussion). The family Rhizobiaceae includes three genera; *Rhizobium, Bradyrhizobium,* and *Azorhizobium. Rhizobium* spp. are differentiated from *Bradyrhizobium* spp. largely on the basis of growth rate and patterns of mannitol utilization. *Rhizobium* spp. have a doubling time of 2-4 hours and produce acidic end products from mannitol catabolism, while *Bradyrhizobium* spp. have a doubling time of 7-20 hours and generate alkaline products from mannitol (Prakash and Atherly, 1985). In addition, there are differences in organization of symbiotic genes between *Rhizobium* and *Bradyrhizobium*. *Rhizobium* spp. tend to have many of their symbiotic genes localized on large plasmids, while symbiotic

genes appear to be chromosomally located in *Bradyrhizobium*. The genus *Azorhizobium* is unique in that, in addition to root nodules, it elicits nodules on the stems of its host plant and is able to fix nitrogen in the free-living state as well as the symbiotic state (de Bruijn *et al.* 1988).

Species designations among rhizobia is primarily dictated by plant species nodulated. Table 1 shows a listing of various rhizobial species and primary host plants nodulated. In general, *Bradyrhizobium* spp. tend to have a broader host range than do *Rhizobium* spp. One notable exception is *Rhizobium* spp. NGR234 (MPIK3030). This particular organism can nodulate about 20 different legume genera, although some interactions are ineffective, (*i.e.* no nitrogen fixation occurs; Stanley and Cervantes, 1991). The mechanisms by which host specificity is achieved have long been a source of intrigue for researchers in the field.

Outline of nodulation

The development of the nitrogen fixing nodule is a complex process and requires both plant and bacterial functions. A critical factor in the establishment of the symbiosis is the exchange of signal molecules between the host plant and the potential symbiont. Colonization of the rhizosphere surrounding the host plant root is presumed to be necessary for further steps of the infection process to take place. Several studies have been done to elucidate a potential role for bacterial chemotaxis to the host plant root in the

Table 1: Examples of rhizobial strains and primary host plants nodulated.

Rhizobial strain	Plant host
Rhizobium meliloti	Alfalfa Sweet clover
Rhizobium leguminosarum	
biovar *viciae*	Peas, Vetch
biovar *phaseoli*	Beans
biovar *trifolii*	Clover
Rhizobium sp. NGR234	Many hosts
Rhizobium fredii	Soybean, Siratro, several others
Bradyrhizobium japonicum	Soybean, Siratro, Mungbean, Pigeon pea, Cowpea
Azorhizobium caulinodans	*Sesbania*

infection process. While it appears that bacterial motility is important for competitiveness for nodule occupancy, a role for specific chemotaxis towards host plants is less clear. *Rhizobium* and *Bradyrhizobium* spp. studied are clearly chemotactic towards plant root exudates, but until chemotactic mutants are isolated, the importance of specific chemotaxis will not be known (reviewed by Roth and Stacey, 1991).

Attachment of rhizobia to root hairs is thought to be essential for successful infection (Roth and Stacey, 1991). An early hypothesis invoked specific binding between plant and bacteria that was mediated by the interaction of plant lectin with a specific bacterial cell surface carbohydrate residue (Bohlool and Schmidt, 1974). It was further hypothesized that different lectins, having different ligand affinities, might be a basis for host specificity. Initial results seemed to support these views, with the finding that in certain cases, only homologous rhizobia bound to root hairs and that the addition of carbohydrate hapten could inhibit bacterial binding. Failure to reproduce these results in other laboratories cast doubt upon the lectin hypothesis (see Halvorson and Stacey, 1986, for review). However, recent work with transgenic plants showed that the addition of a pea lectin gene to white clover plants could enhance nodule formation by the heterologous symbiont *R. leguminosarum* bv *viciae* (Diaz *et al.* 1989). This result suggests that a host

lectin is involved in mediating host specificity, although the exact mechanism remains unclear.

Upon attachment of bacteria to root hair surfaces, marked deformations (curling) of such root hairs occurs. Bacteria then become entrapped within these curls. The infection begins within these curls, with the rhizobia penetrating the cell wall of the root hair. Concomitant to root hair curling is the rapid division of cortical cells in the vicinity of the protruding root hair. An infection thread, formed from an invagination of the root hair cytoplasmic membrane, develops at the site of bacterial penetration. Plant cell wall material is deposited to form the infection thread, and bacteria travel through this structure towards the dividing cortical cells. The infection thread penetrates into the cytoplasm of the cortical cells, where bacteria are released. Upon release, the bacteria are surrounded by a membrane of plant-derived origin termed the peribacteroid or symbiosome membrane. The bacteria undergo poorly understood developmental changes to form bacteroids upon internalization within a cortical cell. Differentiation to the bacteroid state is accompanied by several chemical and morphological changes that include alterations in nucleoid structure, heme content, and cell wall structure (Sutton, 1981, Brewin *et al.* 1993). The bacteroids and the plant-derived membrane that surrounds them have been collectively termed the symbiosome (Roth *et al.* 1988), which can be considered the functional unit of nitrogen fixation.

Nodule development has recently been reviewed by Brewin (1991).

Recent developments in *Rhizobium*-legume research

Research focusing on host-symbiont specificity and the early stages of nodule development has taken advantage of the relative ease with which bacterial genomes can be mutated and manipulated. Such studies have led to the description of many genes in rhizobia that are important or essential for symbiosis. In addition, the nature and function of signal molecules transferred between host and symbiont have been elucidated by studying various bacterial mutants or by analyzing the regulation of both plant and bacterial symbiotic genes thought to take part in initiating the symbiosis (Nap and Bisseling, 1990; Fisher and Long, 1992). Despite these strides made in the knowledge of the symbiotic partners, many relevant details of the interaction need to be worked out. Fine-tuning of the regulation of bacterial symbiotic genes has recently been recognized as being important for optimizing the symbiosis (Kondorosi *et al.* 1989, 1991b). After a decade of investigation, it seems doubtful that all relevant rhizobial symbiotic genes have been identified. Studies on nodulins, plant proteins produced in response to rhizobial infection, have been accelerated with the cloning of nodulin genes, and it has been shown that the addition of bacterial signal molecules induces transcription of some of these genes (Scheres *et al.* 1990; Bisseling *et al.* 1993). Mechanisms by

which signal transduction pathways in host plants trigger the developmental changes necessary for nodule formation are essentially unknown. Identification of additional factors and their functions in both bacteria and plants will be requisite for further insights into development of the *Rhizobium*-legume symbiosis.

CHAPTER II

LITERATURE REVIEW

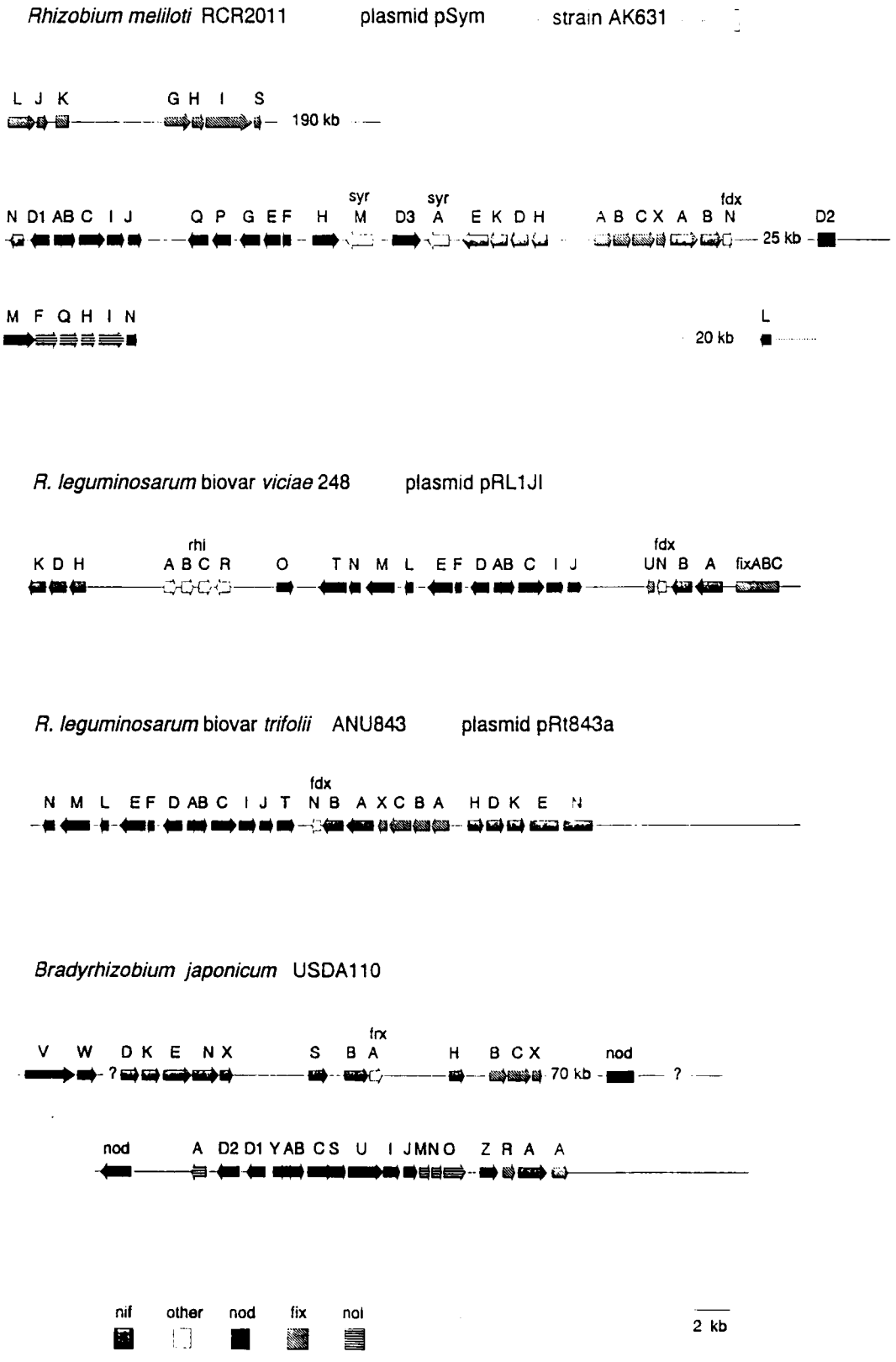
RHIZOBIAL SYMBIOTIC GENES

The development of specialized broad host range vectors, site directed mutagenesis techniques, and gene fusion cassettes, coupled with the relative amenability of bacteria to molecular genetic studies, has facilitated the discovery and analysis of a large number of bacterial genes that are necessary for development of an effective symbiosis. Such rhizobial genes have been collectively termed *sym* genes, and based upon phenotype, can be broken down into three major classes: *i) nif/fix, ii) nod/nol, iii)* others. The following paragraphs will provide a description of each class, with most emphasis placed upon *nod* genes. Figure 1 shows a linkage map of *nod, nif,* and *fix* genes from well-studied rhizobial species. Recently, a compilation of mapped rhizobial genes has become available (Sanjuan *et al.* 1993)

nif/fix genes

Genes designated *nif* or *fix* are necessary for symbiotic nitrogen fixation to occur. Rhizobial *nif* genes are designated as such based upon their homology to *nif* genes cloned and sequenced from the free-living nitrogen fixing bacterium *Klebsiella pneumoniae*. *nif* genes are responsible for the synthesis of nitrogenase, nitrogenase reductase, and cofactors

Figure 1: Linkage map of *nif, fix,* and *nod* genes from various rhizobial species.



necessary for function of these proteins (see Merrick, 1992 for review). The level of sequence conservation among *nif* genes has allowed many rhizobial *nif* genes to be isolated by DNA hybridization using cloned *Klebsiella nif* genes as probes. Genes designated as *fix* are also involved with symbiotic nitrogen fixation, but their functions, other than those acting as regulators of *nifA* (*fixL, fixJ* from *R. meliloti*) are generally not known.

The regulation of *Rhizobium* and *Bradyrhizobium nif* genes shows some similarity to *nif* gene regulation in free-living nitrogen fixing organisms. For example, in both groups of organisms, *nif* transcription requires NifA and is repressed by O_2, but, in contrast to free-living nitrogen fixers, rhizobial *nif* genes are uncoupled from signals produced upon unfavorable glutamate/α-ketoglutarate ratios (See de Bruijn *et al.* 1990 for review). Mechanisms by which O_2 regulates rhizobial *nif* genes are diverse among different rhizobia (Hennecke et al. 1990; David *et al.* 1988; Virts *et al.* 1988; Gilles-Gonzalez *et al.* 1991).

Other genes

A diverse array of gene products that are important for effective symbiosis, yet not involved in eliciting nodule development or nitrogenase activity, are present in rhizobial species. Some of these gene products appear to play a role in cellular maintenance. Certain amino acid and purine auxotrophs

of various rhizobia have been reported to have symbiotic defects (reviewed by Long, 1989a). In *R. meliloti*, but not *B. japonicum*, the *hemA* gene which encodes δ-amino levulinic acid synthetase, the commited step in heme biosynthesis, is essential for nitrogen fixation (Leong, *et al.* 1982; Guerinot and Chelm, 1986; see Sangwan and O'Brian, 1991, for further discussion of *hemA* in *B. japonicum*). Genes involved in the transport of dicarboxylic acids have been identified in *R. meliloti* and *R. leguminosarum* (Watson *et al.* 1988; Ronson *et al.* 1984). These genes are especially important for symbiosis because it is believed that dicarboxylates are the prime source of carbon that bacteroids receive from the host plant (reviewed by Mellor and Werner, 1990). A class of genes that has received more intensive study are the *exo* (*exo*polysaccharide) or *lps* (*l*ipopoly*s*accharide) genes from various rhizobial species (reviewed by Gray and Rolfe, 1990). These genes code for cell surface components necessary for successful symbiosis. Mutations in such genes generally produce an empty nodule phenotype; that is, ineffective (*i.e.* no nitrogen fixation). Nodule structures are elicited on host plants with few or no bacteroids present. Cell surface components may play several important roles in the infection process such as attachment to root hairs (see Diaz *et al.* 1989 for discussion), travel through infection threads (see Kijne, 1992 for review), and in some cases, may be important for bacteroid function (*e.g.* Streeter *et al.* 1992).

nod/nol genes

nod/nol genes are classified on the basis of having an effect on nodule numbers, the time course of nodule development, or by being regulated by NodD in response to host plant flavonoids. NodD is a transcriptional regulator required for expression of many *nod/nol* genes. *nod/nol* genes are, as a whole, essential for eliciting nodule formation. Early stages of the rhizobial infection process include induction of root hair curling and cortical cell division. By complementing a *R. meliloti* strain defective in eliciting root hair curling and nodule development with a *R. meliloti* cosmid clone bank, Long *et al.* (1982) were able to isolate clones encoding nodulation genes. Investigators from other laboratories identified similar clones via analysis of R primes known to contain genes involved in nodulation (Kondorosi *et al.* 1984; Downie *et al.* 1983). Sequence analysis of these genes from *R. meliloti* and *R. leguminosarum* bv *viciae* (Rossen *et al.* 1984; Torok *et al.* 1984), along with hybridization studies (Djordjevic *et al.* 1985b) showed a strong similarity between certain genes. Complementation of heterologous rhizobial mutants defective in eliciting root hair curling with cloned nodulation genes from various rhizobial species (Fisher *et al.* 1985; Marvel *et al.* 1985) supported the idea that certain rhizobial genes involved in early stages of nodulation were allelic. These studies led to the identification of the *nodABC* genes in *R. meliloti* and *R. leguminosarum* biovars *viciae* and *trifolii* (Torok *et al.*

1984; Rossen *et al.* 1984). The high level of homology of these genes allowed other investigators to isolate homologs from other rhizobia such as *B. japonicum* USDA110 (Russell *et al.* 1985). Based upon their presence in all studied rhizobia, *nodABC* have been given the designation "common" *nod* genes.

Host specific *nod* genes

Past experience with R primes had provided information that other *nod* genes were quite closely linked to the common *nod* genes (Kondorosi *et al.* 1984). The use of transposon Tn*5* as an agent of random mutagenesis facilitated mutagenesis of clones carrying common *nod* genes and flanking DNA in *R. meliloti* and *R. leguminosarum* biovars *viciae* and *trifolii* (Debelle *et al.* 1986; Horvath *et al.* 1986; Downie *et al.* 1985; Djordjevic *et al.* 1985a). Such studies led to the identification of genes that, when mutated, produced delays in root hair curling and nodule formation, and/or decreases in nodule number. Sequence analysis of these genes from *R. meliloti, R. leguminosarum* bv *viciae* and *R. leguminosarum* bv *trifolii* showed that some of these genes appeared to be unique, while in other cases, similarities existed between genes from different rhizobial species, suggesting that they were allelic, but the different alleles were unable to complement each other (*e.g. nodE* from *R. leguminosarum* bv *viciae* and *R. leguminosarum* bv *trifolii*, Spaink *et al.* 1989). Such genes have been given the designation host specific *nod* genes, and include *nodFEGPQ, nodH, nodL,* and *nodM* in R. meliloti (Debelle and Sharma, 1986;

Horvath *et al.* 1986; Schwedock and Long, 1989; Baev *et al.* 1991; Baev and Kondorosi, 1992), and *nodFEL*, *nodM*, and *nodO* from *R. leguminosarum* bv *viciae* (Shearman *et al.* 1986; Surin and Downie, 1988; de Maagd *et al.* 1989), and *nodZ* from *B. japonicum* (Nieuwkoop *et al.* 1987; Stacey *et al.* submitted). A number of other rhizobial genes that are regulated in a manner similar to those of the common and host-specific *nod* genes have been isolated, yet individual mutations in such genes do not produce any visible reduction in nodulation efficiency. Such genes include *nodT* from *R. leguminosarum* bv *viciae* and bv *trifolii* strain ANU843 (Surin *et al.* 1990), and *nodY* and *nodSU* from *B. japonicum* USDA110 (Nieuwkoop *et al.* 1987; Göttfert *et al.* 1989, 1990).

Despite the number of *nod/nol* genes isolated, (at current count, 52 genes; *nodA-Z*, *nolA-Z*), biochemical functions for their gene products are largely unknown. Sequence analysis provided some clues (*e.g.* *nodF* and its similarity to acyl carrier protein, Shearman *et al.* 1986). However, such analyses can only provide guesses in absence of direct biochemical experimentation.

Analysis of rhizobial factors that induce early stages of nodulation

Analysis of culture supernatants from various rhizobia showed that such fractions could induce root hair curling (Yao and Vincent, 1969). Further studies with *R. leguminosarum* bv *viciae* showed that the specific activity of the root hair

deforming factor in culture supernatants was enhanced by treating the culture with known inducers of *R. leguminosarum* bv *viciae nod* genes, and that mutations in the common *nod* genes blocked production of this factor (van Brussel *et al.* 1986; Zaat *et al.* 1987a). These analyses showed that rhizobia exported or secreted a factor whose synthesis was dependent upon the common *nod* genes.

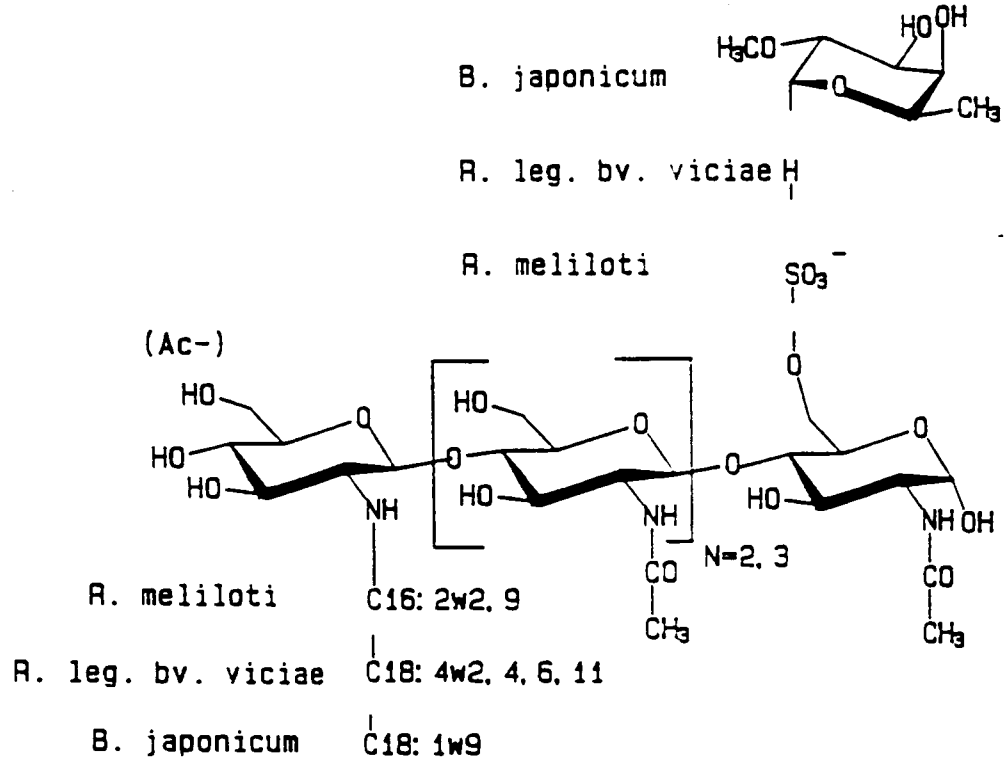
Use of a *R. meliloti* strain (Faucher *et al.* 1988), or *E. coli* containing cloned *R. meliloti nod* genes (Banfalvi and Kondorosi, 1989), demonstrated that the *nodH* gene was essential for provoking root hair curling on alfalfa plants, a normal host of *R. meliloti*. Studies by Faucher *et al.* (1988) showed that, if the *nodH* gene in *R. meliloti* was missing, the induced culture supernatants were unable to induce root hair curling on alfalfa, but could induce hair deformation on vetch, a host on which *R. meliloti* does not normally form nodules or induce root hair curling. Banfalvi and Kondorosi, using clones of *nodABC* and *nodH* under control of *E. coli* promoters, produced similar results; that is, inoculation of *E. coli* cells expressing *nodABC* and *nodH* genes onto alfalfa roots induced root hair deformation. Cell-free filtrates from the same cultures produced the same effect. *E. coli* strains expressing *nodABC* only were unable to promote root hair curling on alfalfa, but could induce curling of root hairs on plants that are not normal hosts of *R. meliloti* [*e.g.* white clover (*Trifolium repens*) and red clover (*Trifolium pratense*)

to a strong extent, and on siratro (*Macroptilium atropurpureum*) to a weaker extent]. Significantly, co-inoculation of alfalfa roots with one strain of *E. coli* expressing *nodABC*, and a second strain that expressed *nodH* was not sufficient for stimulating root hair deformation on alfalfa. This result suggested that the function of NodH was not to act as or produce an additional factor required for alfalfa specificity, but suggested that it might modify a compound produced by the *nodABC* gene products (Banfalvi and Kondorosi, 1989). A similar conclusion and model for NodH function was also devised by Faucher *et al.* (1988).

Subsequent purification of a root hair deforming factor from induced culture supernatants of *R. meliloti* led to the elucidation of the structure of a compound whose synthesis was found to be dependent upon the *nodA* and *nodC* genes (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 non-reducing sugar respectively, and a sulfate group present at C-6 of the reducing sugar. The fatty acyl moiety at the non-reducing sugar was identified as C16:2$\Delta^{2,9}$ (See Figure 2 for structure). This compound was active at inducing root hair deformation at concentrations of 10^{-11}M (Lerouge *et al.* 1990), and its activity was specific to alfalfa, with no activity on a heterologous plant, vetch.

Further studies of this compound showed that it was able to elicit nodule-like structures on the roots of alfalfa plants,

Figure 2: Composite schematic of rhizobial *nod* factors and host specific modifications associated with various species. An acetate moiety (Ac-) on C6 of the non-reducing sugar is associated with *R. meliloti* and *R. leguminosarum* bv *viciae*. Figure generously provided by Dr. R. Carlson, Complex Carbohydrate Research Center, Athens, GA.



and that chemical modifications of the compound reduced or abolished biological activity (Truchet *et al.* 1991). Nitrate has been shown to inhibit nodulation of a number of legumes (Carroll and Mathews, 1990, for review). Truchet *et al.* (1991) showed that addition of 15mM KNO_3 completely suppressed induction of nodule formation, even if 10^{-7}M of the factor was added. The *R. meliloti nod* factor has recently been chemically synthesized, and the synthetic compound possesses the same activity as the compound purified from induced cultures. Such results establish that this factor, and not some other impurity, was indeed responsible for the biological effects seen on alfalfa plants.

Nod factors from other rhizobia

The structure of the nod factor from *R. meliloti* was without precedent. Chitin was previously unreported from prokaryotic sources. Therefore, it was of considerable interest to see if similar compounds were produced by other rhizobia. A compound was subsequently purified from induced culture supernatants of *R. leguminosarum* bv *viciae* by Spaink and colleagues (1991) that had properties similar to that of the factor from *R. meliloti*. Production of this factor was dependent upon the common *nod* genes of *R. leguminosarum* bv *viciae*. It was comprised of an oligomer of N-acetylglucosamine, and was fatty acylated and acetylated at the non-reducing sugar in a manner similar to that of the

factor from *R. meliloti*. This compound differed from the *R. meliloti* factor in that it had no sulfate group at C-6 of the reducing sugar and the fatty acid present was C18:4$\Delta^{2,4,6,11}$ (see Figure 2 for structure). This compound induced root hair curling and nodule formation on pea plants, but not on heterologous hosts (Spaink *et al.* 1991).

A factor from *B. japonicum* USDA110 that is dependent upon NodC function was recently purified and its structure deduced (Sanjuan *et al.* 1992). This factor is a pentamer of N-acetylglucosamine with a fatty acyl substitution (*i.e.* C18:1Δ^{9}) at the non-reducing sugar. A unique feature of this molecule is the 2-*O*-methyl fucose moiety attached to C-6 of the reducing sugar (see Figure 2 for structure).

Functions of Nod proteins

Structural elucidation of *nod* factors has allowed greater insights as to the possible functions of common and host specific *nod* gene products. The conservation in the basic structure of *nod* factors from different species of rhizobia has allowed more sophisticated speculation and biochemical experiments to be performed to find functions for the common *nod* gene products. The commonality of the N-acetylglucosamine backbone and the presence of a fatty acyl substitution would suggest that the common *nod* gene products play a role in the synthesis of the N-acetylglucosamine oligomer and perhaps the addition of the fatty acid. Indeed, similarity between *nodC*

and chitin synthase from *Saccharomyces cerevisiae* has been noted (Bulawa and Wasco, 1991), and recent experiments have shown that NodB from *R. meliloti* is involved with the removal of the acetate moiety from the C-2 residue of the non-reducing sugar (John *et al.* 1993), a prerequisite for N-acylation. No function has yet been ascribed for NodA, but it may play a role in attachment of the fatty acid to the N-acetylglucosamine backbone.

Various modifications of the fatty acid moieties and the different substitutions seen on carbohydrate residues from *nod* factors of different genera/species of Rhizobiaceae would suggest that host-specific nodulation genes are involved with these modifications. This has been shown for several host specific *nod* gene products in *R. meliloti* and *R. leguminosarum* bv *viciae*. The NodPQ and NodH gene products from *R. meliloti* are now known to be involved in the synthesis of a sulfate donor (NodPQ) and sulfate transferase (NodH) (Schwedock and Long, 1990; Roche *et al.* 1991). *nodQP* have similarity to *cysDN* from *E. coli*, genes involved in the synthesis of phosphoadenosine phosphosulfate, a sulfate donor for synthesis of sulfur containing amino acids. NodQP themselves have been shown to be able to synthesize adenosine phosphosulfate (Schwedock and Long, 1990). The *nod* factor produced by *nodZ* mutants of *B. japonicum* USDA110 lack the 2-*O*-methyl fucose substituent, suggesting that NodZ may play some role in the addition of this moiety (Stacey *et al.* submitted). It is not

known if NodZ is involved with the biosynthesis of the fucose residue, or its transfer to the chitose oligosaccharide.

nodE mutants of *R. leguminosarum* bv *viciae* are unable to produce the highly unsaturated fatty acid group seen in the wild-type factor (Spaink *et al.* 1991). With such mutants, a mono-unsaturated fatty acid is associated with the chitose backbone. *nodE* has sequence similarity with β-ketoacylsynthases (Bibb *et al.* 1989), and NodF has a 4'-phosphopantetheine prosthetic group (Geiger *et al.* 1991), analogous to that of acyl carrier proteins involved in fatty acid biosynthesis. Therefore, it seems reasonable to assume that NodE and NodF are responsible for the synthesis of the multiple unsaturations seen in fatty acids associated with nod factors from *R. meliloti* and *R. leguminosarum* bv *viciae*. *nodFE* homologs have not been detected in *B. japonicum* USDA110, as would be expected due to the presence of a mono-unsaturated fatty acid in the USDA110 *nod* factor.

Other genes whose products are known to play a role in *nod* factor biosynthesis include the *nodL* and *nodM* genes from *R. leguminosarum* bv *viciae*. NodL is thought to be involved in the transfer of an *O*-acetyl group to C-6 of the non-reducing sugar (Spaink *et al.* 1991), while *nodM* has been shown to have homology to D-glucosamine synthase, and can complement a *glmS* mutant from *E. coli* (Marie *et al.* 1992). *nodL* and *nodM* are also present in *R. meliloti*. It has been shown that *R. meliloti nodM* also complements an *E. coli glmS* mutation, and

it is presumed that *R. meliloti nodL* and *nodM* carry out similar functions to those seen in *R. leguminosarum* bv *viciae* (Baev *et al.* 1991; Baev and Kondorosi, 1992).

At this time, little is known about the *nod* factor biosynthetic pathway. For example, it is not known how the chitose oligomers are assembled (*i.e.* as polymers that are cleaved to smaller subunits, or synthesized as oligomers). In either scenario, some type of regulatory mechanism would have to be in place to ensure control of oligomer size. Such a function could perhaps come from NodC, which is postulated to be involved in oligomer synthesis, or from some other product common to rhizobia.

It is not known with certainty how *nod* factors are exported from rhizobia, but there is evidence that the NodI and NodJ proteins may be involved (Spaink, 1993). *nodIJ* genes have similarity to a family of ATP binding, membrane associated proteins involved in transport (Higgins *et al.* 1986). NodI is localized to the cytoplasmic membrane in *R. leguminosarum* bv *viciae* (Schlaman *et al.* 1990), which supports a possible transport function. Mutations in *nodIJ* have a spectrum of nodulation phenotypes in rhizobia, ranging from undetectable (Nieuwkoop *et al.* 1987; Göttfert *et al.* 1990) to a slight delay in nodulation (Downie *et al.* 1985; Djordjevic *et al.* 1985a; Debelle *et al.* 1986).

It is presumed that rhizobial *nod* factors interact with some type of receptor associated with host plant root hair

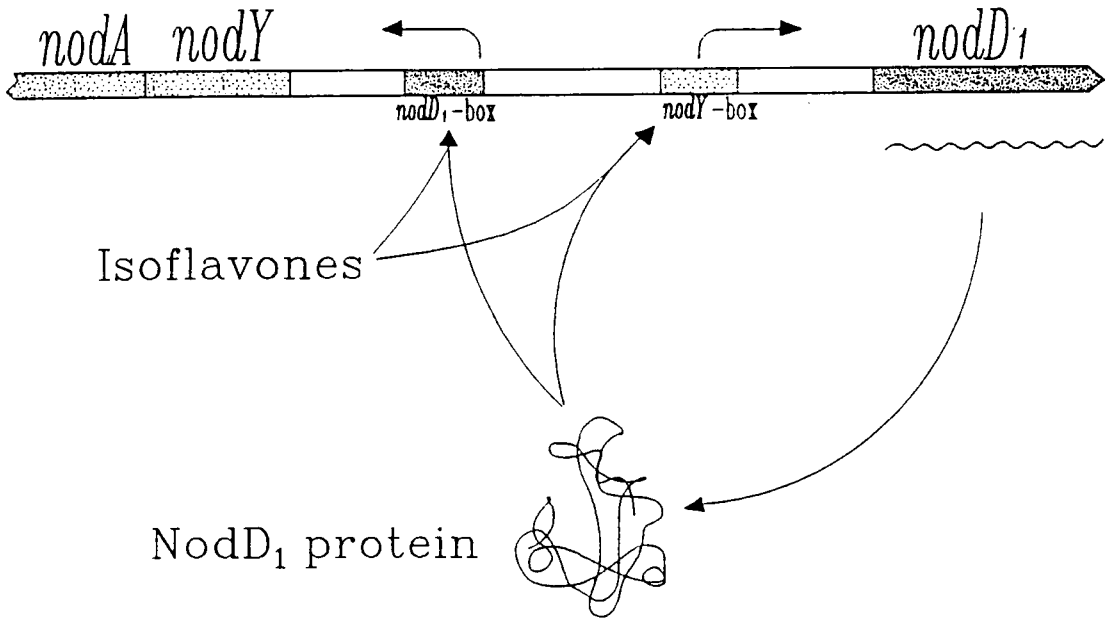
cells, triggering a signal transduction pathway(s). The nature of the putative receptor and signal transduction pathway(s) remains to be elucidated. Likewise, the fate of the *nod* factor itself is unknown. Whether it is internalized, or degraded to produce an active compound also remains to be established. Host specific modifications of the basic structure of *nod* factors would seem to argue that they are involved in recognition by a host plant receptor, but until such a receptor is isolated, the hypothesis cannot be tested.

The structure of rhizobial lipo-oligosaccharide factors is unprecedented in signal transduction. Their structure raises interesting speculation as to their function in plants. It has been known for some time that oligosaccharides have effects on plant hormone balance, which in turn, affects cell growth and development (Darvill *et al.* 1989). A recent report provides evidence that chitinase appears to be important in plant development (de Jong *et al.* 1992). Tobacco plants transformed with *nodAB* have alterations in morphology and growth (Schmidt *et al.* 1991). The *nodAB* gene products may therefore modify chitin-like structures that may exist in plants. Therefore, further studies of the *Rhizobium*-legume interaction therfore would be expected to contribute insights into plant growth and development.

nod GENE REGULATION

Construction of *lacZ* fusions to various *nod* genes in rhizobia has facilitated identification of components necessary for regulation of both common and host specific *nod* genes. These components include flavonoid molecules exuded by host plant roots that act as signal molecules required for induction of common and host specific *nod* genes (Peters and Long, 1986; Redmond *et al.* 1986; Kosslak *et al.* 1987), a set of alleles involved in transcriptional activation of *nod* genes (i.e. *nodD* genes, Mulligan and Long, 1985; Rossen *et al.* 1985; reviewed by Schlaman *et al.* 1992; Györgypal *et al.* 1991), and *cis*-acting upstream sequences that are necessary for NodD binding (*nod* boxes, Rostas *et al.* 1986; Hong *et al.* 1987, Fisher *et al.* 1988). The requirement for these components for *nod* gene induction in all rhizobia studied has led to the proposal of a generalized model for *nod* gene induction. In this model, NodD proteins interact with both the *nod* box and specific flavonoid molecules to stimulate transcription of both common and host specific *nod* genes (See Figure 3 for schematic). The following paragraphs will review current knowledge as to structure and function of NodD proteins and *nod* boxes, and will introduce recently published results that add complexity to the generalized model in specific rhizobial species.

Figure 3: Generalized model for *nod* gene expression in *B. japonicum* USDA110. NodD is thought to interact with isoflavones and specific *cis*-acting sequences (*i.e. nod* boxes) in order to activate transcription of *nod* genes. Similar models have been proposed for other rhizobial species.



NodD proteins

Isolation of mutants that blocked root exudate induction of *nod-lacZ* fusions in *R. meliloti* and *R. leguminosarum* bv *viciae* led to the identification of a set of alleles that were given the designation *nodD* (Mulligan and Long, 1985; Rossen *et al.* 1985). Sequence analysis of *nodD* alleles from a variety of rhizobial species showed that they had a significant level of similarity at the protein level, with the N-terminal portions showing a greater level of similarity than the C-terminal portions (reviewed by Schlaman *et al.* 1992; Györgypal *et al.* 1991). Based upon sequence comparison, NodD was placed in the LysR family of prokaryotic transcriptional regulators (Henikoff *et al.* 1988).

Sequence analysis of the NodD protein revealed a number of motifs postulated to have functional significance. A putative helix-turn-helix motif, suggestive of DNA binding, and a "receiver" motif, purported to be involved in protein-protein interactions, are located in the N-terminal portion of the protein. Studies on sub-cellular localization of NodD suggest that, at least in *R. leguminosarum* bv *viciae*, NodD may be membrane-associated (Schlaman *et al.* 1989). The presence of a hydrophobic alpha helix in NodD proteins support this observation (Györgypal and Kondorosi, 1991). Based on mutational and domain-swapping experiments, the C-terminal portion of the molecule is thought to be involved with flavonoid recognition (Burn *et al.* 1989; Horvath *et al.* 1987).

The requirement for recognition of specific flavonoids may be the basis for the observed sequence divergence among different NodDs in the C-terminus. However, it is worth noting that mutations have been isolated in the N-terminal portion of at least one NodD protein that affect flavonoid recognition and C-terminal mutants have been isolated that affect DNA binding (Burn *et al.* 1989). These results indicate that a view of limited function for each domain in NodD may be too simplistic.

Certain rhizobial species have more than one *nodD* allele. *R. meliloti* strains 1021 and AK631 have three *nodD* genes plus a gene, *syrM*, that has strong similarity to other *nodD* alleles (Honma and Ausubel, 1987; Györgypal *et al.* 1988; Mulligan and Long, 1989; Barnett and Long, 1990; Kondorosi *et al.* 1991a). *R. leguminosarum* bv *phaseoli* has three *nodD* genes (Davis and Johnston, 1990a,b), *B. japonicum* USDA110 has two (Göttfert *et al.* 1989, 1992), while *R. leguminosarum* biovars *viciae* and *trifolii* have only one *nodD* copy (Schlaman *et al.* 1992). The function of each of the extra copies of *nodD* seen in some rhizobial species is not known. Mutations in various combinations of *nodD* alleles in *R. meliloti* strains 1021 and AK631 alter host range of the strains (Honma and Ausubel, 1987; Györgypal *et al.* 1988), and affect the range of inducers of *nodC-lacZ* fusion expression (Györgypal *et al.* 1988; Honma *et al.* 1990). These results are interpreted to suggest that multiple alleles may aid the ability of the bacteria to

nodulate multiple hosts by recognizing subtle differences in flavonoid patterns exuded by various hosts. Mutations that eliminate all three *nodD* genes in *R. meliloti* strain 1021, or the single *nodD* gene in *R. leguminosarum* biovars *viciae* and *trifolii* render the strains Nod$^-$ (Honma and Ausubel, 1987; Downie *et al.* 1985; Shearman *et al.* 1986; Djordjevic *et al.* 1985a). A function for the second copy of *nodD* (*nodD2*) in *B. japonicum* USDA110 is not clear (Göttfert *et al.* 1989, 1992; this study). Residual nodulation of host plants still occurs when both *nodD* alleles from *B. japonicum* USDA110 are removed (Göttfert *et al.* 1989, 1992), making it possible that another positive regulator of *nod* gene expression may still exist in USDA110.

As noted previously, specific, structurally unique flavonoids are responsible for induction of common and host specific *nod* genes in rhizobia. It has yet to be demonstrated by biochemical means that a direct flavonoid-NodD interaction occurs. However, evidence that flavonoids and NodD do interact has come from genetic studies where it has been shown that point mutations in *nodD* can alter the spectrum of flavonoids that are able to induce *nod* gene expression (Burn *et al.* 1989). The transfer of *nodD1* from *R. meliloti* to a NodD$^-$ strain of *R. leguminosarum* bv *viciae* allows *nod* genes in *R. leguminosarum* bv *viciae* to be induced by luteolin, a specific inducer of *R. meliloti nod* genes (Spaink *et al.* 1987). These

experiments suggest that NodD-flavonoid interaction is direct, and not mediated by some secondary factor.

nodD regulation

nodD alleles in rhizobia tend to have a constituitive level of expression that can vary from one species to another (reviewed by Györgypal *et al.* 1991). However, a variety of regulatory mechanisms have been shown to affect this constituitive expression in a species dependent manner. For example, the *nodD* gene in *R. leguminosarum* bv *viciae* is negatively regulated by its own product (Rossen *et al.* 1985), while *nodD1* from *B. japonicum* USDA110 and *R. leguminosarum* bv *phaseoli* are positively regulated by their products in response to flavonoids (Banfalvi *et al.* 1988; Davis and Johnston, 1990b). In *R. meliloti* strain AK631, expression of *nodD1* and *nodD2* is modulated by a repressor protein, the product of the *nolR* gene (Kondorosi *et al.* 1989, 1991b), while no such protein is detected in *R. meliloti* strain 1021 (Kondorosi *et al.* 1989). Regulation of *nodD3* in *R. meliloti* is complex, with positive autoregulation and positive regulation from NodD1 and SyrM occurring (Kondorosi *et al.* 1991a). The need for such complexity of *nodD* regulation is not known, but it has been shown that elevated *nod* gene expression is detrimental for efficient nodulation (Knight *et al.* 1986). Mutations in the *R. meliloti* AK631 *nolR* gene slightly reduce nodulation efficiency on alfalfa compared to a wild type strain (Kondorosi *et al.* 1989). Such results led Kondorosi *et*

al. (1989) to propose that fine-tuning of *nod* gene regulation may be necessary for optimal nodulation proficiency.

nod box studies

Upstream of many flavonoid induced loci is a conserved *cis*-acting sequence of about 50 bp. Deletions removing this segment of DNA render downstream sequences uninducible by flavonoids, confirming its importance as a regulatory sequence (Rostas *et al.* 1986). NodD proteins from *R. meliloti* and *R. leguminosarum* bv *viciae* have been demonstrated to interact directly with *nod* box sequences through the use of gel retardation assays and DNase I footprinting (Hong *et al.* 1987; Fisher *et al.* 1988; Fisher and Long, 1989; Kondorosi *et al.* 1989). These in vitro assays demonstrated the dispensability of flavonoids for DNA-protein interaction, but other reports found flavonoids to enhance DNA binding ability, at least for some rhizobial NodD-*nod* box interactions (Kondorosi *et al.* 1989; Goethals *et al.* 1992). It is not known in what form NodD may bind to *nod* boxes, but based upon footprint patterns, dimers or tetramers of NodD seem likely (Fisher and Long, 1989). For rhizobial strains that have more than one *nodD* allele, it is unknown whether heteromers between different NodD proteins can form. The demonstration that NodD1 and NodD3 from *R. meliloti* strain 1021 can bind to a variety of *nod* boxes from that strain (Fisher and Long, 1989) supports this possibility.

Recently, studies have been undertaken in order to

ascertain what motifs in *nod* boxes may be necessary for function. From these studies, two models have been proposed. One invokes a tetramer of direct repeats as being necessary for function, based upon deletion analysis and gene induction studies done with wild-type and truncated forms of a highly conserved and a degenerate *nod* box (Wang and Stacey, 1991). A second study used results from point mutations in a *nod* box coupled with DNA-protein interaction and gene induction studies to propose a dimer of inverted repeats as being necessary for *nod* box function (Goethals *et al.* 1992). At this time, there is no conclusive evidence that would favor one model over another.

Other regulatory factors

The model for *nod* gene regulation proposed in Figure 3 is a generalization that appears to be true for all rhizobial species so far studied. However, recent work with various rhizobial species has shown that a diverse array of factors, that so far may be species-specific, may play a role in *nod* gene regulation. The level of characterization of these factors varies greatly; some have been cloned, mutagenized, and sequenced, while others are inferred from results of gel retardation assays using extracts from closely related bacterial strains.

As previously described, *R. meliloti* strain AK631 encodes a repressor molecule (NolR) that binds to transcriptional start sites of the *nodD1* and *nodD2* genes (Kondorosi *et al.*

1989, 1991b). Strains with mutations that inactivate this repressor were somewhat less efficient than their wild-type counterparts for alfalfa nodulation. Mutations also had the effect of increasing the activity of common *nod* genes by about 7-fold upon luteolin induction compared to wild-type strains (Kondorosi *et al.* 1989).

A locus in *R. meliloti* strain 1021 has been reported to be essential for efficient luteolin induction of a *nodC-lacZ* fusion. Partial sequencing of this locus has revealed an open reading frame that has significant similarity to the GroEL chaperonin from *E. coli*. Mutations in this locus did not block expression of *nodD1,* but abundance of NodD3 was lessened (Long *et al.* 1991). It was proposed, based upon the strong similarity of this open reading frame with chaperonin-like proteins, that this gene product might be required for NodD function and that the protein may facilitate folding or assembly of NodD into an active form (Long *et al.* 1991).

A gene designated syrM (*sy*mbiotic *r*egulator) has been identified in several *R. meliloti* strains (Mulligan and Long, 1989; Maillet *et al.* 1990; Kondorosi *et al.* 1991a). Initial analysis of this gene showed that its product, along with NodD3, could cause high uninduced levels of *nodC* expression that could not be further enhanced by flavonoid inducers. *syrM* was also found to enhance extracellular polysaccharide production in tandem with a locus designated *syrA* (Mulligan and Long, 1989). Mutations in *syrM* have little effect upon

nodulation of alfalfa (Swanson *et al.* 1987; Kondorosi *et al.* 1991a). Sequence analysis of *syrM* from *R. meliloti* strains 1021 and 41 show that the gene has similarity ranging from 30-60% when compared to NodD proteins from *R. meliloti* (Barnett and Long, 1990; Kondorosi *et al.* 1991a). Gene expression studies with *R. meliloti* AK631 have shown that *syrM* regulation is complex, with the *syrM* gene being under positive control by its own product, NodD2, and NodD3. In addition, based upon results from gel retardation studies with the 5' flanking region of *syrM*, two novel complexes were noted (Kondorosi *et al.* 1991a). One appeared to be common to extracts from both *R. meliloti* strains 1021 and AK631, while a second complex appeared to be specific to AK631. These complexes formed even with extracts from AK631 derivatives that had been deleted of the AK631 *nod-nif* cluster, suggesting that they may identify novel regulators (Kondorosi *et al.* 1991a).

In *B. japonicum* USDA110, the NodD1 protein had been demonstrated to be essential for induction of a *nodY-lacZ* fusion. When a plasmid carrying such a fusion was mobilized into a *nodD1* strain of USDA110, induction with the isoflavone genistein or soybean seed extract was reduced to a fraction of the level seen in the wild-type strain (Banfalvi *et al.* 1988). In addition, the induced levels of activity of the *nodY-lacZ* plasmid fusion were higher with soybean seed extract as an inducer than with purified genistein. Characterization of the components of the soybean seed extract have shown that

malonated, glycosylated forms of isoflavones act as specific inducers of *nodD1*, but not *nodYABC* (Smit *et al.* 1992). It was hypothesized, based upon induction results with a *nodD1-lacZ* gene fusion, that these modified isoflavones acted to increase NodD1 concentration, which would then interact with the *nodYABC nod* box and isoflavones, facilitating activation of the common *nod* genes (Smit *et al.* 1992).

Downstream of *nodD1* in *B. japonicum* USDA110 are two genes, *nodD2* and *nolA*. The USDA110 *nodD2* gene has been characterized by Göttfert and colleagues (1989, 1992). The following information on *nodD2* is condensed from the 1992 work. NodD2 was found to have 62% identity with the USDA110 NodD1 protein. The two genes are separated by 633 bp, which raised the possibility that the *nodD1* and *nodD2* may have separate promoters. A *lacZ* fusion to *nodD2* did not show any detectable inducibility, in contrast to results seen with a *nodD1-lacZ* fusion (Banfalvi *et al.* 1988), which would suggest that the two genes may be differentially regulated.

Initial mutational analysis of *nodD2* (Göttfert *et al.* 1989), using a deletion mutant that had only the *nodD2* gene removed, indicated that it was important for efficient nodulation of soybean and other hosts. However, a subsequent study (Göttfert *et al.* 1992) failed to reproduce these initial observations. This second study did have potential problems in that other possibly important genes were missing via deletion when plant tests were performed. Studies on the importance of

NodD2 in *nod* gene regulation were not reported with the original deletion strain (Göttfert *et al.* 1989). Such studies were done the 1992 paper, with the conclusion that NodD2 played no detectable role in regulation of the *nodYABC genes*. Again, these results are complicated by the fact that other genes were missing as previously described.

Downstream of *nodD2*, separated by 919 bp, is the *nolA* gene of *B. japonicum* USDA110, which was reported to be involved in genotype specific nodulation of soybean (Sadowsky *et al.* 1991). Certain *Bradyrhizobium* strains are blocked from nodulating certain soybean genotypes, yet other strains nodulate such plants normally. Mobilization of the USDA110 *nolA* gene into *B. japonicum* strains blocked for nodulation allowed transconjugants to elicit nodule formation on previously restricted soybean genotypes at levels similar to to those of unrestricted *B. japonicum* strains. Sequence analysis showed that an open reading frame of 711 bp with a predicted translation product of 26871 daltons was present on the complementing plasmid (Sadowsky *et al.* 1991). The translated product had a putative helix-turn-helix motif in its N-terminal portion, which suggested that NolA might have a DNA binding function. *nolA* expression was reported to be induced about three fold with genistein or soybean seed extract. No information was produced as to how NolA might function, but it was hypothesized that a gene regulatory

function was possible, based upon the putative helix-turn-helix motif in the sequence.

For reasons that are not entirely clear, point mutations in *nodD2*, *nolA*, and two open reading frames downstream of *nolA* have been difficult or impossible to obtain (M. Göttfert, M. Sadowsky, pers. comm.). It has been reported that sequences in and around *nolA* render plasmids harboring such sequences refractile to mobilization. Such difficulties have hindered studies of these genes.

A set of genes in *B. japonicum* USDA110 found to be essential for nodulation of cowpea, mungbean, and siratro was characterized by Göttfert and colleagues (1990a). Sequencing of these genes revealed the presence of two open reading frames whose translated products showed strong similarity to other members of the prokaryotic family of two-component regulatory systems. As a generalization, such regulatory systems function with one component acting as a membrane-associated sensor that is capable of detecting some stimulus, and transmitting a signal to a regulatory protein. In many cases, these regulatory proteins are transcriptional activators. Within this regulatory family, certain domains of both sensors and regulators have high levels of similarity. In some cases the membrane sensor protein has been shown to be a histidine kinase that activates the regulatory protein by phosphorylation (see Stock *et al*. 1989, 1990 for reviews). The USDA110 NodV protein had similarity to membrane-bound sensors,

while NodW had similarity to DNA binding regulators. The nodulation phenotype of mutations in these genes on plant hosts (*i.e.* nodulation delay on soybean, Nod$^-$ on all other tested hosts), coupled with the predicted protein structures, led Göttfert *et al.* to propose that NodVW acted as regulators of USDA110 genes involved in host-specific nodulation (Göttfert *et al.* 1990a). However, no NodVW regulated genes were identified, nor was physical evidence for localization of NodVW or phosphotransfer between them presented in their study.

Studies by Sanjuan *et al.* (submitted) have recently demonstrated that the *nodW* gene is essential for efficient induction of *nodYABC* and *nodD1* in USDA110, thus defining a novel class of positive regulators of *nod* gene activation in *B. japonicum* USDA110.

These recent results point to the fact that while there are certain commonalities in rhizobial *nod* gene induction mechanisms (flavonoids, NodD, *nod* boxes), there are also species specific modifications of the general mechanism. There are substantial differences in levels of activity of *nod-lacZ* fusions between different rhizobial species and between different flavonoid induced loci within the same rhizobial species (Mulligan and Long, 1985; Zaat *et al.* 1987b; Banfalvi *et al.* 1988), and it is tempting to speculate that these differences in *nod* gene activity may be due to such modifications. These observations have produced many

unanswered questions. For example, it is not known how widely spread certain regulators may be among rhizobia. Repression of *nod* genes has only been reported in certain *R. meliloti* strains so far, but it is possible that it might exist in other rhizobial species. Three alleles of *nodD* have been reported in *R. meliloti*, and the extra alleles are thought to play a role in flavonoid recognition, facilitating expansion of host range. Results from Göttfert *et al.* (1989) suggest that if NodD2 from *B. japonicum* USDA110 has a role in nodulation, it is apparently not involved in mediating host range. Perhaps USDA110 NodD2 is necessary for *nod* gene activation. For the *B. japonicum* USDA110 NodV and NodW proteins, it is not known whether their effect on *nod* gene expression is direct or indirect. If direct, does NodW bind with *nod* box sequences? Does phosphotransfer between NodV and NodW occur? Is NodV membrane-bound, and if so, does it interact with flavonoids? *nodVW* have so far only been reported in *B. japonicum* USDA110. If NodW binds to *nod* box sequences, which are highly conserved in sequence among rhizobia, it may be that *nodVW*-like genes are present in other rhizobia.

It is becoming clear that other proteinaceous factors, along with NodD, are involved in the modulation of rhizobial *nod* gene expression. *nod* gene regulation appears to be carefully balanced, presumably to optimize production of the lipo-oligosaccharide signal molecules and perhaps unknown

factors necessary for efficient and effective nodulation of host plants.

Rationale for present work

Previously, identification of a new flavonoid induced locus had been reported (Deshmane and Stacey, 1989), and its DNA sequence completed (A. Sharma, pers. comm.). These results led to the construction of mutations within this locus to see if any genes from this locus were critical for nodulation of soybeans and other hosts of USDA110. Analysis of the DNA sequence upstream of open reading frames found within this locus showed that a sequence with similarity to rhizobial *nod* boxes was present, which suggested that downstream genes might be regulated by NodD1 and NodW, which had been shown to be essential for flavonoid mediated induction of other *nod* genes in USDA110. The divergence from consensus of the putative *nod* box led to the proposal that the locus might be regulated by NodD2, a gene product for which function was controversial (Göttfert *et al.* 1989, 1992). Other expression studies were done using various *nod* gene regulatory mutants of USDA110, leading to the discovery of new regulatory mechanisms for USDA110 *nod* genes.

CHAPTER III

MATERIALS AND METHODS

Bacterial strains and plasmids

All strains and plasmids used in this study are listed in Table 2.

Media and Growth Conditions

B. japonicum strains were cultured on modified RDY medium (So *et al.* 1987) for routine growth, β-galactosidase studies, and plant tests. HM salt media (Cole and Elkan, 1973) supplemented with arabinose at 0.1% was used for growth of *B. japonicum* when conducting matings and extracting DNA. Minimal media (Bergersen, 1961) was used to assay for *nod* factor production. *B. japonicum* strains were grown at 30°C. *E. coli* strains were cultured on LB or M9 media (Sambrook *et al.*1989) at 37°C. Antibiotics for selective markers were used in the following concentrations in μg/ml: *E. coli;* ampicillin 100, kanamycin 50, tetracycline 20, streptomycin 20. *B. japonicum;* kanamycin 150, streptomycin 150, spectinomycin 100, tetracycline 150 for plasmid-encoded genes, 100 for chromosome-encoded genes. Unless otherwise stated, all solutions used may be prepared using recipies provided by Sambrook *et al.* (1989).

Table 2: Bacterial strains, plasmids, and bacteriophages

Strains	Relevant characteristics	Source/Reference
B. japonicum		
USDA110	wild type	USDA, Beltsville, MD
AN314	*nodD1*, Km^r, Sm^r	Banfalvi *et al.* 1988
B.j.Δ370	*nodD2*, Km^r, Sm^r	Gottfert *et al.* 1989
B.j.Δ329	*nodD1, nodD2, nolA,* ORF1, ORF2, Km^r	Gottfert *et al.* 1989
B.j.Δ1267	*nodD1, nodD2, nolA,* ORF1, ORF2, Km^r	Gottfert *et al.* 1992
B.j.613	*nodW*, Sm^r, Spc^r	Gottfert *et al.* 1992
B.j.912	*nodD1, nodD2, nolA,* ORF1 ORF2, *nodW*, Sm^r, Spc^r	P. Grob unpublished
WAJ336	*nodD1, nodD2, nolA,* ORF1 ORF2, Sm^r, Spc^r	K. Marcker unpublished
NAD176	Tn*5* in *nolY*, Km^r, Sm^r	Deshmane 1988
NAD177	Tn*5* in *nolY*, Km^r, Sm^r	Deshmane 1988
NAD169	Tn*5* in ORF2, Km^r, Sm^r	Deshmane 1988
NAD181	Tn*5* in ORF2, Km^r, Sm^r	Deshmane 1988
TCD520	*nolY* deletion, Km^r	This study
TCD530	Tn*5* in *nolZ*, Km^r, Sm^r	This study
TCD1000	pND228 in B.j.Δ329 Km^r, Tc^r	This study
TCD1050	pND228 in AN314 Km^r, Sm^r, Tc^r	This study
TCD2000	pND228 in B.j.Δ370 Km^r, Sm^r, Tc^r	This study
TCD3000	pND228 in B.j.613 Sm^r, Spc^r, Tc^r	This study

Table 2 cont

Strains	Relevant characteristics	Source/Reference
TCD910	pTD900 in USDA110, Tc^r	This study
TCD1070	pTD900 in AN314 Km^r, Sm^r, Tc^r	This study
TCD1030	pTD900 in B.j.Δ329 Km^r, Tc^r	This study
TCD2030	pTD900 in B.j.Δ370 Km^r, Sm^r, Tc^r	This study
TCD3010	pTD900 in B.j.613 Sm^r, Spc^r, Tc^r	This study
TCD950	pTD900 in NAD169 Km^r, Sm^r, Tc^r	This study
TCD960	pTD900 in NAD181 Km^r, Sm^r, Tc^r	This study
B.j.912-32	pZB32 in B.j.912 Km^r, Sm^r, Spc^r, Tc^r	J. Sanjuan unpublished
ZB976	pZB22 in USDA110, Tc^r	Banfalvi *et al.* 1988
ZB1027	pZB22 in AN314 Km^r, Sm^r, Tc^r	Banfalvi *et al.* 1988
TCD4000	pZB22 in B.j.Δ370 Km^r, Sm^r, Tc^r	This study
TCD4050	pZB22 in B.j.Δ329 Km^r, Tc^r	This study
TCD5000	pZB22 in WAJ336 Sm^r, Spc^r, Tc^r	This study
TCD6000	pDLG86 in USDA110, Tc^r	This study
TCD6050	pDLG86 in B.j.Δ1267 Km^r, Tc^r	This study
BJS22	pZB22 in B.j.613 Sm^r, Spc^r, Tc^r	J. Sanjuan unpublished
B.j.912-22	pZB22 in B.j.912 Km^r, Sm^r, Spc^r, Tc^r	J. Sanjuan unpublished
B.j.110-573	USDA110 with chromosomal *nodC::lacZ* fusion, Tc^r	P. Grob unpublished

Table 2 cont

Strains	Relevant characteristics	Source/Reference
B.j.Δ1267-573	B.j.Δ1267 with chromosomal *nodC::lacZ* fusion, Km^r, Tc^r	P. Grob unpublished
JSP11	B.j.110-573 with pMSJ12S Sm^r, Spc^r, Tc^r	J. Sanjuan unpublished
JSP12	B.j.Δ1267-573 with pMJS12S, Km^r, Sm^r Spc^r, Tc^r	J. Sanjuan unpublished
TCD330	B.j.Δ1267 with pTD330 integrated, Km^r, Sm^r, Tc^r	This study
TCD340	B.j.Δ1267 with pTD340 integrated, Km^r, Sm^r, Tc^r	This study
TCD350	B.j.Δ1267 with pRj1036 integrated, Km^r, Sm^r, Tc^r	This study
B.j.A3	Tn*5* in *nifD*, Km^r, Sm^r	Hahn *et al.* 1984
E. coli		
JM101	*supE, thi-1 Δ(lac-proAB)* [F' *traD36, proAB, lacI^q ZΔM15*]	Messing, 1983
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F' *proAB, lacI^{q}ZΔM15,* Tn*10* (Tc^r)]	Stratagene
S17-1	RP4 2-Tc::Mu-Km::Tn*7* *thi, pro, hsdR, recA*	Simon *et al.* 1983
Plasmids/ Phages		
M13mp18	M13 sequencing vector	Norrander, 1983
M13mp19	M13 sequencing vector	Norrander, 1983
pUC18	Ap^r	Yanisch-Perron *et al.* 1985
pSUP203	RP4 mob, Tc^r, Ap^r, Cm^r	Simon *et al.* 1983

Table 2 cont

Strains	Relevant characteristics	Source/Reference
pVK100	RP4 mob, Km^r, Tc^r	Knauf and Nester 1982
pRj1035	USDA110 RSα9, RSβ3 Km^r, Tc^r	Acuna *et al.* 1987
pRj1036	Sm^r, Spc^r, Tc^r derivative of pRj1035	This study
pUC4-KIXX	Km^r cassette	Pharmacia
HP45Ω	Sm^r, Spc^r cassette	Prentki and Krisch, 1984
pRK2013	RP4 tra^+, Km^r	Figurski and Helinski, 1979
pND228	*hil5::lacZ* fusion, Tc^r	Deshmane and Stacey, 1989
pZB32	*nodY::lacZ* fusion, Tc^r	Banfalvi *et al.* 1988
pTD900	*nodY::lacZ* fusion, truncated *nodD1*, Tc^r	This study
pZB22	*nodD1::lacZ* fusion, Tc^r	Banfalvi *et al.* 1988
pDLG86	*nolA::lacZ* fusion $NodD1^+$, $NodD2^+$, Tc^r	D. Gerhold 1989
pRj573	integrative *nodC::lacZ* fusion, Tc^r	P.Grob unpublished
pSS5.3	5.3 kb *Eco*R1 fragment of *hil5* in pUC18, Ap^r	A. Sharma unpublished
pTD620	3.2 kb *Eco*R1-*Bam*H1 fragment of *hil5* in pUC18, Ap^r	This study
pTD623	*Xho*1-*Cla*1 deletion from *hil5* ORFA, replaced by KIXX cassette, cloned into pSUP203, Km^r, Tc^r	This study
pTD400	545 bp *Pvu*II-*Cla*I fragment from *hil5* ORFA cloned in pUC18, Ap^r	This study

Table 2 cont

Strains	Relevant characteristics	Source/Reference
pKN7	1.7 kb *Eco*R1 fragment *nodY, nodA, nodB*, in pBR329, Tc^r, Ap^r	G. Stacey unpublished
pTD310	3.0 kb *EcoR1*-BamH1 *nodD1, nodD2* clone in pUC18, Ap^r	This study
pTD320	2.1 kb *Eco*R1-*Hind*III *nolA*, ORF1 clone in pUC18, Ap^r	This study
pTD330	*nodD1, nodD2* from pTD310 cloned into pRj1036 Sm^r, Spc^r, Tc^r	This study
pTD340	*nolA*, ORF1 from pTD320 cloned into pRj1036 Sm^r, Spc^r, Tc^r	This study
pMJS12	2.3 kb *Hind*III fragment with *nolA*, ORF1 cloned into pVK102, Tc^r	Sadowsky *et al.* 1991
pMSJ12S	Sm^r, Spc^r derivative of pMJS12	J. Sanjuan unpublished
pHF360	360 bp *Hind*III fragment of 23S rRNA gene from *P. aeruginosa*	Festl *et al.* 1986

Enzymes and isotopes

Restriction endonucleases, DNA polymerases, and DNA ligase were used following recommended protocols from Sambrook *et al.* (1989). Radioisotopes were obtained from either ICN Biomedical (Irvine, CA) or New England Nuclear (Wilmington, DE).

Genetic techniques

Transformations: Transformation of plasmids into *E. coli* was performed as follows: Competent cells of an appropriate *E. coli* strain were prepared by culturing in LB medium at 37°C until an OD_{600} of 0.5 was reached. Cells were pelleted at 5000*g* for 5 min. at 4°C and resuspended in one fifth volume of 0.1M $MgCl_2$. The cells were again pelleted and then resuspended in 1/50th volume of 0.1M $CaCl_2$. The cell suspension was then placed on ice for 30 min. prior to addition of plasmid DNA. DNA was then added to 100 μl aliquots of cells and the cells were placed on ice for 60 min. The cells were then heat shocked at 42°C for 2 min. One ml of LB medium was then added, and cells were incubated at 37°C for 60 min. Cells were then plated to an appropriate selective medium.

Bacterial matings: Tri-parental matings between *E. coli* donors and *B. japonicum* recipients (Ditta *et al.* 1980) were performed as follows: *B. japonicum* strains were grown in HM medium at 30°C until an OD_{600} of 0.5 to 1.0 was reached. *E. coli* donors and helpers were grown overnight in LB medium. One ml of each culture was pelleted at 4000*g* for 1 min. The cells

were resuspended in 1 ml of HM medium and re-centrifuged. Cells were resuspended in 100 μl of HM medium, mixed, and spotted onto a HM agar plate. The plate was incubated at 30°C overnight. Bacteria were washed from the surface using a HM salt solution (HM medium minus carbon source) and plated to appropriate selective media. Donors, helpers, and recipients were plated individually to selective media as controls.

Construction of the TCD520 deletion mutant: A deletion that removed about 65% of the open reading frame designated *nolY* from the *hil5* region was constructed in the following manner: A 3.2 kb *Eco*RI-*Bam*HI fragment encompassing the *hil5* region was cloned into pUC18. This plasmid was digested with *Xho*I and *Cla*I, releasing a 490 bp fragment from *nolY*. The two fragments were separated by electrophoresis, and the resulting 5.4 kb fragment was recovered. This 5.4 kb fragment had the 5' overhanging ends blunted with Klenow fragment of DNA polymerase I. A 1.6 kb kanamycin resistance cassette (pUC4-KIXX, Pharmacia) was digested with *Xho*I, and it's 5'overhangs were blunted with Klenow. The 5.4 kb fragment and the KIXX cassette fragment were ligated, and Ap^r, Km^r transformants were selected. Plasmid DNA from such clones was digested with *Eco*RI and *Bam*HI to release the pUC18 vector. The 4.3 kb fragment with the *hil5* DNA and KIXX cassette was then ligated to pSUP203 DNA that had been cut with *Eco*RI and *Bcl*I. pSUP203 is a high frequency of mobilization suicide vector (Simon *et al.* 1983). Transformants that were Ap^r, Tc^r, and Km^r were selected

and checked for integrity by restriction analysis. The resulting clone (pTD623) was then mobilized to *B. japonicum* USDA110, and Km^r, Tc^s strains, indicating double crossover events, were selected. Integrity of the strains was confirmed by Southern hybridization.

Nucleic acid manipulation and purification

Plasmid isolation from *E. coli*: Plasmids from *E. coli* strains were isolated using the alkaline lysis protocol 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 extracted as follows: Cultures were grown in HM medium until late log phase was reached. 3 ml of the culture was pelleted, and the pellet was resuspended with 1.5 ml of 0.5% Sarkosyl in TNE buffer (Sambrook *et al.* 1989). The cells were again pelleted, and then resuspended in 0.45 ml of TE buffer. 0.15 ml of a 5% SDS solution in TE buffer and 0.15 ml of a 2.5 mg/ml solution of Proteinase K were then added to the resuspended cells, mixed, and incubated at 37°C for 1 hour. The solution was then extracted with phenol/chloroform several times, and DNA was precipitated by adding two volumes of ethanol.

DNA sequencing and sequence analysis: DNA sequencing was performed by cloning various restriction fragments into

M13mp18 or M13mp19 (Norrander *et al.* 1983). Protocols for phage handling techniques and generation of single stranded phage template DNA can be found in Sambrook *et al.* (1989). Sequencing reactions were done using the chain termination method of Sanger *et al.* (1977), using Sequenase™ or Taquence™ polymerases from United States Biochemical, Cleveland, OH. DNA and protein sequence analyses were done using programs from the University of Wisconsin Genetics Computer Group (UWGCG), Madison, WI. Mapping of Tn*5* insertions from plasmid clones of USDA110 DNA was done by digesting such plasmids with appropriate restriction enzymes to release a fragment that carried the kanamycin resistance marker from Tn*5*, IS50L from the transposon, and flanking USDA110 DNA. Such fragments were cloned into pUC18, selecting for Ap^r, Km^r transformants. The position of the Tn*5* insertion was determined by using a primer homologous to the end of IS50L with the sequence 5'CAGGACGCTACTTGT3', and performing double strand sequencing. Sequencing templates for double strand sequencing were prepared following protocols from Promega (Promega Corporation, Madison, WI).

RNA extractions: Extraction of RNA for use in quantitating levels of *nod* gene transcripts was performed in the following manner: Cultures of the appropriate *B. japonicum* strains were grown in RDY broth with appropriate antibiotics until an OD_{600} of 0.5 to 1.0 was reached. The cultures were then diluted 10-fold to a final volume of 150 ml and 3 ml of soybean seed

extract was then added. The cultures were then incubated for 8 hrs at 30°C. Cells were pelleted and resuspended in 5 ml of 20 mM Na^+ acetate (pH 5.5), 1 mM EDTA and 0.5% SDS. An equal volume of hot phenol (65°C) equilibrated with 20 mM Na^+ acetate (pH 5.5) was added and mixed, and placed at 65°C for 5 min. Organic and aqueous phases were separated by centrifugation, and the aqueous phase was then mixed with phenol/chloroform. Upon recovery of the aqueous phase, 0.1 volume of 3M Na^+ acetate (pH 5.2) was added, followed by two volumes of ethanol. RNA was then examined by gel electrophoresis to monitor integrity of RNA and to ensure that no DNA was present.

Hybridizations: DNA-DNA hybridizations were performed using nylon membranes from Amersham (Amersham Corporation, Arlington Heights, IL). All prehybridization and hybridization steps were carried out at 65°C following recommended protocols from Amersham. High stringency washes were performed as follows: Membranes were washed twice in 2X SSC at 65°C for 15 min each time. The next wash was in 2X SSC, 0.1% SDS at 65°C for 30 min, followed by a final wash in 0.1X SSC at 65°C for 10 min. Membranes were then air dried and put up for autoradiography at -80°C using Kodak X-OMAT AR film. Washing conditions for low stringency blots will be provided in relevant figure legends. RNA-DNA hybridizations were done using nylon filters from Amersham. Conditions for applying and fixing RNA to filters followed protocols from Sambrook *et al.* (1989).

Prehybridization and hybridization of RNA filters was done at 42°C in a solution of 50% formamide, 5X SSPE, and 5X Denhardt's reagent. Washing of RNA filters was done as follows: one 20 min wash at room temperature in 1X SSC, 0.1% SDS, followed by three washes of 20 min each at 68°C in 0.2X SSC, 0.1% SDS (Sambrook *et al.* 1989). Autoradiography was done with Kodak X-OMAT AR film at -80°C. Radioanalytic scanning of RNA dot blots was performed using an AMBIS radioanalytic imaging scanner (AMBIS Corporation, San Diego, CA).

Plant tests

Seeds of *Glycine max* cv Essex, *Macroptilium atropurpureum* (siratro), *Vigna unguiculata* (cowpea, Caloona variety), and *Vigna radiata* (mungbean, King variety) were surface sterilized as follows: Seeds of soybean, cowpea, and mungbean were rinsed in a 20% Chlorox solution for 10 min, rinsed three times with sterile water, placed in 0.01 M HCl for 10 min, and rinsed 5-6 times with sterile water. Siratro seeds were soaked in 95% ethanol for 30 sec., rinsed three times with sterile water, soaked in 0.1% $HgCl_2$ for 5 min, and then rinsed 6 times with sterile water. Upon a 48 hour germination, seedlings were transferred to plastic growth pouches (Vaughan's Seed Company, Downers Grove, IL), inoculated with 10^6 cells of the appropriate bacterial culture, and placed in a growth chamber with a 16 hr photoperiod. Plants were watered as necessary with a nitrogen free nutrient solution (Wacek and Brill,

1976). Plants were examined either every day or every other day for nodule formation beginning at 9 days post-inoculation. Upon completion of the assays with soybean plants, nodules from the plants were surface sterilized (Somasegaren and Hoben, 1985), crushed, and plated to RDY medium with no selection. Isolated colonies were then transferred to RDY medium with appropriate antibiotic selection to ensure that nodules elicted were occupied by the applied bacterial inoculum.

Competition for nodule occupancy assays were performed as follows: Cultures of USDA110, the *nolY* deletion mutant TCD520, and a control strain B.j.A3 (*nifD*) were grown in RDY broth with appropriate antibiotics. OD_{600} measurements were taken and cells were diluted to an OD_{600} of 0.001, which was found to be about 10^6 cells per ml, based upon plate count results. Cultures were mixed in 1:1, 1:10, and 10:1 ratios. A total of $2X10^6$ bacterial cells were then inoculated to a series of soybean plants. Plants were monitored for normal nodulation kinetics compared to control plants inoculated with wild-type bacteria. At 18 days post-inoculation, 20-40 nodules from each treatment were picked, surface sterilized, crushed, and contents streaked to RDY medium. Resulting isolated colonies from each nodule (10 per nodule) were then picked to RDY medium and RDY with kanamycin, and scored for sensitivity or resistance to the antibiotic. Double occupancies where both bacterial genotypes were present in the nodule occurred at a

low frequency (<15%); these were not counted when analyzing the results.

β-galactosidase assays

β-galactosidase activities of strains harboring *lacZ* fusions were assayed essentially following protocols used by Banfalvi *et al.* (1988), and Smit *et al.* (1992). Strains were grown in RDY broth with appropriate antibiotic selection at 30°C until an OD_{600} of 0.4 to 1.0 was reached. Cultures were then diluted to an OD_{600} of 0.05 to 0.1 in RDY broth, without addition of antibiotics. Genistein, dissolved in 100% ethanol, was then added to cultures to a final concentration of 2 μM. Soybean seed extract was prepared by washing *G. max* cv Essex seeds in a 50/50 mix of water and ethanol (1 ml/seed) for 12-24 hours at 37°C. The supernatent was filtered and concentrated to 1/10th of initial volume. 40 μl of this solution was then added to 2 ml of culture as an inducer. Upon induction, cultures were incubated at 30°C for 14-16 hrs.

After incubation, 0.5 ml of each culture was mixed with 0.5 ml of Z buffer (Miller, 1972), 25 μl of 0.1% SDS, and 50 μl of choloroform. This solution was then vortexed vigorously for about 5 seconds, and allowed to set at room temperature for 10 min. 150 μl of a 4 mg/ml solution of CPRG (chlorophenol red-β-D-galactopyranoside, Boehringer Mannheim) in Z buffer was then added as a substrate to each sample. Samples were then incubated for 20-40 min at 30°C. Samples were then centrifuged

for 2 min, and OD_{574} readings were taken. Calculations of activity were performed following the example from Miller (1972). CPRG has a 8-fold greater sensitivity than does ONPG (*ortho*-nitrophenyl-β-D-galactopyranoside). Results presented are averages and standard deviations of three or more independent assays.

Assay for nod factor metabolites

A general protocol devised by Spaink *et al.* (1992) was used. Briefly, *B. japonicum* strains were grown in RDY medium until an OD_{600} of 0.4 to 0.8 was reached. Cells (1-2 mls) were pelleted and resuspended in a minimal salts medium lacking a carbon source. Cells were diluted to an OD_{600} of 0.1 in minimal salts and were incubated at 30°C for 6-8 hrs. ^{14}C-labeled acetate (New England Nuclear) was added to a final concentration of 25 μCi/ml. Inducers of *nod* gene expression were added, and cells were incubated for 20 hrs at 30°C. Cultures were extracted with butanol, and the butanol fraction was then dried down and resuspended in 15-20 μl of butanol. Two to three μl of this solution was spotted onto a reverse phase silica gel plate that had undergone complete octadecylsilanization (Sigma Chemical Co., St. Louis, MO Catalog number T-7145) for thin-layer chromatography. The running solvent was a 50/50 mix of water and acetonitrile. Plates were then put up for autoradiography on Kodak X-OMAT RP film for 8-12 days at room temperature.

CHAPTER IV

RESULTS

CHARACTERIZATION OF A NEW HOST INDUCED LOCUS IN *B. japonicum* USDA110

Random mini-Mu::*lacZ* mutagenesis of a 5.3kb *Eco*R1 fragment adjacent to the *nodD* genes of USDA110 led to the discovery of a single isoflavone inducible fusion (Deshmane and Stacey 1989). The region of DNA surrounding this insertion had been given the trivial designation *hil5* (*hil* - *h*ost *i*nducible *l*ocus). Sequencing of the DNA surrounding this insertion was subsequently performed to facilitate further analysis of this locus (see Figure 4 for a linkage map of the *hil5* region). The sequence revealed the presence of two open reading frames, preceded by a region with significant similarity to *nod* boxes sequenced from other rhizobia. The two open reading frames, designated *nolY* and *nolZ*, were 726 and 225 bp in size and produce predicted translation products of 26581 and 8604 daltons respectively. Analysis of the predicted translation products of *nolY* and *nolZ* using the TFASTA protein analysis program of the University of Wisconsin Genetics Computer Group (UWGCG) did not reveal any significant homologies to existing sequences in the data bank. No notable motifs that could suggest a function (*i.e.* membrane spanning, helix-turn-helix) were seen during the analyses of these open reading frames.

Figure 4: Linkage map of the *B. japonicum* USDA110 *hil5* region. Sequence analysis of this region revealed that *hil5* was comprised of two open reading frames that have now been designated *nolY* and *nolZ*.

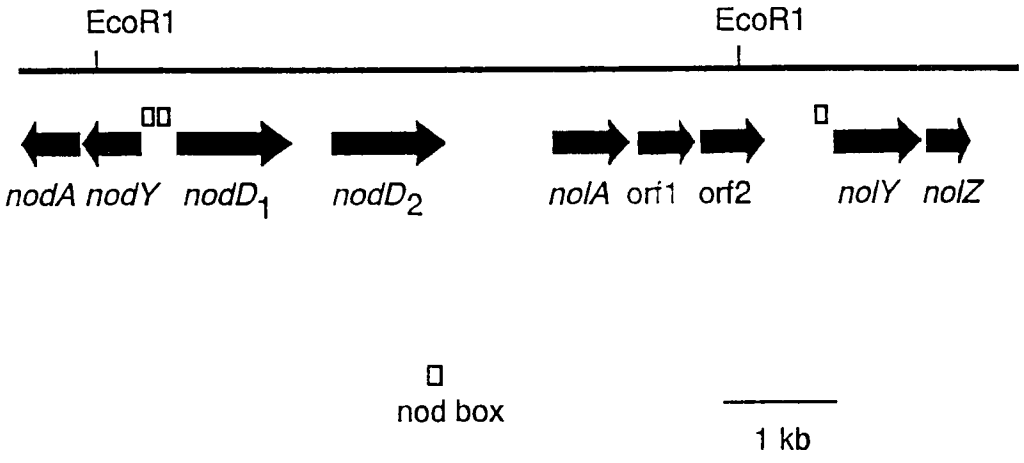


Figure 5 shows the DNA sequence and translation products of the *hil5* region.

A sequence upstream of the two open reading frames was detected that had similarity to *nod* boxes sequenced from other rhizobia. Alignment of this putative *nod* box with a consensus *nod* box sequence showed a 70% sequence similarity. Two models have been proposed to define functional sequences/motifs in rhizobial *nod* boxes. One (Wang and Stacey, 1991), proposes a tetramer of direct repeats as a functional motif. Another, (Goethals *et al.* 1992) proposes two motifs of ATC(N_9)GAT as being necessary for function. A consensus motif has been proposed for LysR type regulators that consists of the sequence T(N_{11})A (Goethals *et al.* 1992). A comparison of the putative *hil5 nod* box to the proposed models shows that it has similarities to all of these models (Figure 6).

Expression of the *hil5* open reading frames

A translational *lacZ* fusion was mapped to *nolZ* of the *hil5* region by cloning the junction of the fusion insertion and surrounding DNA and sequencing through the junction to assess the point of insertion. Mobilization of the fusion plasmid into wild type USDA110 showed that induction of the fusion was dependent upon the addition of the isoflavone genistein, or soybean seed extract (Table 3). This fusion was mobilized to various regulatory mutants of USDA110 to see if other known *nod* gene regulators affected *nolZ* expression. The results

Figure 5: Nucleotide sequence of the *hil5* region. Translated open reading frames *nolY* and *nolZ* are delineated by the single letter amino acid code. An underlined DNA sequence upstream of *nolY* has similarity to rhizobial *nod* boxes. Arrows denote where Tn*5* insertions have been mapped within the locus. The most 5' arrow denotes the insertion point of mutant strain NAD177, while the arrow downstream represents the insertion point of NAD176. An arrow in *nolZ* represents the insertion point of mutant strain TCD530. Amino acids underlined in NolY denote the extent of a *nolY* deletion mutation (TCD520). This sequence information was generously provided by Dr. Arun Sharma.

CCCGGGCATTCCCTATGCTTCCTAGCCCGGATAGCAGGAGAACCAATCAACCGGCTCCGA

TGAAGGCGGTGAGATTGCTCGCCTGCGCTATCAACGAACTGGATGCATCCCATCAACACT

TTCTATTTTACTAATTGAAGCCAAACACCGCATAAGTCGATCGCCGGCTTACGGAAGGCA

GTGTTCACGAATGTCGCGTGATAATGGATCACTTGGTCGCAGAGCAAATCACGTGCTTCT

TGGCTGCTTCGAGCAATGTTTGACGGCAAGACACGTGGGGGCACGAATGCAGTGCGGTGA

CCAGTCGCAGTTGGCGCGCAATCTACACGACGTTGCCTGCCCCTTTGGGCATGATACTTG

ATTGCTCTAAGAAGACAGCTATCACGCACACTCGGCGATGGTAAATGCCTCCGCAGCGTA
M P P Q R

GAACTCGGGCAGGTGAGTTGGCCTTCGCAGGGGTGCGGGCTGGACCTTTCGCTCTGACCA
R T R A G E L A F A G V R A G P F A L T

GGCGGGTCGCCTCAACAGTCCGGCGCTGTTGCCGCGGCTCCAAGCAGAGCAGTACAATCA
R R V A S T V R R C C R G S K Q S S T I

GCTGTGTGGCCCCGAACAGAGTCACCGCTGTTGCCGAACGCACTGGGCGGCTCGAGAATC
S C V A P N R V T A V A E R T G R L E N

CATTCAAATCCCGCGCCATCGGCAACGCTTTTCGTTTTTCCCTAGACGCTCATTGCACTT
P F K S R A I G N A F R F S L D A H C T

TTGCAATCCTTGCAGCAGTCATTTGTCCAGCGAAGCGTCCGCCGTTTGGCGAACGGGAAA
F A I L A A V I C P A K R P P F G E R E

CGATACCTCTACTCCGCCTGTCTTGCATTTCACCACTTTGGAGAAATACCGTGGAACTTG
T I P L L R L S C I S P L W R N T V E L

ACCCTTCCAATTCAAGAATACAAAACCTCGAGGATCGCGTCGACCTGCAACTCGACATCG
D P S N S R I Q N L E D R V D L Q L D I

TGGCTGGGCTCATGCGCGAAGAAGGCAACGAGCTGGAAGCGATCCGATTACTTAATGCCC
V A G L M R E E G N E L E A I R L L N A

TCATAGGCCAAATGATCACCGCGGAACTGGAGCTCGGCCAGCTTGGCGATACAAGAACCG
L I G Q M I T A E L E L G Q L G D T R T

GCCACGGACGAATGCGATTGTCGCAAGACCGTGGCGTTTACCACCCCGAGGACCTTCAGA
G H G R M R L S Q D R G V Y H P E D L Q

TTCTGGGACGCCTTTTTGATCAGACTGTGGCGGCATTACCGGCCGCGCTGCGGACTTCCG
I L G R L F D Q T V A A L P A A L R T S

CCAATCGATTGACGATCGCCAAGCTCATTCTTTCACGTGCGGCAGCGGACTGAGATTTCA
A N R L T I A K L I L S R A A A D U

TTTGCGCTTTTTGGAGATTGATGGCGTTGAGCGGACGTTTATGGCTGGGCTGACAGTTAC

ACTTAGGCCGCACACACCAGGGATCGACATTTGCTGTTTATGGGCTCACCGTCCGTAAGC
M G S P S V S

GCGCGGCTTCCGCTGGCCTTTCATATGGCAACAGTTCGCCACCACTTTCGAAATCAGGCT
A P L P L A F H M A T V R H H F R N Q A

TGCTCTATCTCACGCCGCCGGCGACGCATTCGATCTAGCTGGGCCTTGCTGTCCCTCGAC
C S I S R R R R R I R S S W A L L S L D

GCCTTTCACGAGCTGCAAGTATCCAAAGCTCGGAAGCGCGCCGCCGATCCCACGCTGCGC
A F H E L Q V S K A R K R A A D P T L R

CGGCTTTCCTACGCAACTCCGTATTGAACCCACACCGACGGCGCCCGGCCCTTCTTTGCC
R L S Y A T P Y U

```
[(ATC)CATA..][..G(GAT)G..]....[(ATC)CAAACA][ATC(GAT)TTT]ACCAATC
  :::  :        : ::: :          :::  : ::    ::  :: ::: :: :::
  ATC AACGAA  CTG GAT GCA TCCC  ATC AACACT  TTC TAT TTT ACTAATT
```

Proposed consensus repeat from Wang and Stacey (1991):

$$\begin{array}{c} \text{G C} \quad \text{A A} \quad \text{A} \\ A_{74}T_{90}C_{88}G_{85}A_{93}T_{89}T_{71}Y_{74}T_{74} \end{array}$$

Proposed consensus from Goethals *et al.* (1992):

$$ATC(N_9)GAT$$

Proposed consensus for LysR regulators:

$$T\text{-}(N_{11})\text{-}A$$

Figure 6: Alignment of a proposed *nod* box consensus (top line, Wang and Stacey, 1991) with the putative *nod* box from the *hi15* region (bottom line). Brackets [] indicate the positions of the 9 bp repeats proposed by Wang and Stacey (1991) to be important for function. Parentheses () indicate positions of short inverted repeats proposed by Goethals *et al.* (1992) to be important for function. Nucleotides in bold type denote conserved bases found within promoters regulated by LysR-type proteins (Goethals *et al.* 1992). Subscripts within the proposed consensus from Wang and Stacey (1991) denote percentage of occurrance of that nucleotide, based upon examination of several purported *nod* boxes. Conserved substitutions are noted above this consensus.

Table 3: Expression of a *nolZ-lacZ* fusion plasmid in wild type *B. japonicum* USDA110 and regulatory mutants.

Strain	Units of Activity[a]		
	Control	2μM Genistein	SSE[b]
NAD2021 (wild type)	7±1	110±10	142±20
TCD1050 (*nodD1*)	4±1	6±2	12±1
TCD1000 (B.j.Δ329)	4±1	5±1	8±1
TCD3000 (*nodW*)	6±2	7±2	10±1

[a]Units using CPRG as a substrate

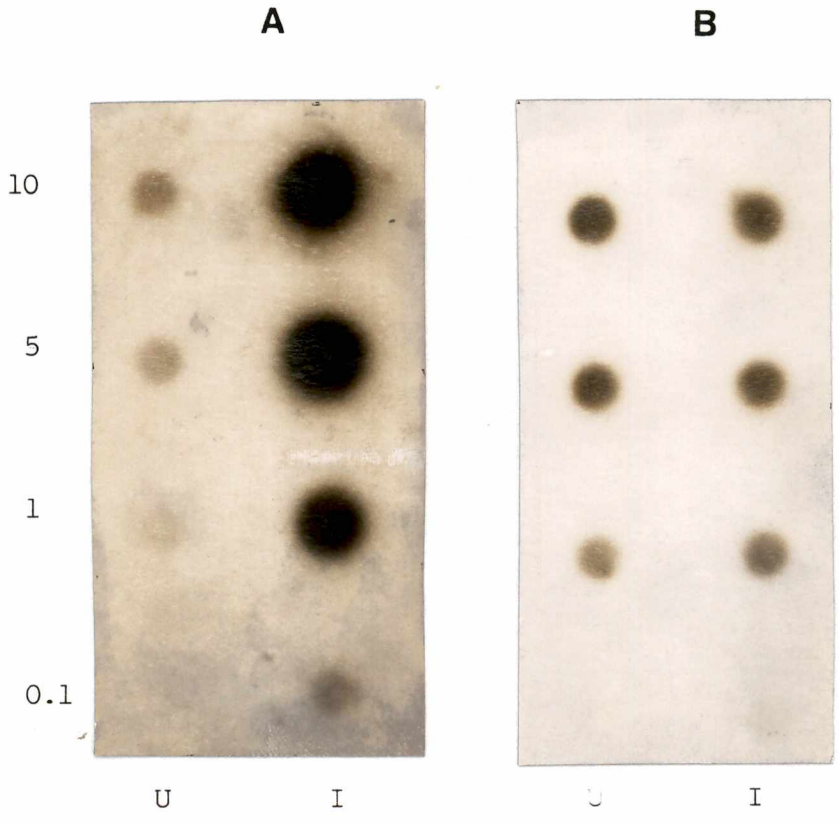
[b]SSE-soybean seed extract

presented in Table 3 show that induction of this fusion is dependent upon the NodD1 protein, similar to the *nodYABC* and *nodD1* flavonoid inducible genes. This result suggests that the putative *nod* box upstream of *nolY* is functional. The fusion plasmid was not induced in a *nodW* mutant of USDA110. The *nodVW* genes of USDA110 have similarity to the prokaryotic family of two-component regulatory systems (Göttfert *et al.* 1990a). Results from J. Sanjaun (this lab) have shown that *nodW* is essential for the induction of the *nodYABC* and *nodD1* genes from USDA110. The *nolZ* fusion plasmid was uninducible in a mutant deleted of *nodD1, nodD2, nolA,* ORF1, and ORF2. In spite of several efforts, an inducible *nolY-lacZ* fusion was not obtained. To quantitate *nolY* expression, RNA was extracted from uninduced and induced cultures of wild type USDA110, blotted to nylon, and hybridized with a *nolY* specific probe. Quantification of counts from the filter showed a 40 to 50-fold increase in expression over uninduced controls after normalization by hybridization with a 23S rRNA gene (Figure 7).

Mutational analysis of *hil5*

Past experiments from Deshmane (1988 Ph.D. thesis) had generated Tn*5* insertions that mapped near the *hil5* region. Cloning of the junctions where Tn*5* and flanking DNA met was accomplished by selection for the kanamycin resistance marker and the junction was sequenced by using an oligonucleotide homologous to IS50L of Tn*5* as a primer. Two insertions in *nolY*

Figure 7: Analysis of *nolY* transcription via RNA dot blots. Total RNA from uninduced and soybean seed extract induced *B. japonicum* USDA110 cultures was spotted onto a filter and hybridized with a *nolY* specific probe as described in Materials and Methods. A) USDA110 RNA from uninduced (U) and induced (I) cultures hybridized with *nolY*. B) Same filter hybridized with a 23S rRNA gene probe as a control for RNA quantity on the filter. Numbers on left indicate quantity of RNA spotted in μg.



(NAD176 and NAD177) and one insertion in *nolZ* (TCD530) were found (Figure 5). NAD176 and NAD177 had been previously reported to be similar to wild type USDA110 in nodulation of soybean (Deshmane, 1988 Ph.D. thesis). To test whether these mutations had any effect on nodulation of alternate hosts, these mutants were inoculated onto siratro and cowpea plants. The results showed that these mutants had no detectable alterations in the time course of nodule appearance or nodule numbers (data not shown). The Tn*5* insertions in *nolY* are quite close to the 5' end of the open reading frame, so there was a chance that they might be leaky mutants due to the presence of promoter activity from within the transposon and the possibility that a fusion protein between the IS element and NolY may retain residual activity. An internal deletion that removed 65% of the *nolY* coding sequence was constructed (see Materials and Methods), and this mutant (TCD520) was tested on various USDA110 plant hosts for nodulation proficiency. No detectable alterations in nodulation kinetics or nodule numbers were seen when this mutant was inoculated onto soybean, cowpea, or siratro (Figure 8). However, a mild reduction in nodule number and reduction in percentage of plants nodulated was noted when the mutant was inoculated to mungbean plants (Figure 9). A *nolZ* mutant had no detectable phenotype on any plant host tested (Figure 8).

Competition studies based upon nodule occupancy of mutant versus wild-type strains have been used to detect otherwise

Figure 8: Nodulation kinetics of *B. japonicum* USDA110 and *nolY* and *nolZ* mutant derivatives TCD520 and TCD530, respectively, on soybean plants. Nodule numbers per plant were essentially the same throughout the assay. Similar results were obtained when the mutants were inoculated onto siratro and cowpea plants. A minimum of 10 plants was used for each assay.

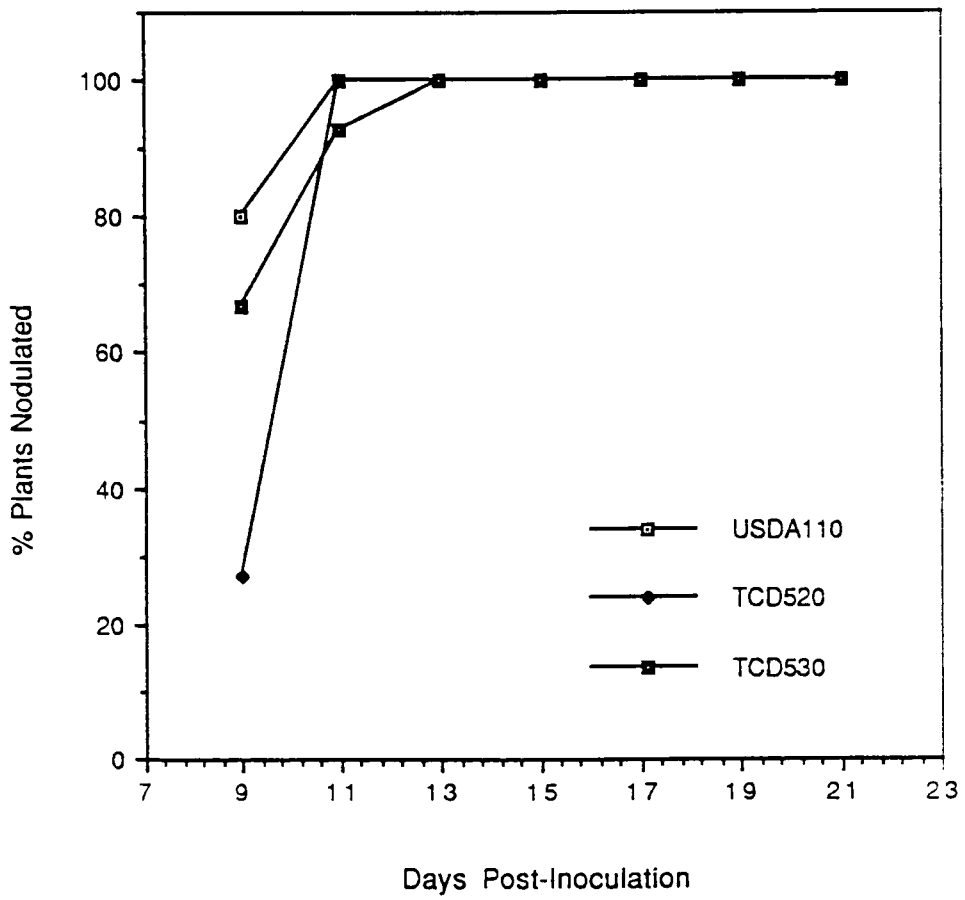
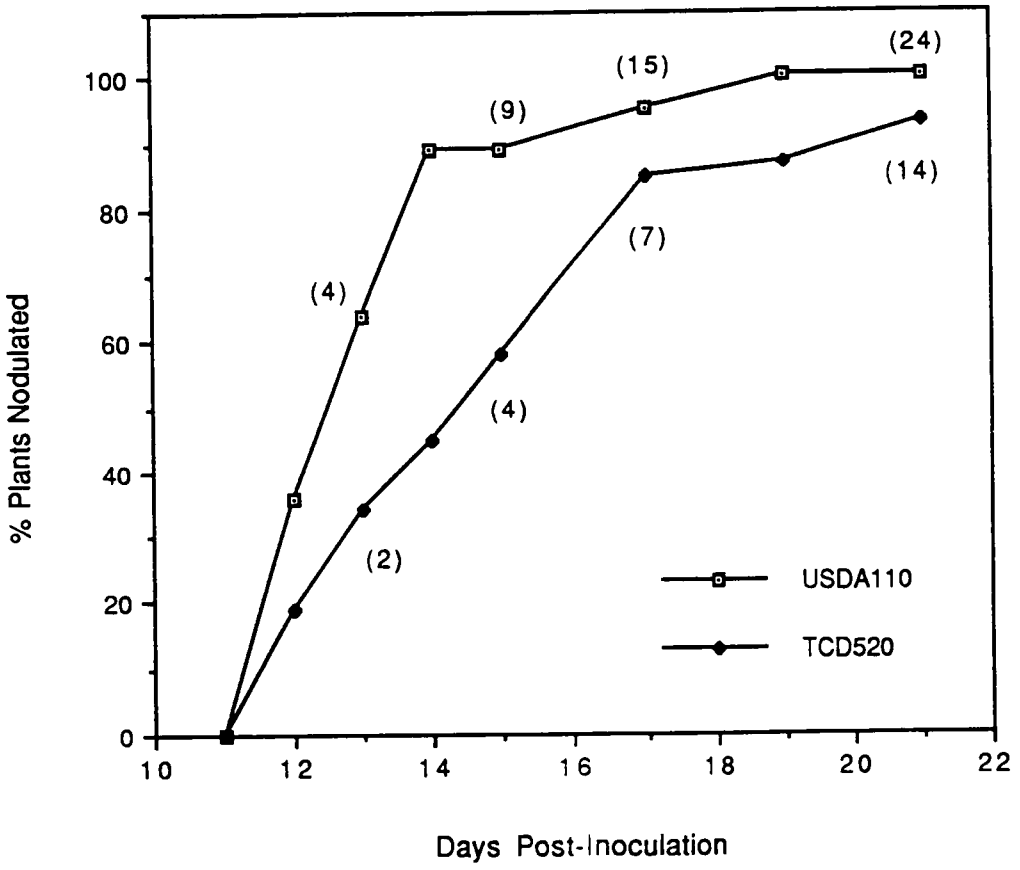


Figure 9: Nodulation kinetics of *B. japonicum* USDA110 and a *nolY* mutant, TCD520, on mungbean plants. Numbers in parentheses indicate the average number of nodules per plant at that time point. The data are the results from tests of 40 or more plants per treatment, spread over three assays.



unnoticeable phenotypic alterations of nodulation proficiency in *R. meliloti* (Sanjuan and Olivares, 1991). A recent study from Bhagwat and Keister (1992) reported that clones from a *B. japonicum* USDA438 subtraction library had been isolated that enhanced the competitiveness of *B. japonicum* USDA110. Moreover, these clones were found to encode genes selectively expressed in a medium that contained soybean seed extract, suggesting that such genes may be transcriptionally regulated by flavonoids. To see if a competitively altered phenotype could be noted for the *hil5* region, competition assays with USDA110 and the *nolY* deletion mutant TCD520 were performed, using a *nifD* mutant of USDA110 as a control (strain B.j.A3, Hahn *et al.* 1984) and soybean as a host. It was hypothesized that the mutant might be less competitive for nodule occupancy when compared to the wild type. At 18 days post-inoculation, nodules were picked, surface sterilized, crushed, and plated onto selective and non-selective media. Counts of kanamycin resistant (TCD520) versus kanamycin sensitive (USDA110) bacteria extracted from crushed nodules indicated that TCD520 was at least equally as competitive with the wild type USDA110 strain (Table 4).

Recently, a protocol has been devised to analyze production of *nod* factors from rhizobia using thin-layer chromatography (Spaink *et al.* 1992). Induced culture supernatants of the TCD520 mutant, along with USDA110, were analyzed by this technique. No detectable alteration was found in the R_f of the

Table 4: Results from nodule occupancy assays done with wild type USDA110 and a *nolY* deletion mutant TCD520. Numbers presented are averages from two experiments. Strain B.j.A3 is a *nifD* mutant of USDA110 that is not expected to be defective for nodule occupancy.

Culture/ Inoculum ratio	Percentage of nodule occupancy	
	USDA110	TCD520 or B.j.A3
USDA110	100	ND[a]
TCD520	ND	100
B.j.A3	ND	100
USDA110/TCD520 1:1	17	83
USDA110/TCD520 10:1	71	29
USDA110/TCD520 1:10	6	94
USDA110/B.j.A3 1:1	54	46
USDA110/B.j.A3 10:1	70	30
USDA110/B.j.A3 1:10	11	89

[a]ND-not determined

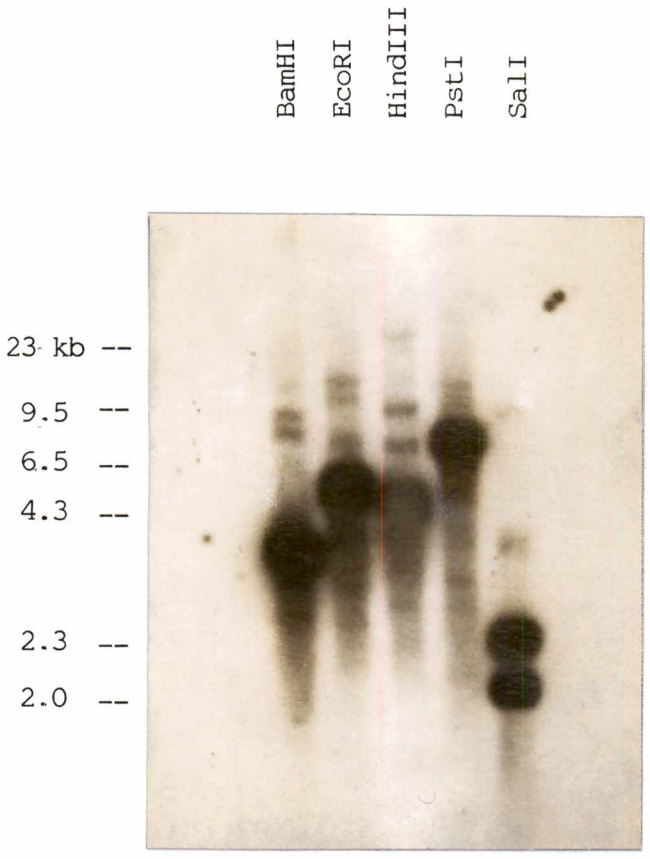
factors from the mutant when compared to the wild type. In addition, the amount of factor produced by the mutant appeared to be similar to that of the wild type, which would suggest that NolY is neither a regulator of common *nod* gene expression, or a transporter of the factor (data not shown).

Analysis for functional reiteration of *hil5*

Functional homologs of *nod* genes are known to exist in the genomes of various rhizobia. These homologs usually code for proteins involved in a biosynthetic reaction required for cellular maintenance. Examples include the *nodPQ* genes, involved in nod factor biosynthesis in *R. meliloti,* which are homologous to *cysDN* from *E. coli*, a set of genes involved in the synthesis of a sulfate donor (Schwedock and Long, 1990), and *nodM* in *R. leguminosarum* bv *viceae* which is homologous to *glmS* from *E. coli*, a gene involved in the synthesis of glucosamine (Marie *et al.* 1992). Mutations in these *nod* genes have little or no detectable effect on nodulation of their respective plant hosts. Studies of these genes have shown that they do cross-hybridize with their homologs, and in some cases, complement each other. In order to see if this was a possible explanation for the lack of an altered phenotype with *hil5* mutants, chromosomal DNA from USDA110 was digested with a variety of restriction enzymes, separated by electrophoresis, and blotted onto a filter for Southern analysis, using an internal fragment of *nolY* as a probe. A low stringency wash revealed the presence of very faint bands

cross-hybridizing with an *nolY* specific probe (Figure 10). The weak hybridization signal renders this experiment inconclusive. The possibility of functional reiteration of *nolY* was not further pursued.

Figure 10: Low stringency hybridization of a *nolY* probe to *B. japonicum* USDA110 chromosomal DNA. The filter was washed in the following manner: two washes in 2X SSC, 15 min. per wash, followed by one wash in 2X SSC, 0.1% SDS for 30 min. This was followed by one wash in 0.1X SSC for 10 min. All washes were done at 45°C. The filter was overexposed (20 hours) to visualize cross-hybridizing bands.



STUDIES ON *nod* GENE REGULATION IN *B. japonicum* USDA110

nod gene induction studies with a *nodD2* deletion mutant

As previously mentioned, it was unclear from early studies (Göttfert *et al.* 1989) whether a *nodD2* deletion mutant (strain B.j.Δ370) had any effect upon *nod* gene regulation in USDA110. Therefore, various *nod-lacZ* fusions were mobilized into this strain and tested for inducible expression. Results from the induction studies are presented in Table 5. The results suggest that *nodD2* is required for maximal induction of *nodY*, *nodD1*, and *nolZ*. However, characterization of mRNA from uninduced cultures of the B.j.Δ370 strain showed that the downstream *nolA* gene (see map of the USDA110 regulatory locus, Figure 11) had a 15-20 fold increase in expression compared to USDA110 (Figure 12). This effect is most likely due to the positioning of the antibiotic resistance cassette, which reads towards *nolA* (M. Göttfert, pers. comm.). This result raises the question as to whether it is the loss of NodD2 or the potential increase in NolA that is responsible for the decrease in *nod* gene expression noted in Table 5.

NodD1-independent expression of *nodYABC* and *nodD1*

Previously published results had demonstrated that NodD1 from *B. japonicum* USDA110 was necessary for maximal induction and expression of *nodYABC* and *nodD1* by isoflavonoids (Banfalvi *et al.* 1988). A low level of induction was still detected using soybean seed extract as an inducer when *nodD1* strains

Table 5: Expression of *nodY*, *nodD1*, and *nolZ-lacZ* fusion plasmids in a *nodD2* mutant of *B. japonicum* USDA110.

	Units of Activity[a]		
Strain/fusion	Control	2μM Genistein	SSE[b]
TCD910::*nodY-lacZ* (wild type)	25±2	1630±192	2635±269
TCD2030::*nodY-lacZ* (*nodD2*)	23±4	80±11	115±15
ZB976::*nodD1-lacZ* (wild type)	23±3	47±8	61±10
TCD4000::*nodD1-lacZ* (*nodD2*)	11±2	10±2	20±2
NAD2021::*nolZ-lacZ* (wild type)	7±1	110±10	142±20
TCD2000::*nolZ-lacZ* (*nodD2*)	10±1	54±11	69±9

[a]Units using CPRG as a substrate

[b]SSE-soybean seed extract

Figure 11: Linkage map of the *nodD1, nodD2, nolA* regulatory locus from *B. japonicum* USDA110.

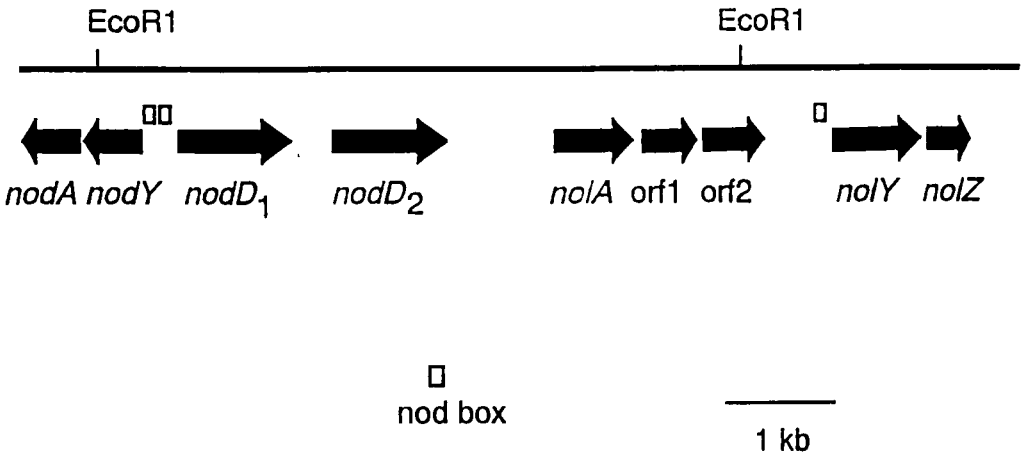
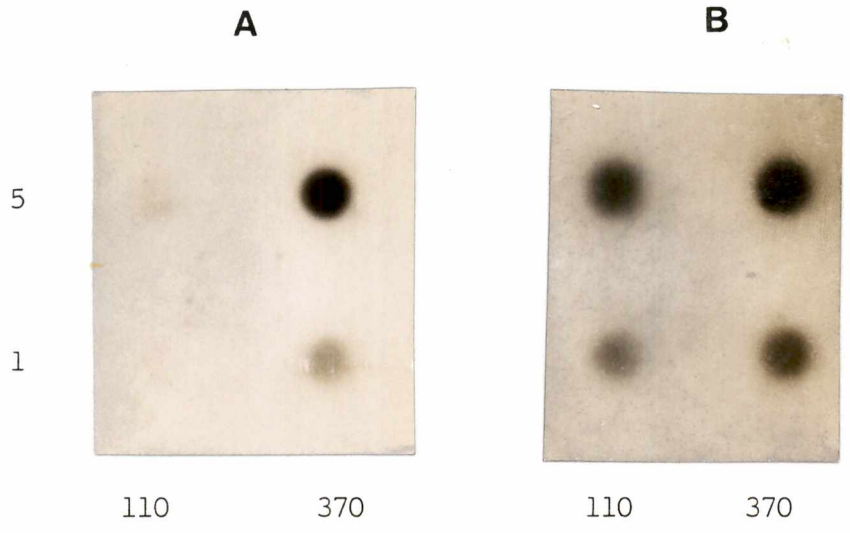


Figure 12: Analysis of *nolA* transcription from *B. japonicum* USDA110, and the USDA110 *nodD2* deletion mutant B.j.Δ370. Total RNA from cultures of each strain was extracted and fixed to filters as described in Materials and Methods, and then hybridized to: A) a *nolA probe*. B) a 23S rRNA gene probe to ensure that RNA levels were constant. Numbers on left indicate amount of RNA spotted in μg.



harboring either a *nodY-lacZ* or *nodD1-lacZ* fusion were tested. It was proposed that this residual induction was possibly mediated by NodD2. To test this hypothesis, plasmids encoding either a *nodY-lacZ* or *nodD1-lacZ* fusion were mobilized into *B. japonicum* strain Δ329, which had been deleted of nodD1, nodD2, nolA, ORF1, and ORF2. Since this strain lacked both *nodD1* and *nodD2*, it was expected that the *nod-lacZ* fusions would not be inducible. Surprisingly, induction of the *nodY-lacZ* and *nodD1-lacZ* fusions occurred to an extent equal to or greater than seen in the wild type strains (Table 6, Table 7). This induction has been reproduced using other independently derived strains that remove the same segment of DNA (strains B.j.Δ1267 and WAJ336, see Table 6 and Table 7). To ensure that observations made were not due to some copy number effect of the fusion plasmid, a *nodC-lacZ* fusion was integrated into the chromosome of strain B.j.Δ1267 (construct generously provided by P. Grob, ETH-Zentrum, Zurich, Switzerland). Results from induction studies with this strain (*i.e.* B.j.Δ1267-573) and a similar fusion in the wild-type strain (*i.e.* B.j.110-573) indicated that a significant level of induction still occurred in the deletion strain (Table 6).

Identification of activators and repressors of *nod* gene expression

Two immediate conclusions can be made from the results presented in Tables 6 and 7. One is that there must be some other positive regulator of *nod* gene expression that can

Table 6: Expression of *nodY* and *nodC-lacZ* fusions in wild type *B. japonicum* USDA110 and regulatory mutants.

	Units of Activity[a]		
Strain/fusion	Control	2μM Genistein	SSE[b]
TCD910::*nodY-lacZ* (wild type)	14±3	859±123	1407±280
TCD1070::*nodY-lacZ* (*nodD1*)	27±4	36±8	59±7
TCD1030::*nodY-lacZ* (B.j.Δ329)	9±2	897±127	1533±354
TCD3010::*nodY-lacZ* (*nodW*)	12±1	13±1	25±6
B.j.110-573::*nodC-lacZ* (wild type)	9±1	418±30	578±41
B.j.Δ1267-573::*nodC-lacZ* (B.j.Δ1267)	6±1	280±27	366±32

[a]Units using CPRG as a substrate

[b]SSE-soybean seed extract

Table 7: Expression of a *nodD1-lacZ* fusion plasmid in wild type *B. japonicum* USDA110 and regulatory mutants.

Strain	Units of Activity[a]		
	Control	2μM Genistein	SSE[b]
ZB976 (wild type)	23±3	47±8	61±10
ZB1027 (*nodD1*)	9±1	7±1	16±2
TCD4050 (B.j.Δ329)	13±3	60±6	84±5
TCD5000 (WAJ336)	10±1	58±4	89±3
BJS22 (*nodW*)	11±3	8±3	15±5

[a]Units using CPRG as a substrate

[b]SSE-soybean seed extract

function in the absence of the *nodD1* and *nodD2* genes deleted in strains B.j.Δ329, B.j.Δ1267, and WAJ336. The second is that deletion of the DNA in strains B.j.Δ329 and B.j.Δ1267 appeared to remove a negative effector of *nod* gene expression. An immediate candidate for the *nod* gene activator was the *nodW* gene product, which had been previously shown to be essential for maximal induction of *nodYABC* and *nodD1* (Sanjuan *et al.* submitted, see Table 6). To see if NodW was acting in the absence of NodD1 and NodD2, strain B.j.Δ1267 was deleted of the *nodW* gene to create strain B.j.912 (courtesy of P. Grob). Plasmids encoding either a *nodY-lacZ* or *nodD1-lacZ* fusion were mobilized into this strain and inducibility by isoflavones was tested. The results, shown in Table 8, indicated that essentially no induction of the *nod* gene fusions occurred. Therefore, NodW is essential for the *nod* gene induction previously seen in the B.j.Δ1267 mutant. This result does not exclude the possibility that other unknown positive regulators of *nod* gene expression may be present.

A likely possibility for a represor of *nod* gene expression is the *nolA* gene product. This hypothesis is supported by the presence of a putative helix-turn-helix motif in the NolA sequence, which is suggestive of a DNA binding function. In addition, *B. japonicum* strain SD6-1c (Sadowsky *et al.* 1991) harboring *nolA* and ORF1 on a multicopy plasmid showed a significant decrease in *nod* factor production, as judged by

Table 8: Expression of *nodY* and *nodD1-lacZ* fusions in *B. japonicum* strain 912.

Strain/fusion	Units of Activity[a]		
	Control	2μM Genistein	SSE[b]
TCD910::*nodY-lacZ* (wild type)	14±3	859±123	1407±280
TCD1030::*nodY-lacZ* (B.j.Δ329)	9±2	897±127	1533±354
B.j.912-32::*nodY-lacZ* (B.j.Δ1267, *nodW*)	11±1	10±1	14±2
ZB976::*nodD1-lacZ* (wild type)	23±3	47±8	61±10
TCD4050::*nodD1-lacZ* (B.j.Δ329)	13±3	60±6	84±5
B.j.912-22::*nodD1-lacZ* (B.j.Δ1267, *nodW*)	13±1	13±1	13±1

[a]Units using CPRG as a substrate

[b]SSE-soybean seed extract

analysis of ^{14}C acetate labeled compounds produced upon isoflavone induction of *nod* genes (Sanjuan, pers. comm.).

Complementation of B.j. Δ1267 with *nolA* and *nodD1 nodD2*

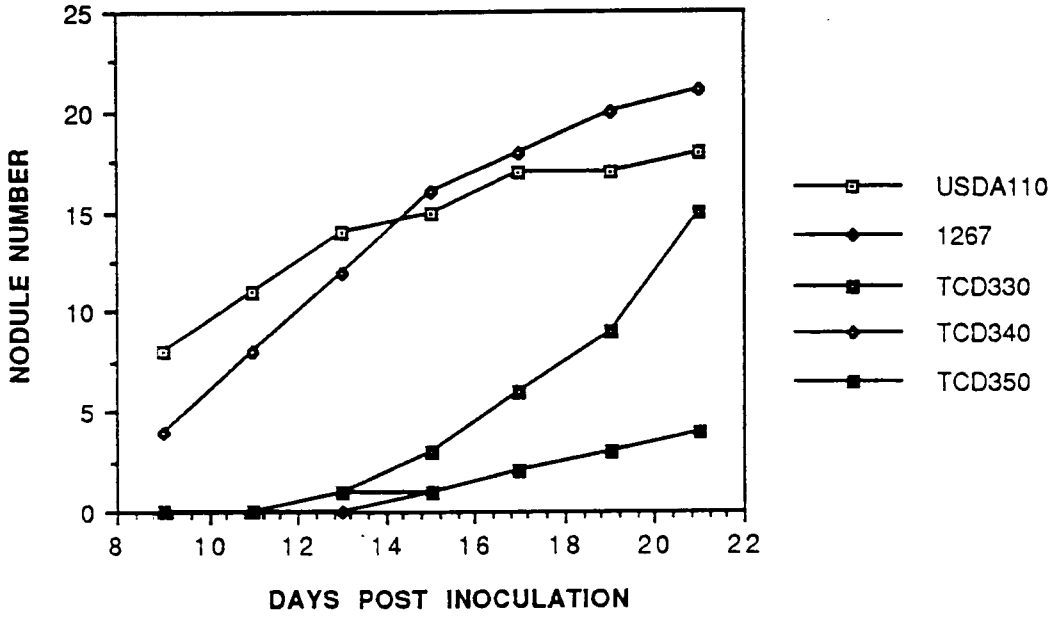
As previously mentioned, point mutations in and around *nolA* have been largely unobtainable, making analysis of individual genes in this region difficult. The B.j.Δ1267 and B.j.Δ329 strains had been initially reported to have a substantial reduction in nodule numbers on soybeans and other USDA110 hosts (Göttfert *et al.* 1989, 1992), yet paradoxically, they have a wild-type level of *nod* gene expression. To find what genes were responsible for the B.j.Δ1267 phenotype (*i.e.* high level of *nod* gene induction, poor nodulation ability), complementation experiments were performed. Cloned fragments of *nodD1 nodD2* or *nolA* ORF1 were integrated into the B.j.Δ1267 chromosome using a streptomycin/spectinomycin resistant derivative of the integration vector pRj1035 (Acuna *et al.* 1987). pRj1035 is unable to replicate outside of *E. coli*, and has repetitive elements found in the USDA110 chromosome cloned into it as portable regions of homology for integration. It was expected that, with the addition of *nodD1 nodD2* to B.j.Δ1267, nodulation proficiency would return to normal levels and nod gene expression levels might be enhanced beyond the levels seen with the parent B.j.Δ1267 strain. Addition of *nolA* ORF1 to a B.j.Δ1267 strain with a chromosomal *nodC-lacZ* fusion was expected to return the level of *nod* gene induction

to that expected of a *nodD1* mutant (*i.e.* little to no induction).

Plant tests with complemented B.j.Δ1267 strains

Past results using either the B.j.Δ329 or B.j.Δ1267 *nodD1 nodD2 nolA* ORF1 ORF2 deletions had shown that these strains exhibited delayed nodulation and a significant reduction in nodule numbers on soybeans and other hosts (Göttfert *et al.* 1989, 1992). It was assumed that the loss of *nodD1* and *nodD2* were responsible for this phenotype. B.j.Δ1267 strains with either *nodD1 nodD2* or *nolA* ORF1 integrated into their genomes were inoculated onto soybean plants to test nodulation proficiency (Figure 13). The B.j.Δ1267 strain complemented with *nodD1 nodD2* (strain TCD330) showed a delay in nodule appearence and reduction in nodule number compared to wild type, although more nodules appeared at the end of the assay compared to the parent strain B.j.Δ1267. A control strain that had the vector pRj1036 integrated into its genome (strain TCD350) nodulated soybean in a manner similar to that of the parent strain B.j.Δ1267. Addition of *nolA* ORF1 to B.j.Δ1267 (strain TCD340) allowed the strain to nodulate at essentially wild-type levels. Nodules induced by the Δ1267-*nolA* ORF1 strain had an appearence similar to those formed by the wild type. Nodules elicited by the B.j.Δ1267 strain and the B.j.Δ1267-*nodD1 nodD2* strain (TCD330) had an abnormal appearence compared to wild type in that some of the nodules exhibited a "collar" of tissue surrounding the junction of the

Figure 13: Nodulation kinetics on soybean of *B. japonicum* USDA110, B.j.Δ1267 and B.j.Δ1267 derivatives complemented with *nodD1 nodD2* (TCD330), *nolA*-ORF1 (TCD340) or the integrative vector pRj1036 only, as a control (TCD350). Results are from a minimum of 30 plants spread over three assays.



nodule and the root. In addition, these strains induced the formation of many necrotic bumps on the roots of the host plants. These results suggested that the *nolA* or ORF1 may code for a protein important for nodulation, or a regulator of other unknown genes important for effective nodulation. The results with strain TCD330 (B.j.Δ1267-*nodD1 nodD2)* were surprising, but could be explained by the fact that other downstream genes that could be important for NodD function are missing in this construct.

nod gene expression in B.j.Δ1267 strains complemented with _nodD1 nodD2_ or _nolA_ ORF1

Based upon the results seen with *nod* gene induction assays in the B.j.Δ1267 strain, it was assumed that addition of *nodD1 nodD2* would enhance expression of *nod* gene-*lacZ* fusions. However, due to constraints caused by a lack of a suitable antibiotic selection, this experiment could not be performed. Instead, *nodYABC* expression was monitored in a B.j.Δ1267 strain complemented with nodD1 *and nodD2* by measuring mRNA transcripts, using a 1.7 kb *Eco*RI fragment that carried the 3' end of *nodY*, all of *nodA*, and the 5' portion of *nodB* as a probe. Results from this experiment showed that *nodYAB* was not expressed at a higher level in either uninduced or soybean seed extract induced cultures (data not shown). Addition of a *nolA* ORF1 containing plasmid to the B.j.Δ1267 strain with a chromosomal *nodC-lacZ* fusion was predicted to reduce *nod* gene induction to a level seen in *nodD1* strains. However, results

from the expression studies showed that *nod* gene induction was reduced only to a small extent (Table 9).

The results from the gene expression studies with the complemented B.j.Δ1267 strains was somewhat surprising. A possible explanation for the results is that with the complemented strains, other genes were still missing, (*i.e. nolA*, ORF1, ORF2) whose products might contribute to function of NodD1, NodD2, or NolA.

Studies on *nolA* regulation

Previous results (Sadowsky *et al.* 1991) showed that the *nolA* gene was induced about 3-fold upon addition of genistein or soybean seed extract. The availability of a *B. japonicum* strain that lacked *nolA* (B.j.Δ1267) and a plasmid that had a *nolA-lacZ* fusion provided an opportunity to see if NolA was autoregulatory. Autoregulation is not an uncommon occurance among prokaryotic transcriptional regulators (Raibaud and Schwartz, 1984; Maloy and Stewart, 1993). Mobilization of pDLG86 (*nolA-lacZ*, NodD1$^+$, NodD2$^+$) into B.j.Δ1267 allowed a comparison of *nolA* expression levels in this strain compared to the levels seen in the wild type USDA110. Results showed that *nolA* expression was reduced about 25-fold in the B.j.Δ1267 strain compared to the USDA110 strain (Table 10). This result indicates that NolA positively autoregulates its own synthesis and suggests, although not confirms, that NolA has DNA binding properties.

Table 9: Induction of *B. japonicum* USDA110 and Δ1267 strains having a *nodC-lacZ* chromosomal fusion in the presence or absence of plasmid-borne *nolA*-ORF1.

	Units of Activity[a]		
Strain	Control	2μM Genistein	SSE[b]
B.j.110-573	5±1	780±66	1416±136
JSP11 (110-573, *nolA*-ORF1)	5±1	510±51	1134±56
B.j.Δ1267-573	6±1	505±90	1111±14
JSP12 (Δ1267-573, *nolA*-ORF1)	4±1	399±49	710±68

[a]Units using CPRG as a substrate

[b]SSE-soybean seed extract

Table 10: Expression of a *nolA-lacZ* fusion plasmid in a *nolA* background.

Strain/plasmid	Units of Activity[a]
TCD6000 (wild type, pDLG86)	2191±145
TCD6050 (B.j.Δ1267, pDLG86)	79±8

[a]Units using CPRG as a substrate

CHAPTER V

DISCUSSION

Results from Chapter IV describe characterization of a new flavonoid induced locus in *B. japonicum* USDA110. Sequence analysis delineated two new open reading frames that have been designated *nolY* and *nolZ*. No truly significant phenotypic alteration in nodulation proficiency was noted when *nolY* or *nolZ* mutants were inoculated onto soybean, siratro, or cowpea plants. A slight delay in nodulation and reduction in nodule number was seen when a *nolY* deletion (TCD520) was inoculated onto mungbean plants (Figure 9). The lack of a strong altered nodulation phenotype is surprising, based upon the substantial level of *nolZ-lacZ* induction in the wild type upon addition of genistein or soybean seed extract (see Table 3). However, such a result is not without precedent. For example, although regulated by NodD, mutations in *nodT* in *R. leguminosarum* bv *viciae* and *R. leguminosarum* bv *trifolii* ANU843 (Surin *et al.* 1990), and *nodSU* from *B. japonicum* USDA110 (Göttfert *et al.* 1990b) do not result in a measureable nodulation phenotype. These results are unexplained but it is possible that laboratory assays to monitor nodulation efficiency do not provide a high enough level of sensitivity to detect subtle defects (see Göttfert *et al.* 1992 for discussion). In some cases, the lack of a significant altered nodulation phenotype in specific *nod* gene mutants has been correlated with the

presence of a functional homolog of such a gene elsewhere in the genome. This has been shown to be the case for *nodPQ* mutants of *R. meliloti* (Schwedock and Long, 1989, 1990) and *nodM* mutants of *R. leguminosarum* bv *viciae* (Marie *et al.* 1992). For *nolY*, it is not clear whether there is a functional homolog elsewhere in the USDA110 genome based upon results from hybridization experiments. It should be noted that the two copies of glutamine synthetase in *B. japonicum* USDA110 have no significant homology with each other, yet both copies must be inactivated to see glutamine auxotrophy and a Fix$^-$ phenotype on soybean (Scott-Craig and Chelm, 1992). Thus, the possibility of functional reiteration of *nolY*, and perhaps *nolZ*, remains as an explanation for the lack of a significant nodulation phenotype associated with these mutations.

It was reported in Chapter IV that no visible differences were noted in the R_f of *nod* factors from USDA110 and the *nolY* mutant TCD520, based upon analysis by thin-layer chromatography. It is possible that very subtle differences in *nod* factor structure would not be revealed by TLC analysis. S. Luka (pers. comm.) noted that a mutation in the *nolO* gene of USDA110 caused production of a spectrum of both wild-type and structurally altered *nod* factors, and that these differences could be resolved only after HPLC separation. Thus it is possible that further analysis of the *nod* factors from *nolY* and *nolZ* mutants could show a similar result.

An important observation that enabled experiments to be

designed to ascertain *R. meliloti* NodPQ function came when it was found that *nodPQ* hybridized to a region on the Kohara ordered library of *E. coli* where the *cysDNC* genes mapped (Schwedock and Long, 1990; Kohara *et al.* 1987). At this time, biochemical functions for the CysDNC gene products were known, but the genes had not been sequenced in *E. coli*, so of course, comparisons of the *nodPQ* sequence with existing DNA sequence databases would have not revealed this similarity. Taking advantage of the extensive body of genetic and biochemical information on *E. coli* by screening a Kohara library with a *nolY* probe or probes from other *nod/nol* genes that have no apparent function in nodulation could provide useful clues that could lead to the discovery of a function for such genes.

Regulation of *nolYZ* expression

In addition to NodD1, NodW was found to be essential for flavonoid induction of a *nolZ-lacZ* fusion. NodW was shown to be necessary for flavonoid induction of the *nodYABC* and *nodD1* genes in USDA110 (Sanjuan *et al.* submitted). This result suggests that NodW may be a general component involved in activation of isoflavone induced genes in *B. japonicum* USDA110.

A sequence that has substantial similarity to previously characterized rhizobial *nod* boxes at the nucleotide sequence level was identified 5' to *nolY*. This sequence also contained the various proposed motifs for *nod* box function. The dependence of *nolZ* expression on NodD1 and isoflavones suggest

that this putative *nod* box is functional. A study by Göttfert and colleagues (1989) used a synthetic oligonucleotide that had sequence similarity to rhizobial *nod* boxes to probe for cross-hybridizing clones from a *B. japonicum* USDA110 gene library. Such clones might be expected to encode genes involved in nodulation. Several clones were found and the DNA surrounding these putative *nod* boxes was deleted. Surprisingly, none of the mutations generated had any effect on nodulation of soybean, and only one showed a nodulation defect on an alternate host, mungbean. The sequence of the *nod* box on each of the clones was determined and were found to vary in their similarity to a consensus *nod* box sequence from about 50 to 80% at the nucleotide level. None of these putative *nod* boxes were tested to see if they controlled transcription of any downstream genes.

Interestingly, none of the clones obtained by Göttfert *et al.* (1989) cross-hybridized to the *nolYZ* region. Although the *nolYZ* operon does not appear to be critical for nodulation, it is induced to a significant extent by the addition of isoflavones. These observations raise the obvious possibility that there may be other undiscovered loci present in *B. japonicum* USDA110 whose transcription is flavonoid and NodD dependent and that may be necessary for nodulation.

Studies on *nodD2* function

Previous results from Göttfert *et al.* (1989, 1992) were contradictory as to a function for the *nodD2* gene product from *B. japonicum* USDA110 (see Literature Review). A major difference in the two studies was that two USDA110 mutants were used. In the 1989 study, a *nodD2* deletion was constructed (strain B.j.Δ370) that did not remove *nodD1* or *nolA*; while in the 1992 study, a large deletion that removed *nodD1, nodD2, nolA*, and two open reading frames downstream from *nolA* was constructed. Both *nodD1* and *nodD2* were separately conjugated into this deletion strain to ascertain possible function. Using the B.j.Δ370 *nodD2* mutant, the results presented here indicate that *nodD2* is important for maximal induction of the *nodYABC* and *nodD1* genes, and the *nolYZ* operon. Interestingly, the effects of the *nodD2* mutation varied considerably between these three operons, with *nolZ* and *nodD1* affected relatively less than *nodYABC*, which showed a 20-fold loss of inducibility as compared to wild-type levels (see Table 5). It is not known why these differences exist, but it may be due to subtle differences in *nod* box structure found in the respective promoters. It is possible that both homomers and heteromers of NodD bind to *nod* boxes in USDA110. For example, NodD1 homomers may bind to *nodD1* and *nolYZ nod* boxes with reasonable efficiency, while heteromers of NodD1 and NodD2 are required for efficient expression of *nodYABC*. In this model, the slight loss of inducibility seen with the *nolZ* and *nodD1-lacZ* fusions

in the *nodD2* background could be due to the loss of NodD2; thereby, preventing optimal expression.

It was noted in Chapter IV that the construction of the B.j.Δ370 *nodD2* deletion resulted in an elevation of *nolA* mRNA, presumably resulting in an increase in NolA. NolA has been postulated to be a repressor of *nodD1* and *nodYABC* expression. This result raises the possibility that the effect of the B.j.Δ370 mutation on *nodD1* and *nodYABC* expression may be due to an increase in NolA levels. Such a possibility seems to be countered by the observation that addition of *nolA* ORF1 on a multi-copy plasmid had relatively little effect on the inducible level of a *nodC-lacZ* chromosomal fusion (see Table 9). However, the same plasmid transferred into wild type strain SD6-1c resulted in a significant reduction in the production of *nod* factors, a result supportive of the presence of a repressor function. Thus, the role of *nodD2* as an important component of *nod* gene expression in *B. japonicum* remains to be unequivocally shown.

nod gene expression in strains B.j.Δ329, B.j.Δ1267, and WAJ336

Conventional models of *nod* gene expression in *B. japonicum* USDA110 have NodD1 as an essential component of the regulatory circuitry (Smit *et al.* 1992). Thus, it was of considerable surprise to see that *nodYABC* and *nodD1* were induced to an extent equal to or greater than found in the wild type in strains (*i.e.* B.j.Δ329, B.j.Δ1267, WAJ336) that had been deleted of *nodD1, nodD2, nolA,* ORF1, and ORF2. Such results

led to the conclusion that an additional regulatory protein must allow *nod* gene expression in the absence of NodD1 and NodD2. Furthermore, the action of this protein was only readily detectable in the absence of a repressor.

Curiously, the *nolYZ* operon is not expressed in a B.j.Δ329 background. Therefore, *nolYZ* expression is regulated differentially from the *nodD1* or *nodYABC* operons. The mechanism allowing this differential regulation is not readily apparent. One possibility is that the repressor of *nodD1* and *nodYABC* transcription (*e.g.* NolA) does not act on *nolYZ*. However, this fails to explain why NodW cannot fully activate *nolYZ* expression in the B.j.Δ329 background. Perhaps an additional repressor exists that maintains low levels of *nolYZ* expression in B.j.Δ329. Another possibility is that NodW cannot act directly to activate *nolYZ* expression, but acts by stimulating *nodD1* expression, with NodD1 then activating *nolYZ* transcription. This model would require that an explanation be provided as to why NodW can independently activate *nodYABC* and *nodD1* expression, but not *nolYZ*. An analysis of NodW binding to respective promoters may provide this explanation.

Complementation of B.j.Δ1267 with *nodD1 nodD2* or *nolA* ORF1

Given the suspected role of NolA as a repressor, it was of interest to see if addition of *nolA* ORF1 to B.j.Δ1267 would result in a significant reduction in the levels of a chromosomal *nodC-lacZ* fusion. However, the results were surprising in that *nolA* ORF1 had little effect upon the

induction of the fusion by genistein or soybean seed extract. An argument can be made that ORF2 is somehow required for such repression, but this does not seem likely based upon the observation that a Tn*5* insertion in ORF2 has no discernable effect upon the expression of a *nodY-lacZ* fusion (data not shown). Alternatively, it is possible that the presence of ORF1 somehow antagonizes NolA function. What is needed is a plasmid encoding only *nolA* so that these experiments can be repeated and NolA can be unequivocally tested as a repressor.

It is also possible that NolA requires interaction with NodD1, NodD2, or both to act as a repressor in *nod* gene expression. However, the strains required for testing this hypothesis have proved impossible to construct because of an inability to mutate only *nolA*. This inability could suggest that NolA is essential for growth. However, *nolA* can be deleted along with adjacent genes, and therfore, is not essential. It could be that NolA is required for modulating expression or activity of unknown genes or proteins and that the loss of regulation of such genes or proteins could be detrimental to the cell.

There is evidence that suggests that NolA is a repressor of *nod* gene expression. For example, the addition of *nolA* ORF1 to *B. japonicum* SD6-1c (a wild-type strain lacking *nolA*, Sadowsky *et al.* 1991) causes a substantial decrease in the production of *nod* factors (J. Sanjuan, pers. comm.). Further circumstantial evidence for a NolA repressive function comes

from studies with *B. japonicum* strain USDA135. This wild type strain lacks a copy of *nolA* as judged by hybridization (Sadowsky *et al.* 1991). A *nodD1-lacZ* fusion plasmid mobilized into this strain is induced 80-fold by soybean seed extract, while the same fusion is induced only 3-fold in *B. japonicum* USDA110 (Banfalvi *et al.* 1988).

Plant tests with complemented B.j.Δ1267 strains

Nodulation by the B.j.Δ1267 strain was severely inhibited during the early period of nodule formation (Göttfert *et al.* 1992). This lack of nodulation proficiency may be due to the loss of *nodD1* and perhaps *nodD2*, genes thought to be essential for *nod* gene expression. In strain B.j.Δ1267, common *nod* genes are expressed at wild-type levels. Furthermore, based upon thin-layer chromatography analysis. *nod* factor profiles from induced B.j.Δ1267 cultures are similar to those from induced USDA110 cultures. These results suggest that there are other unknown functions missing from strain B.j.Δ1267 that are important for efficient nodulation. DNA fragments encoding *nodD1 nodD2* or *nolA* ORF1 were conjugated into strain B.j.Δ1267 in an attempt to restore nodulation proficiency.

The results from the plant tests were surprising in that addition of *nolA* ORF1 to strain B.j.Δ1267 restored nodulation to near wild-type levels, while the addition of *nodD1 nodD2* showed a significant nodulation improvement only in the later stages of the assay (see Figure 13). These results suggest that NolA or ORF1 carries out some important function involved

with nodulation, or that they are regulators of other unknown genes important for nodulation. In Table 10, it was shown that NolA appears to regulate its own expression, supporting the idea that NolA can act as a transcriptional regulator. Therefore, considerable evidence supports the idea that NolA may be a regulator of other necessary nodulation genes.

The *nod* genes in *B. japonicum* USDA110 were originally isolated by a strategy of cloning DNA that hybridized to common *nod* genes from *R. meliloti* (Russell *et al.* 1985). The cloned region was then mutagenized and the mutant phenotypes tested. Such an approach takes advantage of the observation that genes involved in similar functions tend to be clustered on prokaryotic genomes. However, this approach may not allow identification of all functionally related genes. An example are the *nodVW* genes which are distant from the *nodYABC* genes on the *B. japonicum* chromosome (Kundig *et al.* 1993). Therefore, it is possible that not all relevant nodulation genes in *B. japonicum* USDA110 have been identified.

If there are other unidentified *B. japonicum* nodulation genes, they could be isolated by a couple of approaches. One approach would be to randomly mutate the genome of USDA110 to an extent that all genes should be detected. However, screening for nodulation defective bacteria is a laborious process. The observation that NolA may complement nodulation defects seen in strain B.j.Δ1267, and that it may do so by regulating other unknown nodulation genes, opens the

possibility that NolA regulated genes could be isolated by using subtractive hybridization procedures (see Sambrook *et al.* 1989 for discussion of the technique). Such a technique has been used to isolate *B. japonicum* genes that are expressed differentially (Scott-Craig *et al.* 1991; Bhagwat and Keister, 1992). In this case, a comparison of mRNA from induced cultures of B.j.Δ1267 cells to uninduced wild-type cells should detect genes that are relesed from NolA repression or independently regulated by NodW. Furthermore, a similar experiment could be done using induced cells of a B.j.Δ1267 strain harboring *nolA* and uninduced B.j.Δ1267 cells to detect genes that might be activated by NolA. All classes of genes isolated would be of interest to study.

Summary

Results from this study have characterized a new locus in *B. japonicum* USDA110 that is comprised of two open reading frames designated *nolY* and *nolZ*, and an upstream region that has strong similarity to *nod* boxes analyzed from other rhizobia. The expression of the *nolYZ* operon was found to be dependent upon isoflavones, NodD1, and NodW. Mutations within *nolYZ* had little to no effect on nodulation efficiency on host plants tested. The basis for the lack of a significant nodulation phenotype is not known.

Important observations pertaining to *nod* gene expression in *B. japonicum* were made. A role for NodD2 in regulation of *nod* gene expression is proposed based upon results from the assay

of *nod-lacZ* fusions into strain B.j.Δ370, a *nodD2* mutant. However, a conclusive role for NodD2 in *nod* gene regulation cannot be asserted due to concomitant elevated expression of *nolA* in strain B.j.Δ370. It appears likely that a repressor is encoded downstream of *nodD2* in *B. japonicum* USDA110. NolA is the most likely candidate for this repressor. This is based upon the presence of a putative DNA-binding helix-turn-helix motif in the protein and the observation that conjugation of *nolA* ORF1 into *B. japonicum* SD6-1c drastically reduces the level of *nod* factor synthesis. However, a repressive effect by NolA ORF1 could not be demonstrated in strain USDA110. Comparison of *nod* gene induction profiles with nodulation proficiency (Table 11) suggests that there may be unknown genes present within the USDA110 genome that are important for nodulation and that they may have novel mechanisms of regulation. A comparison of the expression levels of *nodD1, nodYABC*, and *nolYZ* in both wild-type and mutant backgrounds indicates that each operon is differentially regulated. For example, *nodD1* is specifically expressed by isoflavone glucosides that do not induce *nodYABC* (Smit *et al.* 1992), while *nolYZ* does not appear to be under control of a repressor that acts upon *nodYABC* and *nodD1*. The reasons for these differences are not known. Modulation of host range determination or control of nodulation proficiency in a soil environment are two possible explanations.

Results presented here and elsewhere (Sanjuan *et al.*

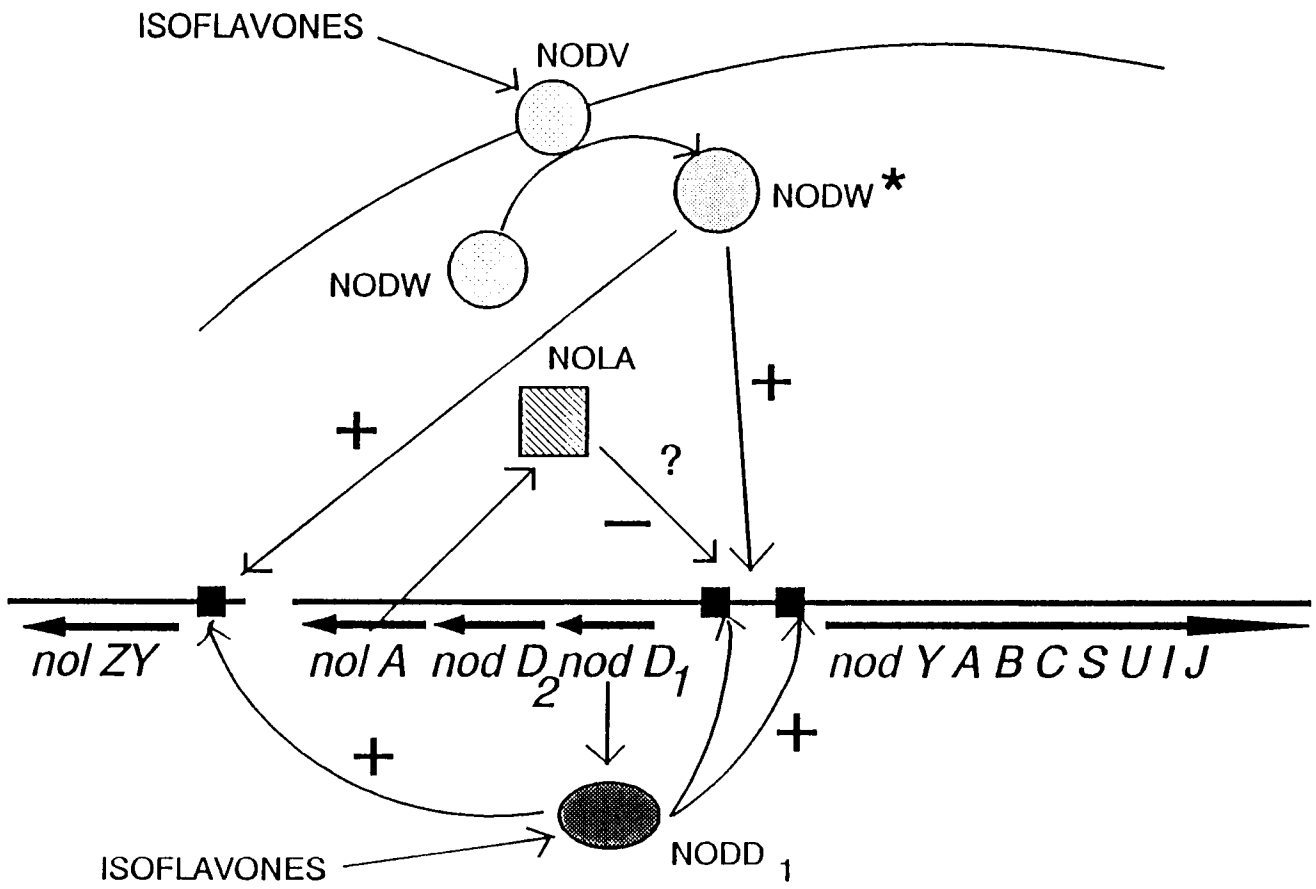
Table 11: *B. japonicum* USDA110 *nod* gene regulatory mutants and their plant nodulation phenotypes.

Mutation	Nodulation Phenotype on:		*nod* gene induction
	Soybean	Other Hosts	
nodD1	slight delay[a]	delay[a]	none[b]
nodD2	delay?[c]	delay?[c]	weak[d]
B.j.Δ1267	strong delay[a,c]	strong delay[a,c]	wild type[d]
nodW	delay[e]	Nod^{-e}	none[f]
B.j.912	Nod^{-g}	Nod^{-g}	none[d]

[a]Göttfert *et al.* 1992
[b]Banfalvi *et al.* 1988
[c]Göttfert *et al.* 1989
[d]This study
[e]Göttfert *et al.* 1990a
[f]Sanjuan *et al.* submitted
[g]P. Grob, pers. comm.

submitted) have expanded upon the generalized model for *nod* gene induction presented in Figure 3. In addition to NodD1, NodW, NodD2, and perhaps NolA, all appear to modulate *nod* gene expression in USDA110. A model that attempts to summarize the USDA110 *nod* gene regulatory scheme is presented in Figure 14. In this scheme, NodV, NodD1, and perhaps NodD2, are postulated to interact with host-derived isoflavones. NodV is proposed to transduce a signal to NodW, perhaps via phosphotransfer as seen in other two-component regulatory cascades (see Stock *et al.* 1989 for review). Upon activation, NodW would then interact with *cis*-acting sequences within the *nod* gene promoters and activate transcription. NodD1 and/or NodD2 would be expected to interact with *nod* boxes in a fashion similar to that seen in other rhizobia (Hong *et al.* 1987; Fisher *et al.* 1988). NolA is postulated to be a repressor of USDA110 *nod* gene expression, but this has not been clearly demonstrated. The biological need for such complexity in *nod* gene expression may be to balance the production of *nod* factor signals to optimize nodulation efficiency.

Figure 14: Summary of *nod* gene regulatory circuitry in *B. japonicum* USDA110. +, -, indicate positive or negative effect. * indicates covalent modification. The placement of NodV, and it's interaction with isoflavones, and the presumed covalent modification of NodW are speculations based upon predicted properties that arose from sequence analysis (Göttfert *et al.* 1990a). NolA is thought to have a negative effect upon USDA110 common *nod* gene expression, but evidence so far is inconclusive.


ISOFLAVONES
NODV
NODW*
NODW
NOLA
+
+
?
—
nol ZY
nol A
nod D2
nod D1
nod Y A B C S U I J
+
+
ISOFLAVONES
NODD 1

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VITA

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